STUDIES ON NANOCURCUMIN BY UTILISING ITS BIOAVAILABILITY AND BIO-DISTRIBUTION AGAINST NICOTINE INDUCED TOXICITY AT CELLULAR LEVEL UNDER PROTEIN RESTRICTED CONDITION

THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY (SCIENCE)

Under the supervision of

Prof. Brajadulal Chattopadhyay

Professor

Department of Physics

Jadavpur University

BY

SOMASHREE BISWAS

Department of physics

Kolkata-700032

2024

Dedicated

To

My Beloved Parents

And Daughter

JADAVPUR UNIVERSITY

KOLKATA-700032, INDIA

CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled "**Studies On Nanocurcumin By Utilising Its Bioavailability And Bio-Distribution Against Nicotine Induced Toxicity At Cellular Level Under Protein Restricted Condition**" submitted by Somashree Biswas who got her registered on 20.07.2017 for the award of Ph.D.(science) degree at Jadavpur university, is absolutely upon her own work under the supervision of Prof. Brajadualal Chattopadhayay, professor, Department of Physics, Jadavpur university and that neither thesis nor any part of it has been submitted for either any degree/diploma or any other academic award anywhere before.

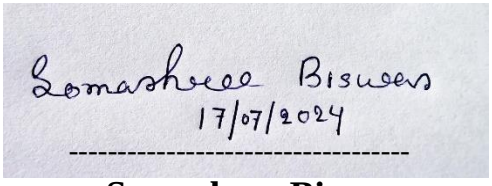
B Chattopadhyay 17/12/2024

Signature of the supervisor with seal

Dr. Brajadulal Chattopadhyay
Professor
Department of Physics
Jadavpur University
Kolkata - 700 032

DECLARATION

I do hereby declare that the work embodies in this thesis entitled "**Studies On Nanocurcumin By Utilizing Its Bioavailability And Bio-Distribution Against Nicotine Induced Toxicity At Cellular Level Under Protein Restricted Condition"** which is being submitted for the Degree of Doctor of philosophy (science) has been carried out by me in the Biophysics Laboratory, Department of Physics, Jadavpur University, Kolkata. Neither the thesis nor any part thereof has been presented anywhere earlier for any degree/diploma or academic award whatsoever.



Date:
Kolkata, India

Somashree Biswas

ACKNOWLEDGEMENT

First and foremost, I want to express my gratitude to God for making it possible for me to successfully do this assignment. Life is an ongoing adventure filled with challenges, victories, and setbacks. We can never truly know what the future has in store for us because every moment is uncertain. This is my chance to express my profound gratitude to everyone who has helped, supported, and contributed in any kind to my doctoral studies. Under these kinds of situations, my supervisor, Professor **Brajadulal Chattopadhyay** of Jadavpur University, Department of Physics has been there for me, supporting me the entire way and offering me guidance. Your assistance has been enormously appreciated.

I would like to express my gratitude to my research work's supervisor, Prof. **Brajadulal Chattopadhyay**, for his support throughout the entire process for his politeness, and never-ending excitement and direction. I am really appreciative of his constant flow of thoughts, which led to numerous new research directions, and his thorough proofreading of this thesis. I've had the good fortune to receive his unwavering support and encouragement.

I place on record, my sincere heartfelt thanks to Dr. Abiral Tamang, Assistant professor, Department of physics, Jadavpur university for his constants support, valuable speech and motivation to take up this area as perspective research topic for my Ph.D.

In my Ph.D. tenure, I got the opportunity to work in collaboration with Assistant professor Arunima Biswas, Molecular and cell Biology Laboratory, Department of Zoology, University of Kalyani. I would like to extend special acknowledgement to her.

I owe a debt of gratitude to Dr. Krishna Chattopadhyay for his insightful criticism, unwavering encouragement, insightful recommendations, and assistance in helping me get over obstacles I faced while working on my project.

Many thanks to my lab colleagues, seniors, and well-wishers for providing me with invaluable assistance throughout my entire research. They provide me with a helpful network and a welcoming lab setting so that I can easily carry out my studies. I was too fortunate to be included in them.

I am not sure how to acknowledge my family. I have to admit that without their unwavering love and support, I could not have progressed to this point. I can never adequately convey my gratitude to my parents and brother for what they have given up for me.

I consider myself extremely fortunate to be married to Dr. Biswajit Jana, without whose unwavering support and cooperation in all aspect of my life, I could not have persevered long enough to complete this happy task today.

Special thanks to my daughter Ahitangni who is the reason I have so much more energy. My pillars of support are indescribable.

I vote of thanks to all the office staff for their help in regards to all officials' activities related directly or indirectly to my research work during these years.

Finally, I would like to express my profound sense of gratitude to the University grant commission (UGC-NET), Govt of India for providing me financial support. Again, Thanks to all.

Somashree Biswas

Department of physics

Jadavpur University

Kolkata-700032

CONTENTS

CONTENTS

CONTENTS

CONTENTS

CONTENTS

CONTENTS

CONTENTS

CONTENTS

CONTENTS

List of Figures

List of Tables

List of Symbols and Abbreviations

- **Ach :** Acetylcholine
- **ALP :** Alkaline Phosphatise
- **ALT :** Alanine Aminotransferase
- **AST :** Aspartate Aminotransferase
- **CAT :** Catalase
- **CRT :** Chemo Radiotherapy
- **CVD :** Cardiovascular Disease
- **DSBs :** Double-Strand Breaks
- **EMT :** Epithelial-Mesenchymal-Transition
- **FMO3 :** Flavin-Containing Monooxygenase 3
- **GGT :** Gamma-Glutamyl Transferase
- **GR :** Glutathione Reductase
- **GXp :** Glutathione Peroxidase
- **H_2O_2 :** Hydrogen Peroxide
- **HDL :** High Density Lipoprotein
- **IFN-g :** Interferon Gamma
- **IL-4 :** Interleukin-4
- **IL-6 :** Interleukin-6
- **LDH :** Lactate Dehydrogenase
- **LDL :** Low Density Lipoprotein
- **MDA :** Malondialdehyde
- **nAChRs :** Nicotinic Acetylcholine Receptors
- **NK :** Natural Killer
- **NKT :** Natural Killer T
- **NNK :** 4-(Metylnitrosamino)-1-(3-Pyridyl)-1-Butanon
- **NNN :** N′-Nitrosonornicotine
- **NRT :** Nicotine Replacement Therapy
- **NTS :** Nucleus Tractus Solitaries
- **PAH :** Poly Cyclic Hydrocarbons
- **PFC :** Prefrontal Cortex
- **ROS :** Reactive Oxygen Species
- **RT :** Radiotherapy
- **SOD:** Superoxide Dismutase
- **SSBs :** Single Strand Breaks
- **TC :** Total Cholesterol
- **TG :** Triglyceride

- **VP :** Ventral Pallidum
- **VTA :** Ventral Tegmental Area
- **µg :** Microgram
- **µL :** Micro-Litre
- **NFHS :** National Family Health Survey
- **COPD :** Chronic Obstructive Pulmonary Disease
- **NSCLC :** Non -Small Cell Lung Cancer
- **WHO :** World Health Organization
- **GC :** Gas Chromatography
- **HPO :** Hypothalamic Pituitary Ovarian
- **HPA :** Hypothalamic Pituitary Adrenal
- **HPT :** Hypothalamic Pituitary Thyroid
- **AHA :** American Heart Association
- **TNF-α:** Tumour Necrosis Factor Alpha
- **TSNA:** Tobacco-Specific N-Nitrosamines
- **UGT :** Uridine Diphosphate-Glucuronosyltransferase
- **µM :** Micro Molar
- **NTS :** Nucleus Tractus Solitaries
- **PFC :** Prefrontal Cortex
- **CNS :** Central Nervous System
- **CKD :** Chronic Kidney Disease
- **GFR :** Glomerular Filtration Rate
- **NAFLD :** Non Alcoholic Fatty Liver Disease
- **PEM :** Protein Energy Malnutrition
- **CF :** Cystic Fibrosis
- **CFTR :** Cystic Fibrosis Transmembrane Conductance Regulator

Research Proceedings

Seminars and conferences attended:

- Oral presentation on "potential amelioration of nanocurcumin against nicotine-induced toxicity on rats", at International Conference on Nanotechnology and Nanomaterial, Midnapur, 2018.
- Poster presentation on "The challenging efficacy of nano formulated curcumin against nicotine induced toxicities in female albino rats and protein malnutrition." at International Conference on Nanotechnology (ICNT-2018) Brajalalchak, Haldia,Purba Medinipur,16th -17th November,2018.
- Poster presentation on "Rapid synthesis of Bio-inspired nanoparticles for various biological Applications" at seminar on twists and Turs in Physics Research: special Emphasis on condensed Matter and Biophysics (TTPR_2017) Jadavpur University, kolkata-700032, 21st-22nd February.
- Poster presentation on "Efficacy of Nano-Curcumin on Nicotine-Induced Genotoxicity and Immunomodulatory Disruption in Protein-Malnourished Female Rats." International Conference on Energy-Efficient Materials (ICEEM-2024) and Workshop on solar cell simulation software SCAPS-1D), Mizoram University, Aizawl, India,13th-14th June,2024.

LIST OF PUBLICATIONS

- **Somashree Biswas,** Krishna Chattopadhyay, Brajadulal Chattopadhyay. Efficacy of nano-curcumin on nicotine-induced genotoxicity and immunomodulatory disruption in protein-malnourished female rats. *Indian Journal of physiology and allied sciences*. **2024**; 76(1):25-31.

- **Somashree Biswas**, Krishna Chattopadhyay, Solanki Sarkar, Arunima Biswas., Brajadulal Chattopadhyay. Study the comparative efficacies of curcumin and Nanocurcumin on human cancer cells. *Journal of Xi'an University of Architecture & Technology*. **2024**; 7: 294-323.

- Krishna Chattopadhyay. **Somashree Biswas**., Subrata Mukhopadhyay and Brajadulal Chattopadhyay. Amelioration of nicotine induced toxicity by nanocurcumin in protein malnourished condition. *Scientific journal of biology*. **2018**; 1(1):1-8.

- Anwesha Samanta, Krishna Chattopadhyay, **Somashree Biswas**, Bhola Nath Paul and Brajadulal Chattopadhyay. Promising Efficacy of nanocurcumin in comparison to curcumin against nicotine-induced complications. *Journal of biologically active products from nature*. **2022**; 12(4): 366-377.

CHAPTER I

INTRODUCTION

1.1. Introduction

The widespread use of tobacco products has been shown to have multiple harmful effects on health, making it a serious global health concern with dire ramifications. The World Health Organization estimates that tobacco-related illnesses killed more than 8 million people in 2017. If current trends continue, the group forecasts that number will reach over 1 billion by the 21st century. [1]. One of the biggest threats to public health is tobacco dependency, which can lead to a number of life-threatening illnesses that affect the heart, lungs, kidneys, and other organs in addition to cancer. In India, tobacco-related malignancies contributed to 27% of the nation's cancer burden in 2020 [2,3]. 6.6% of women and 32.7% of men all over the world who are at least 15 years old smoke tobacco. Despite being a poisonous and addictive substance, nicotine is widely used in almost all nations, cultures, and faiths [4]. According to the Global Adult Tobacco Survey (GATS), India total prevalence of tobacco use in India is 10.38% for smokers and 21.38% for non-smokers between 2016 and 2017. 28.6% of adults (42.4% of males and 14.2% of women) presently use tobacco in either smoke or smokeless form. The Demographic Health Survey, commonly referred to as the National Family Health Survey (NFHS), is one of India's largest health surveys. It gives disaggregate estimates of a variety of health and demographic factors. [5]. Compared to men, women smokers are more likely to develop smoking-related illnesses like heart disease, stroke, reduced lung function, COPD and lung cancer at younger ages [6]. Surprisingly, women have particular issues related to their biological/reproductive life cycle because of tobacco use. These difficulties include women's malignancies (such as cervical cancer), early menopause, menstruation (such as irregular cycles and dysmenorrhea), osteoporosis, impacts on fertility, pregnancy, and fetus/child development, as well as the negative effects of nicotine on neurodevelopment. Furthermore, Extrauterine Pregnancies, abruptio/previa placenta, premature

membrane rupture, miscarriage, stillbirth, low birth weight, preterm delivery, and tiny gestational age have all been associated to maternal smoking [5]. Congenital anomalies, including cleft lip, cardiac septal defects, pulmonary, tricuspid valve and great arteries malformations, pyloric stenosis and clubfoot, as well as various malformations, have also been linked to maternal smoking [7]. Postmenopausal smokers who smoke cigarettes have greater levels of testosterone, androstenedione, estrone, and 17-hydroxy-progesterone [8]. Nicotine has been shown to enhance the growth and spread of lung cancer cells. It can activate pathways that lead to enhanced cell proliferation, angiogenesis (the development of new blood vessels to feed tumors) and metastasis. There is a clear correlation between smoking cigarettes and the development of non-small cell lung cancer (NSCLC) [9]. Lung cancer is a serious global health concern, being the second most frequent cancer and leading cause of cancer-related death globally. According to the Centers for Disease Control and Prevention (2022), the primary cause of lung cancer is tobacco smoke and nicotine is the major contributor for 80% to 90% of lung cancer-related fatalities [10]. Nicotine promotes lung cancer progression and metastasis through the activation of the PI3K/Akt/mTOR signaling pathway and nicotine may influence the activity of enzymes involved in carcinogen metabolism or modulate inflammatory responses, which could exacerbate lung cancer risk [11]. Because of high mortality rates and great prevalence, Lung and cervical cancers continues to be the leading cause of death. are extremely frequent and difficult to treat. It has also been demonstrated that in a same way nicotine affects cervical cancer cells by encouraging cell invasion and proliferation by modifying signaling pathways like the MAPK/ERK pathway, which can alter the behavior of these cells. [12]. Nicotine can influence the immune response by impacting different types of immune cells, such as T cells, B cells, and macrophages. It typically inhibits immunological processes, resulting in heightened vulnerability to infections and compromised

immune responses [13]. Nicotine has a substantial influence on the cardiovascular system. It raises the heart rate, blood pressure, and promotes the development of atherosclerosis. Nicotine promotes the release of catecholamines, which may contribute to these effects [14]. It can change immunological responses, which may result in an increase in inflammation as well as a suppression of immune function [15]. It affects mortality and illness risk in the digestive system and exacerbates respiratory disorders including asthma and COPD. It affects the central nervous system, which results in altered thought processes and addiction [16]. Nicotine induces oxidative stress by activating signaling pathways like the NF-kB pathway, which can increase the expression of pro-inflammatory cytokines and ROS-producing enzymes. Nicotine generates ROS through a variety of mechanisms, including NADPH oxidase activation, mitochondrial dysfunction, and poor antioxidant defenses. This increased ROS generation is linked to oxidative stress, which plays a role in the development and progression of different diseases [17]. Nicotine easily enters the placenta, and the fetuses of mothers who smoke are exposed to higher nicotine amounts than their mothers. Nicotine's strong lipophilicity allows it to easily pass the placental barrier. According to studies, nicotine levels in fetal blood can be much greater than in mother blood. This is related to the placenta's ability to carry nicotine from mother to fetus [18]. Long-term impacts of nicotine consumption during pregnancy on a child's neurodevelopment and health outcomes. In cell cultures, nicotine has been demonstrated to promote chromosome aberrations and sister chromatid exchange (SCE). Nicotine can also damage chromosomal integrity and perhaps have mutagenic consequences. In cell models, nicotine has been shown to function as a clastogen, causing an increase in sister chromatid exchange and chromosome abnormalities [19].

Protein Energy Malnutrition (PEM) remains a significant public health issue in developing nations, affecting individuals of all ages including newborns, young children, pregnant and

breastfeeding mothers, and lower-income populations. Low dietary protein limits the toxicity, disposition, and biosynthetic activity. Protein Energy Malnutrition (PEM) is a disorder caused by insufficient protein and energy intake, which can lead to health difficulties [20]. Though it is well known that nicotine causes many health issues in normal dietary situations, the noxious implications of nicotine, predominantly in protein-restricted diets, are a source of worry.

Curcumin is a yellow polyphenolic pigment which is obtained from the rhizome of Curcuma longa L. (turmeric), has been used in Chinese and Ayurvedic medicine, as well as culinary applications. It exerts a wide range of biological and pharmacological actions, including anti-inflammatory, antioxidant, antibacterial, antiviral, anti-fungal, anti-hepatotoxic, hypolipidemic, and anticancer activities. Curcumin contains immunomodulatory and anti-allergic properties and has been utilized as a chemo preventive drug against several cancers, including liver, gastric, breast, prostate, and cervical cancer [21-23]. In addition, several in vitro and in vivo studies on curcumin demonstrate its anti-carcinogenic effects on lung cancer through inhibition of cell proliferation and induction of apoptosis. Curcumin's preventive function against nicotine-induced toxicities has been thoroughly shown in protein-restricted conditions. Curcumin's role in nicotine-induced toxicity has been well established, including hepatotoxicity, cardiorespiratory illness, renal toxicity, and immunological effects. Curcumin's primary limitations against clinical application include poor water solubility, limited bioavailability, poor absorption, and quick elimination from our bodies. Animal research on curcumin absorption, metabolism, and excretion provide useful information about its bioavailability and elimination. Curcumin is digested, and a significant amount of the orally supplied curcumin is eliminated in feces. Curcumin's low systemic bioavailability limits its therapeutic applicability, and understanding its pharmacological effects in vivo is difficult.

The advancement of nanotechnology is one of the most significant achievements of the twenty-first century. Applications of nanoparticle in medical research are getting increasingly popular. Nanoparticles provide significant advantages over regular therapeutic molecules, including a higher drug loading rate, increased biocompatibility, and tailored administration, which allows for more effective treatment of various ailments. Nanomaterials with a wide surface area enhanced the solubility of indissoluble medications with a high absorption rate, resulting in a lower total drug dosage [24]. This could lessen the negative side effects of conventional compounds. The current study has been conducted to investigate the protective effects of nanocurcumin against nicotine induction under regular protein diet conditions. In this study, nanoparticles of curcumin were produced by ultrasonication and characterized by FE-SEM, UV-Visible spectrophotometer, FTIR, and XRD. This produced nanocurcumin were employed against nicotine-induced female albino rats at a dose of 4 mg/kg body weight. The parameters of various groups of animas were analyzed, including SGOT, SGPT, ACP, ALP, Urea, Creatinine, MDA, SOD, Catalase, GPX, GSH, IL-4, IL-6, IFN-γ, TNF-α, BCL-2, BAX, Estrogen, and Progesterone. This research also included a DNA damage study and DNA content analysis. A further study was conducted to investigate the antiproliferative and anticarcinogenic properties of nanocurcumin on two different cancer cells through MTT assay, cell morphology assay, DAPI staining ROS generation and DNA laddering analysis.

Previous research suggests that oxidative stress is a key component in the development and progression of many problems [25]. It was found that the liver, renal enzymes and lipid component of the blood were significantly increased as the result of nicotine induced adverse effect in serum which can be ameliorated by curcumin itself and strikingly it was also observed that nanocurcumin is much more potent than curcumin. This study established the fact that nanocurcumin has the

better efficiency to increase the intracellular ROS supporting apoptosis of both cervical cancer and lung cancer cells. There is increase in the levels of ROS in the treatment group indicating the apoptic mode of cell death by nanocurcumin through ROS generation. It has been also evident that nanocurcumin doses are harmless and causes no-side effect to normal human body cells. It has also been observed that curcumin needs higher doses compared to nanocurcumin to cause apoptosis of the same cancer cells and more time to initiate therapeutic activities. This study was paying attention on investigating the effects of nicotine on immunomodulatory and genotoxicity disruptions in female rats kept on a protein restricted diet, and find out the ameliorative effectiveness of nanocurcumin after 1 hr of nicotine intoxication. Nanocurcumin ameliorates the nicotine-induced genotoxic effects, keeps the female sex hormone levels and reestablishes the normality of immune responses in protein-restricted diet rats with higher efficiency than curcumin.

1.2. Aims and Objectives

The tobacco pandemic is a significant public health threat globally. Women are increasingly using tobacco products in various forms, which has emerged as India's largest public health issue. One key health risk factor that dramatically raises the incidence of malignancies is cigarette smoke. Nitrosonornicotine, a tobacco-specific nitrosamine, is one of the strong carcinogens and is accused of the addictive potential of smoking.

Curcumin is a natural biphenolic compound derived from curcumin longa which significantly ameliorates the nicotine induced toxicity. The main disadvantages of curcumin that prevent its application in clinical practice are its poor water solubility, limited bioavailability, poor absorption, and quick elimination from our bodies. Nano-particles of curcumin are therefore proposed as nano-drugs for oral supplementation to increase their bio-availability against nicotine-induced toxicities under protein-restricted conditions.

The aims and objectives of this work are:

- To preparation and characterization of Nanocurcumin for its increased bio-availability and bio-distribution.
- Studies on the ameliorative efficacies of nanocurcumin against various nicotine-induced physicochemical stresses (oxidative, hepatotoxicity, inflammatory, genotoxicity) and in female population under protein restricted condition.
- To examine the anticarcinogenic and antiproliferative properties of nanocurcumin on two different cancer cells.

REFERENCES

1. Sansone L, Milani F, Fabrizi R, Belli M, Cristina M, Zaga V, Lure AD, Cicconi L, Bonassi S,Russo P. Nicotine: From Discovery to Biological Effects. Int J Mol Sci. 2023; 24(19): 14570.

2. Danaei G, Vander Hoorn S, Lopez AD, Murray CJ, Ezzati M, Comparative Risk Assessment collaborating group (Cancers). Causes of cancer in the world: comparative risk assessment of nine behavioural and environmental risk factors. Lancet. 2005; 366: 1784–1793.

3. Sirur S. 27.1% of India's all cancer cases in 2020 will be tobacco-related, ICMR report estimates. ThePrint. 2020.

4. GBD 2019 Tobacco Collaborators. Spatial, temporal, and demographic patterns in prevalence of smoking tobacco use and attributable disease burden in 204 countries and territories, 1990–2019: a systematic analysis from the Global Burden of Disease Study 2019. Lancet. 2021; 397: 2337–2360.

5. Rai B, Bramhankar M. Tobacco use among Indian states: Key findings from the latest demographic health survey 2019–2020. Tob. Prev. Cessation. 2021; 7:19.

6. Young RP, Hopkins RJ Christmas T. COPD prevalence is increased in lung cancer, independent of age, sex and smoking history. Eur Respir J. 2009; 34(2): 380-386.

7. Öberg M, Jaakkola MS, Woodward A, Peruga A, Prüss-Ustün A. Worldwide burden of disease from exposure to second-hand smoke: a retrospective analysis of data from 192 countries. Lancet. 2011; 377(9760):139–146.

8. Brand JS, Chan M-F, Dowsett M, et al. Cigarette smoking and endogenous sex hormones in postmenopausal women. J Clin Endocrinol Metab. 2011; 96(10):3184–92.

9. Dasgupta P,Rizwani W,Pillai S,Kinkade R,Kovacs M,Rastogi S,Banerjee S,Carless M,Kim E,Coppola D,Haura E, Chellappan.Nicotine induces cell proliferation, invasion and epithelial-mesenchymal transition in a variety of human cancer cell lines.Int J Cancer.2009; 124: 36–45.

10. Walser T,Cui X,Yanagawa J, Lee JM, Heinrich E, Lee G, Sharma S, Dubinett SM. Smoking and Lung Cancer. Proc Am Thorac Soc. 2008; 1; 5(8):811–815.

11. He Z, Xu Y, Rao Z, Zhang Z, Zhou J, Zhou T, Wang H.The role of α7-nAChR-mediated PI3K/AKT pathway in lung cancer induced by nicotine. Sci Total Environ. 2024; 912: 169604

12. Kyriakis JM, Avrich J. Mammalian mapk signal transduction pathways activated by stress and inflammation: a 10-year update. Physiol. Rev. 2012; 92 (2): 689–737.

13. Qiu F, Liang CL, Liu H , Zeng YQ, Hou S, Huang S, Lai X, Dai Z. Impacts of cigarette smoking on immune responsiveness: Up and down or upside down? Oncotarget. 2016; 25:8(1):268–284.

14. Benowitz NL, Burbank AD. Cardiovascular Toxicity of Nicotine: Implications for Electronic Cigarette Use. Trends Cardiovasc Med. 2016; 26(6):515–523.

15. Sopori M. Effects of cigarette smoke on the immune system. Nat Rev Immunol. 2002; 2(5):372-7.

16. Dahdah A, Jaggers RM, Sreejit G, Johnson J, Kanuri B, Murphy AJ, Nagareddy PR.Immunological Insights into Cigarette Smoking-Induced Cardiovascular Disease Risk.Cells. 2022; 11(20): 3190.

17. Barr J, Sharma CS, Sarkar S, Wise k, Dong L, Periyakaruppan A, Ramesh GT. Nicotine induces oxidative stress and activates nuclear transcription factor kappa B in rat mesencephalic cells. Mol Cell Biochem. 2006; 297(1-2):93–99.

18. Aycicek A, Varma M, Ahmet K, Abdurrahim K, Erel O. Maternal active or passive smoking causes oxidative stress in placental tissue. Eur J Pediatr. 2011; 170(5):645–651.

19. Sen S, Sharma A. Inhibition of clastogenic effects of nicotine by chrorophyllin in mice bone marrow cells in vivo. Phytother Res. 1991; 6:130–133.

20. Musa TH, Akintunde TY, Musa HH Ghimire U, Gatasi G, Malnutrition Research Output: A Bibliometric Analysis for articles Index in Web of Science between 1900 and 2020. Electron. J. Gen. Med. 2021; 18(3): 2516-3507.

21. Panahi, Y,Hosseini, MS, Khalili N, Naimi E, Majeed M, Sahebkar A. Antioxidant and anti-inflammatory effects of curcuminoid-piperine combination in subjects with metabolic syndrome: Arandomized controlled trial and an updated meta-analysis. Clin. Nutr.2015; 34: 1101−1108.

22. Yue GG, Chan BC. Hon PM, Lee MY, Fung KP, Leung PC, Lau CB. Evaluation of in vitro anti-proliferative and immunomodulatory activities of compounds isolated from Curcuma longa. Food Chem. Toxicol. 2010; 48:2011−2020.

23. Paul S, Sa G. Curcumin as an Adjuvant to Cancer Immunotherapy. Front Oncol.2021; 16:11-675923.

24. Patra JK, Das G, Fraceto, LF, Campos EVR, Rodriguez-Torres MdP, Acosta-Torres LS,Diaz-Torres LA. Grillo R, Swamy MK,Sharma S,Habtemariam S, and Shin HS. Nano-based drug delivery systems: recent developments and future prospects. J. Nanobiotechnology.2018; 16(1): 71.

25. Taniyama Y, Griendling KK. Reactive oxygen species in the vasculature. Hypertension. 2003; 42:1075–1081.

CHAPTER II

REVIEW & LITERATURE

2. Literature Review

Nicotine is the principle harmful ingredient in tobacco products which is a parasympathetic alkaloid seen in high amounts mainly in tobacco plant leaves and in slightly in other plants like the Solanaceae family [1]. The most widespread usage of tobacco in the world is cigarette smoking. There is no degree of tobacco exposure that is safe, and all kinds of tobacco use are dangerous. Cigars, Waterpipe tobacco, cigarillos, pipe tobacco, heated tobacco, roll-your-own tobacco, bidis, kreteks, and smokeless tobacco products are other examples of tobacco products. Tobacco-derived nicotine is the second most commonly used substance worldwide, behind caffeine from tea and coffee. Almost all faiths, all nations, and all civilizations frequently use nicotine [2]. With more than a billion smokers globally, tobacco is currently the second most widely used psychoactive drug. Smokers who consume cigarettes or cigars develop a nicotine addiction. More than 4,000 distinct chemicals make up the complex chemical combination that is tobacco smoke. More than fifty of them are known to be mutagens, cocarcinogens, or carcinogens [3]. Nicotine usage increases vulnerability to a number of infectious diseases and causes terrible health issues including heart disease, cancer, lung illness, cardiovascular disease. Almost every organ in the body is harmed by smoking. The International Classification of Diseases, Tenth Revision (ICD-10) refers to this addiction as tobacco dependence, whereas the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, refers to it as tobacco use disorder. Besides nicotine, additional components found in cigarettes, like non-nicotine compounds and flavorings, can stimulate the addictive effect of tobacco [4]. Today, tobacco smoking is the world's greatest cause of death. Of those mortality rate, nearly 7 million are directly linked to tobacco use, and over 1.3 million are related to secondhand smoke exposure for nonsmokers [5]. Approximately 80% of the 1.3 billion tobacco users worldwide stay in low-and middle-income nations, which bear the

brunt of the cost of tobacco-related death and morbidity [6]. In 2020 nearly 23% of the world's population used tobacco: 36.7% of which are men and 7.8% are women. The Global Adult Tobacco Survey India, 2016–17, found that about 267 million adults in India who are 15 years of age or older use tobacco, accounting for 29% of all adults. According to WHO data, the overall economic expenditures of tobacco use-related illnesses in India for those 35 and older in 2017–18 came to INR 177 341 crore (USD 27.5 billion) [6].

2.1. Over View of Nicotine

2.1.1. History of Nicotine

Nicotine is a naturally occurring chemical substance found in the leaves of plants belonging to the genus Nicotiana, which includes over 60 species and is often distinguished by its large leaves and tubular flowers. The main alkaloid found in tobacco is nicotine, an organic substance. Nicotine occurs throughout the tobacco plant and particularly in the leaves. By weight, the chemical makes up around 5% of the plant. Tobacco was first grown in America's temperate regions. The native populations chewed and smoked the leaves, and they spread the plant throughout the Americas. Tobacco had spread over the continent and neighboring islands by the time Columbus landed on San Salvador in 1492, and the leaves were being traded. As Spanish, Portuguese, and eventually English sailors began using tobacco in ports in the 1500s [7].

2.1.2. The Tobacco Timeline

The tobacco plant (Nicotiana tabacum) and its related compound are named after Jean Nicot, a French diplomat to Portugal, who carried tobacco seeds to Paris in 1550. John Rolfe started growing tobacco in Jamestown, the first prosperous English settlement in the modern-day United

States, in 1612. Tobacco quickly became the main cash crop and influenced the colonial and early American currencies. James Bonsack invented the cigarette-rolling machine in 1880, which led to the widespread use of commercial cigarettes. Billions of cigarettes and cigars were sold annually at the turn of the century. The public's opinion of tobacco and nicotine underwent a dramatic change in the middle of the 20th century. In 1964, the U.S. Surgeon General's report highlighted the health risks associated with smoking, including lung cancer and heart disease. Nicotine was later shown to be the addictive ingredient in tobacco. The latter half of the 20th century saw increased regulation of tobacco products, including advertising restrictions and public smoking bans. Research on nicotine addiction and its negative health implications also increased. About 21% of adults in the USA smoked in 2006, while smoking rates have usually decreased in industrialized nations [8]. The early 21st century introduced new nicotine delivery systems, such as electronic cigarettes (e-cigarettes) and vaping devices. These products have been marketed as alternatives to traditional tobacco products, but their health impacts are still being studied.

2.1.3. Nicotine Intake

India stands second in tobacco production in the world. Smoking tobacco products included Cigarettes, cigar, Hookah, Chillum, Beedi, ganja, Cohutta and Mava etc. Numerous forms of smokeless tobacco like khaini, betel quid, snuff, gutkha mishri are used in different parts of the world. There are many ways to consume tobacco such as chewing, hooking, cigar and sniffing.

2.1.3.1. Smoked Tobacco Products

Cigarettes: Cigarettes a French word for "small cigar", are a product used up through smoking and made out of cured, finely cut tobacco leaves and reconstituted tobacco, often mixed with other flavors and then rolled or stuffed into a paper-wrapped cylinder [9]. Often additional additives are

added for flavor and burn control. When smoked, the tobacco burns and produces a smoke that is inhaled into the lungs. Cigarette smoke leads to various diseases like cancer, pulmonary disease, cardiovascular disease, and various health problem that affect every organ of the body [10].

Cigar: Cigars are firmly rolled bundles of dried out and fermented preserved tobacco leaves which are burned so as to smoke may be drawn into the mouth of the smoker. Generally, cigars are not inhaled for the reason of the high alkalinity of smoke, which can quickly turn out to be irritating to the lungs and trachea. Cigars contain many cancer-causing chemicals besides nicotine and these are harmful not only for smokers but also for nonsmokers. Smoking cigars can lead to nicotine addiction and can cause cancers of the larynx (voice box), mouth, lung, esophagus, and pancreas. Heavy cigar smoking can also surge the risk of heart and lung diseases, like chronic bronchitis and emphysema [11].

Hookahs (Water Pipes): Hookah are generally glass-based single or multi-stemmed water pipe for smoking. Hookah originated from India, was a symbol of honor and pride for kings, landlords, and other high-class people. These days, hookah has gained huge popularity, particularly in the Middle East. Hookah use has been there for a long time, emerging in the North Western provinces of India, spreading to Arab world, Iran and Turkey and nowadays gaining acceptance in the Europe and USA [12]. Hookah consumers are at risks of lung disease, infections, cancers, and other medical illnesses. Long-term effects comprise impaired pulmonary disease, pulmonary function, chronic obstructive, gastric cancer and esophageal cancer [13].

Snus: The origin of snus is Sweden and other Scandinavian countries and it is one type of smokeless tobacco product. Snus is not like moist snuff or chewing tobacco because it is not made

from fire-cured tobacco leaves, it is made from steam-cured tobacco leaves. It develops a lower rate of cancer and other tobacco-related disease than cigarettes [14].

Chewing Tobacco: Chewing tobacco is a smokeless form of tobacco that involves chewing the leaves to release nicotine. It is available in various forms, including loose leaf, plug, and twist. It is carcinogenic and responsible for oral cancer [15].

Bidi: These are handmade; sun-cured tobacco which are further roll in non-permeable tendu leaves. Because of the non-permeable nature of tendu leaf, beedi transfers more nicotine into the body compared to cigarettes. It typically has a mixture of cultivars to make it ignite for a longer period. It accounts for about 40% of the tobacco intake and generally a rurally consumed tobacco variety. Bidis are far more injurious compared to other tobacco-containing products such as cigarettes as it is in raw form. It alone produces three times more carbon monoxide and five times more tar than the cigarettes and cigarettes. Studies indicate that bidi smokers have a higher risk of developing oral, esophageal, and lung cancers compared to non-smokers [16].

Vaping Products: These include electronic cigarettes (e-cigarettes) and vaping devices that vaporize liquid solutions containing nicotine, flavorings, and other chemicals. While marketed as a less harmful alternative to smoking, vaping is still linked to health risks [17].

Snuff: Snuff is finely ground tobacco that can be inhaled (nasal snuff) or placed in the mouth (oral snuff). It provides a smokeless alternative but still poses significant health risks [18].

Kretek: Kretek, also known as clove cigarettes, are a popular form of tobacco product originating from Indonesia. They are made from a blend of tobacco and crushed cloves, often infused with additional flavorings. Kretek are widely consumed in Indonesia and have gained popularity in

other countries as well. Kretek typically contains a mix of tobacco, cloves, and various flavorings. The clove content is what distinguishes them from regular cigarettes and gives them a distinct aroma and taste. Kretek smoking can lead to chronic respiratory issues, such as chronic obstructive pulmonary disease (COPD) and asthma. The combustion of cloves can also produce additional harmful substances [19]. The use of kretek is linked to oral health issues, including gum disease and oral cancer, due to the carcinogenic compounds present [20].

Dissolvable Tobacco: Dissolvable tobacco products, which include items like orbs, strips, and lozenges that dissolve in the mouth, are often marketed as alternatives to traditional tobacco products. They are designed to provide a discreet way to consume nicotine without smoking [21]. Dissolvable tobacco products contain carcinogenic substances, which can increase the risk of oral cancers, including cancers of the mouth, throat, and esophagus [22]. These products can lead to gum disease, tooth decay, and other oral health problems. Users may also develop conditions such as leukoplakia, which are precancerous lesions in the mouth [23].

2.1.3.2. Smokeless Tobacco Products

India is the fifth largest exporter of smokeless tobacco and numerous tobacco products. Smokeless tobacco contains gutkha, betel quid or pan, mishri, loose tobacco, zarda, khaini and chewed loose tobacco. 60% of the Indian population consumes smokeless tobacco products. Commonly among both men and women living in rural areas having low income. Gutkha is vastly promoted among smokeless tobacco derivatives. Roasted or raw, and packed forms of numerous number of smokeless tobacco are easily available. Smokeless tobacco comprises of toxic chemicals and carcinogens which responsible for causing cancer. These products also cause many

reproductive problems such as maternal anaemia, premature birth, placental development and low birth weight.

Masheri: Masheri or otherwise known as mishri is an alternative for cleaning teeth and is prepared by burning or partially roasting tobacco powdered. High sugar content in masheri can cause tooth decay and cavities, because of bacteria in the mouth feed on sugar, producing acids which harm tooth enamel [24]. Regular consumption of sugary products like masheri can contribute to weight gain and obesity, particularly if not balanced with physical activity [25]. High sugar intake is associated with an increased risk of gum disease, as sugar can lead to plaque buildup, which affects gum health [26].

Mava: Mava typically contains a blend of tobacco, slaked lime (calcium hydroxide), and sometimes additional flavoring agents or sweeteners. Its appearance resembles gutka but mava is white in colour. It is chewed or held in the mouth, allowing nicotine to be absorbed through the oral mucosa. Use of mava is linked to an increased risk of various cancers, particularly oral, esophageal, and pancreatic cancers, due to the carcinogenic substances present [27]. Regular use can lead to severe oral health problems, including gum disease, tooth decay, and lesions in the mouth [28].

Paan along with tobacco: Most frequently used in rural part of India where they coat betel leaf with areca nuts, lime, sweetening agents, spices condiments and tobacco. We also known this alternately as betel quid. It's a form of chewing tobacco and it causes cancer. Paan use is common in South Asian communities. The combination of areca nut and tobacco significantly increases the risk of oral cancers, including cancers of the mouth, throat, and esophagus. Regular use can lead to gum disease, tooth decay, and bad breath. Staining of teeth is also common [29].

Khaini: It is more like mava without having areca nut. It is mainly a combination of sun-dried tobacco, slaked lime, flavored with cardamom, methanol or some other flavoring. It is also chewed like mava.

Gutka: Ghutka, a chewable form of tobacco mixed with areca nut and other flavorings, poses significant health risks. The effects of ghutka on oral health, linking it to conditions such as leukoplakia and oral squamous cell carcinoma. The use of gutkha has been shown to have genotoxic and clastogenic properties [30].

The widespread usage of smokeless tobacco in diverse forms leads to oral sub mucous fibrosis (OSMF) that possibly converts into a malignant state in all age groups. The carcinogenic effects of smokeless tobacco in oral cavity are because of the formation of ROS and free radicals. Low cost, pleasant taste and easy availability are the reasons for the extensive use of these tobacco derivatives by adolescents, and even children. The use of these smokeless tobacco products precisely among people having lower socioeconomic, are increasing oral cancer rates.

2.1.4. Physical & Chemical Data of Nicotine

Nicotine is a chiral alkaloid that accounts for 2-8% of the dry mass of tobacco leaves and more than 90% of the tobacco alkaloid content [31].

Chemical formula: Nicotine has a molecular formula of $C_{10}H_{14}N_2$. IUPAC name: 3-[2-(N-methyl pyrrolidinyl)]pyridine (Figure 2.1). Appearance: colourless, oily, hygroscopic liquid, with distinctive odour, turns brown on exposure to air. Solubility in water: miscible. Boiling point (decomposes): 247 °C. Vapour pressure at 20 °C: 0.006 kPa, Density: 1.01 g cm^{-3}. Octanol/water

partition coefficient as log Pow: 1.2. pK_a = 8.5, Nicotine is a weak base and turns brown through oxidation processes while exposed to air and light.

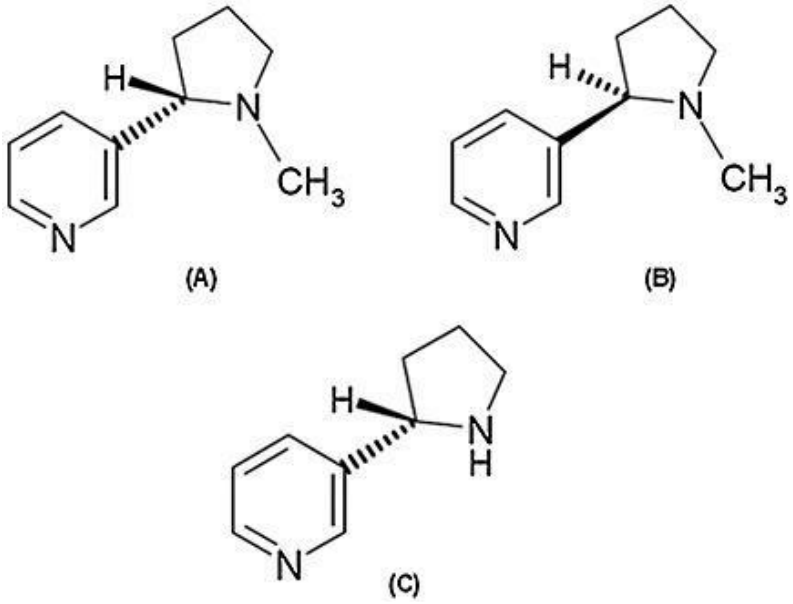


Figure 2.1: The Chemical structure of (A). (S)- (−)-nicotine, (B). (R)- (+)-nicotine (C). (S) (−)-nornicotine

The majority of total nicotine found in tobacco exists in the S(−) form. A racemic R(+) form occurs upon pyrolysis [32]. The S(−) form of nicotine readily binds to nicotine receptors, whereas the R(+) form is a weaker agonist. The pyrrolidine nitrogen ionize at pH 7.4 (37°C), whereas the pyridine nitrogen remains unionized, forming a compound which exists in two different states, protonated and non-protonated hydrophilic form. Connelly, Willits, Brice and Swaim were the first to designate the ultraviolet spectrophotometric determination of nicotine from tobacco, tobacco extracts and distillates. Since that time, researchers have created a multitude techniques for the analysis of nicotine. Due to its comparative thermal stability and volatility, nicotine can be effectively analyzed across various matrices using gas chromatography (GC) techniques. Liquid chromatography coupled with mass spectrometry (MS) and various

immunoassay methods, such as radioimmunoassay and enzyme-linked immunosorbent assay, are also employed for the detection of nicotine and its metabolites. [33].

2.1.5. Toxicokinetics

2.1.5.1. Absorption of Nicotine

Nicotine mainly absorbed through the Skin, urinary bladder, oral cavity, lung, gastrointestinal system and mouth. pH affects nicotine's ability to pass through cellular membranes. Nicotine does not quickly pass through membranes when it is ionized, as it is in acidic conditions. Between 60% - 80% of nicotine is absorbed by the respiratory system. The large surface area of the alveoli and the breakdown of nicotine at physiological pH (about 7.4), promotes transfer across cell membranes, allow nicotine from cigarette smoke to be rapidly absorbed into the lung. The absorption process via the alveoli is influenced by the concentration of nicotine present in the smoke. Nicotine is effectively absorbed in the small intestine, which has a larger surface area and a higher alkaline pH, but it is poorly absorbed from the stomach because of the acidity of the gastric fluid [34].

2.1.5.2. Metabolism of Nicotine

Nicotine is a potent psychoactive neurotoxin that functions as both a stimulant and a sedative, with its absorption and metabolism occurring in the liver, but some transformation also occurs in the lung and kidneys. Through a two-step process, around 70-80% of nicotine is converted to cotinine by C-oxidation. The initial phase, catalyzed by the cytochrome P450 2A6 enzyme, generates the nicotine-iminium ion through the hydroxylation of nicotine, resulting in the formation of 5-hydroxynicotine. The subsequent step is facilitated by aldehyde oxidase, resulting

in the formation of cotinine [35]. In animals, the di-astronomers, the 1′-(R)-2′-(S)-cis and 1′-(S)-2′-(S)-trans-isomers, are formed when 4–7% of nicotine is converted to another metabolite, Nicotine N′-oxide [36], through the action of a flavin-containing monooxygenase 3 (FMO_3) [37]. Approximately 3–5% of nicotine undergoes transformation through glucuronidation, a process catalyzed by uridine diphosphate-glucuronosyltransferase, resulting in the formation of nicotine glucuronide [38]. A minor proportion of nicotine (0.4–0.8%) is also transformed into nornicotine, a process believed to be facilitated by the cytochrome P450 system [39]. Alternative routes of nicotine metabolism include the production of nornicotine, trans-3-hydroxy-cotinine, demethylcotinine, and δ-(3-pyridyl)-γ-methylaminobutyric acid, as well as the processes of N-oxidation and N-methylation of nicotine. The phase II metabolic process encompasses the N- and O-glucuronidation of nicotine along with its metabolites.

2.1.5.3. Distribution of Nicotine in Body Tissues

Approximately 69% of nicotine remains in its ionized state and 31% in its nonionized form after entering the circulation (pH 7.4), while it has a modest affinity for plasma proteins (5%) [40]. Due to its lipophilic nature of nicotine quickly crosses the blood-brain barrier. Nicotine and cotinine concentrations in skeletal muscle are comparable to those in whole blood. Smokers have a higher receptor binding capacity than nonsmokers due to nicotine's strong affinity for brain tissues [41-43]. Within minutes, nicotine concentrations in the brain can exceed those in plasma, contributing to its psychoactive effects. Liver and Kidneys accumulate nicotine and play crucial roles in its metabolism and excretion. The liver is where nicotine undergoes substantial biotransformation. Being highly lipophilic nature of nicotine, leading to significant accumulation in adipose tissue. This can result in a reservoir effect, where nicotine is released slowly into the bloodstream, prolonging its effects and potentially increasing the risk of dependence. Nicotine

accumulates markedly in gastric juice and saliva [44]. The ratios of gastric juice/plasma and saliva/plasma concentrations are 61 and 11, respectively, when nicotine is administered transdermally, while these ratios are 53 and 87, respectively, in the case of smoking. Nicotine accumulation occurs due to ion-trapping in gastric juice and saliva. Additionally, nicotine is found to concentrate in breast milk, with a milk/plasma ratio of 2.9 [45]. Nicotine readily penetrates the placental barrier, and studies indicate that it accumulates in fetal serum and amniotic fluid at slightly higher levels than in maternal serum [46]. The rate and method of nicotine administration significantly influence the timeline of its accumulation in the brain and other organs, as well as the associated pharmacological effects. Inhaling a cigarette allows nicotine to swiftly enter the pulmonary venous circulation, subsequently traveling rapidly to the left ventricle of the heart, and then into the systemic arterial circulation and the brain. The interval between inhaling a cigarette and the arrival of nicotine in the brain is approximately 10 to 20 seconds. The delivery of nicotine to the brain occurs swiftly; however, there is considerable uptake in the lungs and a delayed release of nicotine. This is supported by pulmonary positron emission tomography data and the gradual decline in arterial nicotine concentrations observed between puffs [47]. Nicotine levels in arterial blood following cigarette smoking can be significantly elevated, potentially reaching as high as 100 ng/ml, although they typically fall within the range of 20 to 60 ng/ml [47-50]. The analysis of tissue absorption, conducted on rabbit tissues by assessing nicotine concentrations following a 24-hrs continuous intravenous infusion, revealed that the spleen, liver, lungs, and brain exhibit a strong affinity for nicotine, while adipose tissue demonstrates a comparatively lower affinity. Nicotine easily penetrates the placenta, resulting in foetuses of smoking mothers being exposed to nicotine levels that are higher than those found in their mothers.

2.1.5.4. Nicotine Excretion

Nicotine has been shown to be eliminated by perspiration, breast fluid, bile, saliva, gastric juice, urine, and feces [51-53]. It has been demonstrated that around 55% of the radioactivity released by an animal when fed 14C-nicotine is eliminated in the urine. Nevertheless, just 1% of the radiation was detected as unaltered nicotine. This outcome shows that nicotine is eliminated after undergoing a thorough metabolism. In human, nicotine has a half-life of 2 hr to 3 hr and disappeared quickly from the body. Infants whose moms smoke also have nicotine and cotinine found in their urine, suggesting that the newborn is exposed to tobacco smoke through the mother [54]. In a different study, researchers found that those with higher nicotine excretion had a daily nicotine intake that was 18% higher and concluded that the rate of elimination of nicotine affects the rate of consumption [55]. The pH of the urine has an impact on the rate of nicotine excretion as well. When the urine's pH is raised to an alkaline level, more uncharged nicotine is present, nicotine is reabsorbed, and less nicotine is expelled [56].

2.1.6. Neuropharmacology

Nicotine is a tertiary amine that comprises both a pyridine ring and a pyrrolidine ring. The (S)-nicotine variant, which is present in tobacco, exhibits stereoselective binding to nicotinic cholinergic receptors (nAChRs). (R)-nicotine, present in minimal amounts in cigarette smoke due to racemization occurring during pyrolysis, acts as a weak agonist at nicotinic acetylcholine receptors (nAChRs). Cigarette smoke extracts nicotine from tobacco and carries it through smoke particles to the lungs, where it is rapidly absorbed into the pulmonary venous system. After then, it swiftly travels to the brain via the arterial circulation. Nicotine easily penetrates brain tissue and attaches itself to ligand-gated ion channels called nAChRs. The channel opens when a cholinergic

agonist attaches to its outside, letting cations like calcium and salt in. Further calcium entry is made possible by these cations' activation of voltage-dependent calcium channels. Both the peripheral and central nervous systems include the five subunits that make up the nAChR complex [57]. There are three β subunits (β2 to β4) and up to nine α subunits (α2 to α10) in the mammalian brain. In human brains, the most prevalent receptor subtypes are α4β2, α3β4, and α7 (homomeric). The α4β2∗ (possible presence of other subunits too in the receptor) receptor subtype is the most prevalent in the human brain and is thought to play a key role in the mediation of nicotine dependence. When the β2 subunit gene is knocked out in mice, nicotine's behavioral effects are eliminated, preventing it from releasing dopamine in the brain or sustaining self-administration [58]. β2 deletion mouse's behavioral responses to nicotine are restored when the β2 subunit gene is reinserted into the ventral tegmental region. It seems that the α4 subunit plays a significant role in determining susceptibility to nicotine [59]. In mice, a single nucleotide point mutation within the pore-forming region leads to the development of a receptor that exhibits heightened sensitivity to nicotine [60]. This mutation significantly increases the sensitivity of mice to reward behaviors induced by nicotine, along with its impacts on tolerance and sensitization. It is thought that the α3β4 nAChR plays a crucial role in mediating the cardiovascular effects associated with nicotine [61]. The homomeric α7 nicotinic acetylcholine receptor (nAChR) is believed to contribute to fast synaptic transmission and may be significant in processes related to learning [62] and sensory gating. [63]. The α5, α6, and/or β3 subunits that may be present in the α4 β2* receptor may alter the receptor's sensitivity and functionality. α5 knockout mice, for instance, are less susceptible to hypolocomotion and seizures brought on by nicotine [64].

2.1.7. Neurocircuitry of Nicotine Addiction

Depending on the dosage and related variables like inter-individual sensitivity and tolerance, nicotine exhibited both positive effects (buzz or "high") and negative effects (dizziness and nausea) [65]. The combination of disparate impulses from several brain areas that result in reward and aversion is how nicotine's addictive qualities are displayed. Among the first smokers' both the unpleasant and reinforcing effects of nicotine combined to produce a comparable manifestation of long-term usage. Nicotine causes pharmacodynamic alterations in brain circuits when people become habitual smokers. As a result, the unpleasant effects of the medicine are less sensitive. Nicotine is a potent reinforcer that causes conditional secondary effects associated with past drug use. Nicotine withdrawal symptoms, such as irritability, agitation, memory loss, absent-mindedness, and anxiety, occur when such people go through an abstinence period. Such unfavorable emotional feelings boost the desire to take nicotine again. The mesolimbic pathways, which are composed of dopaminergic nerve cells located in the prefrontal cortex and ventral tegmental area (VTA), are responsible for the pleasant effects of nicotine [66]. VTA expresses several kinds of nAChR on glutamatergic, dopaminergic, and GABAergic neurons [67,68]. Administration of nicotine can increase dopamine levels by activating dopaminergic neurons. Nicotine's activity on α4 and β2-riched nAChRs is primarily responsible for this rewarding effect [69]. Conversely, the negative effects of nicotine reduce drug use and ease withdrawal symptoms related to the projection of the fasciculus retroflexus. Numerous facets of nicotine dependency have also been linked to other parts of the brain, including the insula, ventral pallidum (VP), nucleus tractus solitaries (NTS), and prefrontal cortex (PFC). These brain areas regulate drug-taking and drug-seeking behaviors and are either directly or indirectly related. A number of somatic symptoms, including as scratching, shaking, and head nods, as well as emotional

indicators, such as increased anxiety-related behaviors, may be used to quantify nicotine withdrawal in animal models [70, 71].

2.1.8. Psychoactive Effects of Nicotine and Nicotine Withdrawal

Nicotine from tobacco lowers tension and anxiety while stimulating and enhancing pleasure in humans. Smoking may enhance one's ability to focus, respond quickly, and complete certain activities. Smokers utilize nicotine to regulate their degree of arousal and mood regulation in everyday life. Symptoms of nicotine withdrawal appear when a person quits smoking. These include irritation, depression, restlessness, anxiety, issues with relationships, difficulties focusing, increased appetite and eating, sleeplessness, and nicotine cravings [72]. Nicotine withdrawal in untreated smokers causes mood changes equivalent to those reported in psychiatric outpatients [73]. Withdrawal from nicotine and other addictive substances can cause hedonic dysregulation, which is the perception that life is not very enjoyable and that once-pleasurable activities are no longer appealing [74]. It is proposed that a relative lack of dopamine release resulting from prolonged nicotine exposure contributes to various mood disorders and anhedonia, along with the persistent tobacco cravings that many smokers experience long after they have ceased smoking. The pharmacological foundations of nicotine addiction can be understood as a blend of positive reinforcements, including enhanced mood or performance, alongside the desire to evade the adverse effects associated with previous drug use, specifically the alleviation of withdrawal symptoms in circumstances where nicotine is inaccessible. In addition to these direct pharmacological processes, conditioning plays a major role in the development of nicotine addiction.

2.1.9. Genetics Impact of Nicotine

According to Hopkins and Evans and Jones et al., [75 76], smoking is a powerful inducer of genetic damage in both human and rodent cells and nicotine enhance the production of aneuploidy [77] polyploidy, and DNA strand breaks in human spermatozoa [77,78]. In human gingival fibroblasts, nicotine promotes the development of micronuclei which is a sign of DNA damage [79] Nicotine induced DNA damage was evaluated using the comet test in human neuroblastoma cells [80], human leucocytes [81], human buccal cells [82], and in vitro culture of peripheral rat blood [83]. The comet assay is a common technique for assessing genotoxicity [84]. Nicotine increases oxidative damage and inflammation by activating the NF-kB pathway, which might exacerbate DNA damage and raise the risk of mutagenesis [85]. Nicotine promotes oxidative stress, which can cause DNA damage via reactive oxygen species (ROS). This oxidative stress is related to DNA strand breakage and base alterations, increasing the chance of mutations and cancer formation [86]. According to Huang et al. female smokers had higher levels of DNA damage indicators, such as 8-OHdG, than male smokers, which may indicate that females are more susceptible to oxidative stress as a result of tobacco use [87].

2.1.10. Negative Effect of Nicotine

The addictive substance in tobacco is nicotine which has negative health effects in adults include cancer in almost every peripheral organ exposed to tobacco smoke, as well as long-term conditions like diabetes mellitus, rheumatoid arthritis, cardiovascular disease, stroke, eye disease, periodontal disease, and immune system disorders. The harmful impact of nicotine and tobacco smoke on the central nervous system (CNS) is attributed to the neurotoxic influence of nicotine on the permeability of the blood-brain barrier (BBB), the expression of nicotinic acetylcholine

receptors, and the functioning of the dopaminergic system. Nicotine affects the central nervous system by attaching to nicotinic acetylcholine receptors, causing the release of dopamine, which adds to its addictive properties. This neuroadaptive alteration may eventually impact cognitive performance and raise the likelihood of mental health issues [88]. Nicotine raises blood pressure and heart rate via boosting the production of catecholamines (such as adrenaline), which can cause hypertension and increase the risk of cardiovascular disease and Chronic exposure can cause endothelial dysfunction and increased atherosclerosis, which are major risk factors for heart attacks and strokes [89]. Smoking during pregnancy accounts for 20% of low birth weight in newborns, 8% of preterm births, and 5% of all perinatal fatalities. Maternal smoking is associated with reduced fetal development and premature delivery, miscarriage owing to nicotine's vasoconstrictive effects on uterine blood flow Tobacco poisoning reduces macrophage phagocytic capability, modifies mucous membrane immunoglobin levels, and disrupts local infection control systems. Babies born to smoking moms had considerably lower birth weights and were more likely to be born preterm than those born to nonsmoking mothers [90]. Nicotine is quickly absorbed into the circulation and can pass across the blood-brain barrier within 10-20 seconds. This rapid transit is helped by its high lipophilicity, which allows it to easily permeate cell membranes and the blood-brain barrier [91]. In rats, nicotine exposure reduces hemoglobin concentration and red blood cell count, which reduces the blood's ability to transport oxygen and the oxidative characteristics of nicotine can damage erythrocyte membranes and cause early cell death [92, 93].

2.1.11. Nicotine Negatively Impacts on Various Organ

Nicotine has detrimental effect on different types of organs such as kidney, liver, and ovaries.

2.1.11.1. Kidney: Nicotine has been proven to exacerbate chronic kidney disease in persons with diabetes and polycystic kidney disease, hypertension and post-kidney transplantation by raising oxidative stress and causing inflammation. Nicotine causes oxidative stress in kidney tissues, resulting in elevated amounts of reactive oxygen species that hampers cellular activity in renal tissues, perhaps contributing to kidney damage and increasing the chance of chronic kidney disease (CKD). Chronic nicotine exposure in rats caused glomerular damage and elevated levels of pro-inflammatory markers that caused higher risk of renal dysfunction and the advancement of chronic kidney disease [94]. Nicotine binds to non-neural nAChRs found in kidney tissue, activating pathways that cause inflammation and fibrosis and nicotine activation of these receptors causes the production of pro-inflammatory cytokines and profibrotic markers, which contribute to kidney damage over time [95]. Nicotine-induced inflammation in renal tissues causes fibrosis and scarring in the kidneys which can cause long-term structural damage that reduces kidney filtration capacity [92]. Nicotine induces vasoconstriction, reducing blood flow to the kidneys and glomerular filtration rates (GFR) which is a marker for healthy kidney function and potentially impairing renal function over time [96]. Nicotine exposure lowers serum protein content by causing damage to the glomerular structures that filter proteins, which results in proteinuria, or the loss of proteins in the urine due to impaired kidney function [97].

2.1.11.2. Liver: Nicotine causes hepatic steatosis, cellular damage, and decreased liver enzyme function by increasing the formation of reactive oxygen species in liver cells. The chance of developing nonalcoholic fatty liver disease (NAFLD) and other liver disorders is raised by this damage [98]. In liver cells, nicotine produces reactive oxygen species, which damages cellular components, especially mitochondria. According to Zhang et al. [99] mice exposed to nicotine showed elevated oxidative stress indicators and mitochondrial malfunction in their liver cells,

which aided in tissue damage and liver cell death. Over time, nicotine exacerbated inflammation and contributed to liver damage by upregulating pro-inflammatory cytokines including TNF-α and IL-6 in liver tissues [100]. Long-term nicotine use caused lipid accumulation in liver cells, interfering with normal liver function and perhaps accelerating the development of more serious liver illnesses such as nonalcoholic fatty liver disease (NAFLD). According to research, smoking increases the likelihood of liver fibrosis, higher histological activity index scores, and the development of cirrhosis in individuals with chronic hepatitis C [101]. Smokers who are exposed to nicotine have higher amounts of carboxyhemoglobin, which reduces the blood's capacity to transport oxygen by competing with oxygen for hemoglobin binding sites. Chronic nicotine consumption dramatically raises carboxyhemoglobin levels, which causes the body to manufacture more red blood cells in reaction to perceived hypoxia, resulting in secondary polycythemia [102]. Blood viscosity rises as a result of the compensatory increase in red blood cells, potentially putting stress on the heart. This impact is linked to increased risks of blood clots, hypertension, and other cardiovascular problems in persistent smokers [103]. Nicotine-induced rat's livers exhibited histopathological alterations such as mononuclear cell infiltration, hyperchromatic nucleus, and expanded sinusoids [104-108]. Nicotine also affects the synthesis of different metabolic enzymes in the liver. Cigarette smoking was notably linked to elevated levels of gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP), while showing an inverse relationship with increased aspartate aminotransferase (AST) [109]. Increased levels of alanine aminotransferase (ALT) following nicotine intake among HCV-positive patients was also been reported [110,111]. An elevated level of alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were reported among smokers with reduced total bilirubin compared to

non-smokers [112-115]. Previous studies demonstrated that nicotine causes a significant reduction of total protein [116,117].

2.1.11.3. Ovary: Nicotine has a negative influence on ovarian function, including fertility, general ovarian health and hormone regulation. Nicotine exhibits anti-estrogenic properties that disrupt the equilibrium of the reproductive and hormonal systems, thereby diminishing the likelihood of successful pregnancy in both healthy women and those receiving assisted reproductive treatments. Tobacco smoke negatively affects folliculogenesis and development by elevating apoptosis or autophagy, causing DNA damage, and disrupting the interactions between oocytes and granulosa cells, all of which contribute to the death of ovarian follicles. Nicotine treatment in rats caused considerable follicular depletion and elevated rates of follicular atresia (degeneration), reducing reproductive capacity and hastening ovarian aging [118]. Nicotine-induced follicular depletion and ovarian aging in rodent models. Hormone production (estrogen and progesterone) and regulation in ovary are disrupted by nicotine that affect the hypothalamic-pituitary-ovarian (HPO) axis, resulting in changed sex hormone levels that may impact fertility and the regularity of menstrual cycles [119]. Previous study showed that Nicotine induced oxidative stress harms ovarian cells, lowering viability and resulting in a depleted ovarian reserve, which can impair fertility and reproductive health [120]. Cigarette smoking and its association with liver enzymes: Increased gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP) levels and an inverse relationship with aspartate aminotransferase (AST) [121]. Nicotine stimulates the sympathetic nervous system, which increases the production and release of adrenaline and norepinephrine. These effects may include weight loss, higher heart rate, elevated blood glucose, and increased activation of the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-thyroid (HPT) axis, which in turn may result in elevated amounts of thyroid hormone and plasma cortisol.

2.1.11.4. Nicotine's Effects on Lipid Profiles and Cardiovascular Disorders

Nicotine has been found to have a wide range of impacts on both multicellular creatures and individual cells. Nicotine, the main component of cigarette smoke, is linked to the development of cardiovascular disease and lung cancer [122,123]. Nicotine is linked to an adverse lipid profile that includes raised triglycerides, reduced HDL (high-density lipoprotein) cholesterol, and elevated LDL (low-density lipoprotein) cholesterol in both human and animal models, which raises the risk of atherosclerosis by causing plaque to develop in arteries [124]. Singh et al. (2016) observed a substantially higher level of VLDL cholesterol [125]. Nicotine causes oxidative stress in blood vessels, which leads to endothelial dysfunction and impairs nitric oxide synthesis, a key element in cardiovascular disease development [126]. It has been shown that the level of lipid peroxidation increases in smokers [127]. Nicotine administration raised free fatty acid concentrations as well as levels of malondialdehyde, hydroperoxides, and conjugated dienes. Nicotine-treated rats' tissues may contain more free fatty acids, which can act as a substrate for lipid peroxidation. It has also been demonstrated that nicotine treatment reduces the activity of free radical scavenging enzymes superoxide dismutase, catalase, and glutathione reductase [128]. Nicotine accelerates lipid accumulation within arterial walls, creating plaque deposits that restrict blood flow, increasing the risk of coronary artery disease and other cardiovascular complications [129]. According to the American Heart Association (AHA, 2023) Smoking continues to be a substantial factor to cardiovascular mortality, accounting for over 30% of all cardiovascular deaths in the United States [130].

2.1.11.5. Nicotine and Cancer

Nicotine, the primary alkaloid found in tobacco plants (Nicotiana), is the key factor in tobacco addiction. This addiction contributes significantly to the alarming rate of smoking-related premature deaths, totaling approximately 435,000 annually in the United States. The majority of these fatalities are attributed to diseases such as cancer, cardiovascular conditions, and respiratory illnesses, all of which are linked to the harmful substances present in tobacco smoke. [131]. Nicotine has an important role in cancer development and progression through mechanisms such as tumor growth stimulation, angiogenesis, apoptosis suppression, and increased metastasis. Tobacco smoke contains toxins that can harm or alter a cell's DNA. DNA is a cell's "instruction manual" that governs its regular growth and function. When DNA is damaged, a cell can grow out of control, resulting in a cancer tumor. Tobacco contains many chemicals, including aromatic amines, polycyclic aromatic hydrocarbons, heterocyclic amines, and Nitroso compounds, which contribute to DNA damage through single and double-strand DNA breaks, bulky adduct formation, production of a basic sites, and base alterations. All tobacco products include a variety of carcinogenic chemicals, including as polycyclic hydrocarbons (PAH) and TSNA, which clearly play an essential part in the development of cancer. The main way that nicotine works is via activating nicotine acetylcholine receptors (nAChRs), which nicotine binds to more strongly than acetylcholine. Additionally, the TSNA compounds NNN (N′-nitrosonornicotine) and NNK (4-(metylnitrosamino)-1-(3-pyridyl)-1-butanone) can be generated from nicotine following oral intake [132]. The significance of nicotine in the process of carcinogenesis is crucial when assessing the potentially harmful impacts of non-tobacco sources of nicotine, including e-cigarettes and nicotine replacement therapies (NRT). Cervical cancer is recognized among women globally and is related with Human papillomavirus (HPV) [133]. Conventional treatments (surgery,

chemotherapy, radiotherapy etc.) are only efficient in the early stages of cervical cancer as a result, it is imperative to find new methods for the treatment of cervical cancer [134]. HPV-16 and HPV-18 are two ‘‘high risk’’ subgroup of HPVs [133] and encode viral proteins E6 and E7 respectively. Cellular tumor suppressor proteins p53 and the retinoblastoma susceptibility gene product Rb can be disrupted by these factors. [135]. HPV infections are typically transient and do not usually involve to clinically considerable lesions of the cervical mucosa apart from persistent infection over a significant period of time [134]. Higher number of HPV infection compared with the small incidence of cervical cancer cofactors like oral contraceptive use, smoking, infection with other sexually transmitted diseases such as Herpes simplex and host factors are involved in the malignant transformation of the cervical mucosa [136].

2.1.11.6. Effect of Nicotine on Antioxidants Level

More than 4,000 compounds included in cigarette smoke have the ability to produce free radicals and are extremely oxidative and carcinogenic [137,138]. Total antioxidant capacity (TAC) represents the total quantity of antioxidants in the body and is a biomarker of antioxidant defense against free radicals. There are studies that support the idea that these free radicals have negative consequences in both smokers and secondhand smokers, inducing oxidative stress [139]. Increase lipid peroxidation and reduce the radical detoxifying enzymes SOD, CAT, and glutathione reductase, while raising GSH levels, which may be linked to the glutathione synthesis feedback mechanism. Nicotine can increase oxidative stress, creating an imbalance between oxidants and antioxidants. This oxidative stress is linked to a variety of conditions, including cardiovascular disease and cancer [140]. The high blood level of LDH is an indicator of acute or chronic cell damage like stroke, blood flow deficiency, heart attack, hepatitis, and tissue death [141]. Catalase (CAT) is another antioxidant enzyme found in all organisms that converts hydrogen peroxide into

water and oxygen. Catalase is produced and localized in H_2O_2-producing cellular settings such as peroxisomes and mitochondria [142,143]. Another major antioxidative enzyme is Superoxide Dismutase (SOD) that catalyzes the transformation of superoxides to oxygen and hydrogen peroxide, therefore playing a critical part in the antioxidant defense mechanism at the cellular level [144]. Glutathione Peroxidase (GXp) is a peroxidase enzyme that protects cells from oxidative damage [143]. GPx converts lipid hydroperoxides to their respective alcohols and hydrogen peroxide to water. $2GSH+H_2O$ yields $GS-GS+2H_2O$ [145]. Nicotine-treated albino rats showed significantly lower levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) [146]. Aspera-Werz et al. and Raddam et al. have noted a similar finding in cigarette smokers [147,148]. On the other hand, tobacco chewers and smokers had higher levels of GPx and lower levels of SOD and Catalase [149]. Delijewski et al. observed that nicotine increased SOD levels and CAT decreased GPx activity in cell culture [150]. Nicotine produces malondialdehyde (MDA) via peroxidation, damages lipids, and releases free radicals. A significantly higher level of MDA (marker of lipid peroxidation) was measured in nicotine-induced rat granulose cells [151]. Similar findings have also been documented in murine plasma [152], rat liver and erythrocytes [153], and human primary endometrial cells [154].

2.1.12. Nicotine Regulates Inflammatory Cytokines, Pro-Apoptotic Indicators, and Female Hormones

Nicotine and other particulate matter can stimulate the immune system, causing the production of pro-inflammatory cytokines such TNF-α and IL-6. Chronic exposure can cause a persistent inflammatory state, which contributes to conditions such as chronic obstructive pulmonary disease (COPD) and cardiovascular disease, as well as an increased risk of several autoimmune diseases (including, systemic lupus erythematosus, rheumatoid arthritis, and multiple

sclerosis) [155-158]. Nicotine causes an inflammatory response in the respiratory tract by activating macrophages and eosinophils, neutrophils, monocytes, and lymphocytes. In terms of apoptosis, nicotine can influence pro-apoptotic indicators such as Bax and caspase. While it may increase survival in some cell types (for example, by activating survival pathways), it may also cause apoptosis in others, particularly under stressful situations. Nicotine stimulates the development of pro-apoptotic proteins while inhibiting the creation of anti-apoptotic proteins, influencing the body's inflammation [159]. The female reproductive system can be significantly impacted by nicotine. In women it can disrupt the normal hormonal balance, affecting levels of progesterone and estrogen, which are essential for regulating the menstrual cycle and ovulation. This hormonal imbalance can result in irregular menstrual cycles and decreased fertility [160].

2.1.12.1. Interleukin-6 (IL-6): The two glycoprotein chains that make up the polypeptide IL-6 are an alpha chain with a molecular weight of 80 kDa and a beta chain with a molecular weight of 130 kDa (Figure 2.2). By lacking the intracellular region, the α-chain can only attach to IL-6 with low affinity and complex molecule then instantly binds to the high-affinity β-chain and uses the β-chain to send information to the cell [161]. Figure 1 illustrates the up-up-down-down structure of the four lengthy alpha-helical chains that make up IL-6. IL-6 is involved in numerous biological processes, including inflammation and immune regulation. Unbalanced immune regulation of the IL-6/IL-6 receptor axis can result in a number of inflammatory disorders, including chronic hepatitis and rheumatoid arthritis. It can trigger the downstream classical signaling pathways, including the JAK/STAT3 and PI3K/Akt signaling pathways, after it has specifically bound to sIL-6R. The process can subsequently activate T cells, leading to the release of inflammatory substances by immune cells such as neutrophils, fibroblasts, and macrophages, which in turn facilitates the development of inflammation [162]. Inflammatory responses, immune cell

activation and regulation, and information transmission are all mediated by these interleukins, chemokines, and other inflammatory-related cytokines, all of which are strongly correlated with tobacco smoke use. Previous research has highlighted the connection between tobacco consumption and various health issues. For instance, smoking is recognized for its effects on the activation and levels of specific white blood cells, including leukocytes, which are associated with increased concentrations of inflammatory markers such as IL-6 and C-reactive protein (CRP). Nicotine affects the synthesis of IL6. Through AP-1 and STAT-3-activated pathways, nicotine unregulates the production of IL-6 in human endothelial cells [163]. Current smokers have higher levels of IL-6 than non-smokers [164-166].

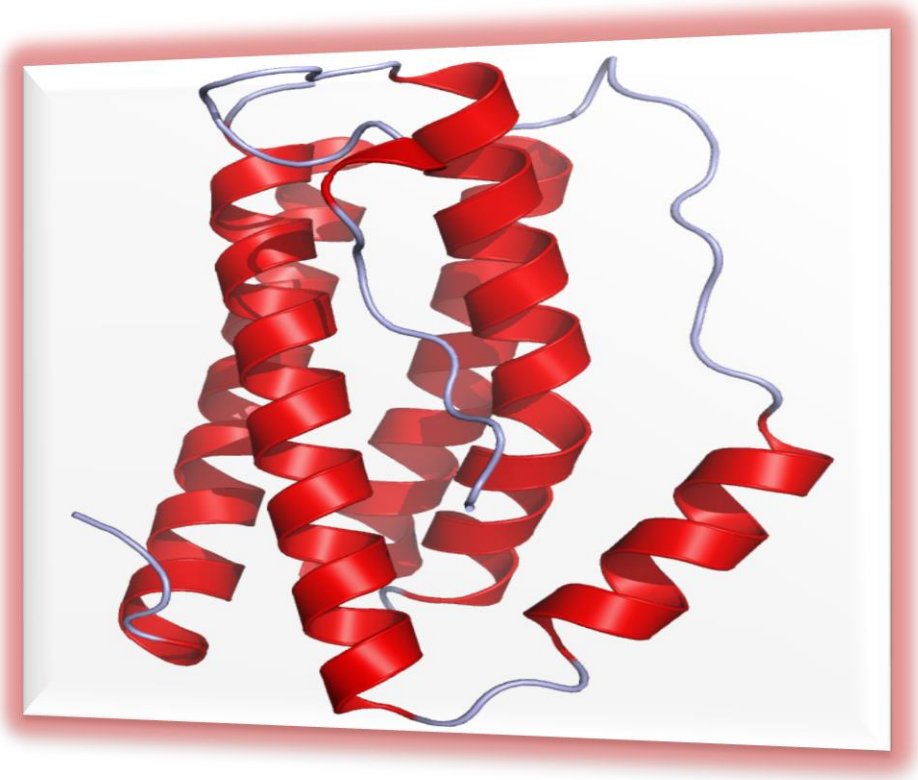


Figure 2.2: Crystallographic structure of IL-6.

2.1.12.2. Interleukin-4 (IL-4): Interleukin 4 (IL-4) is a cytokine that promotes the differentiation of naive helper T cells (Th0 cells) into Th2 cells. Mast cells, Th2 cells, eosinophils, and basophils are the main producers of IL-4 [167] (Figure 2.3). Among the various biological functions of interleukin-4 are the promotion of T and activated B cell proliferation as well as the conversion of B cells into plasma cells. It is an important modulator of both adaptive and humoral immunity [168]. IL-4 increases the synthesis of MHC class II and induces B cell class flipping to IgE. IL-4 reduces the production of Th1 cells, macrophages, IFNγ, and dendritic cells. The development of several immunological illnesses, including allergies and some autoimmune diseases, is significantly influenced by IL-4. By promoting tumor development, IL-4 can innately stimulate tumor cells and strengthen their resistance to apoptosis. Rheumatoid arthritis patients produced more IL-4 after being exposed to nicotine [169]. Similarly, smokers have greater levels of IL-4 than nonsmokers [170-173]. According to Olsson et al., there was no difference in serum IL-4 levels between those who had never smoked and those who had [174].

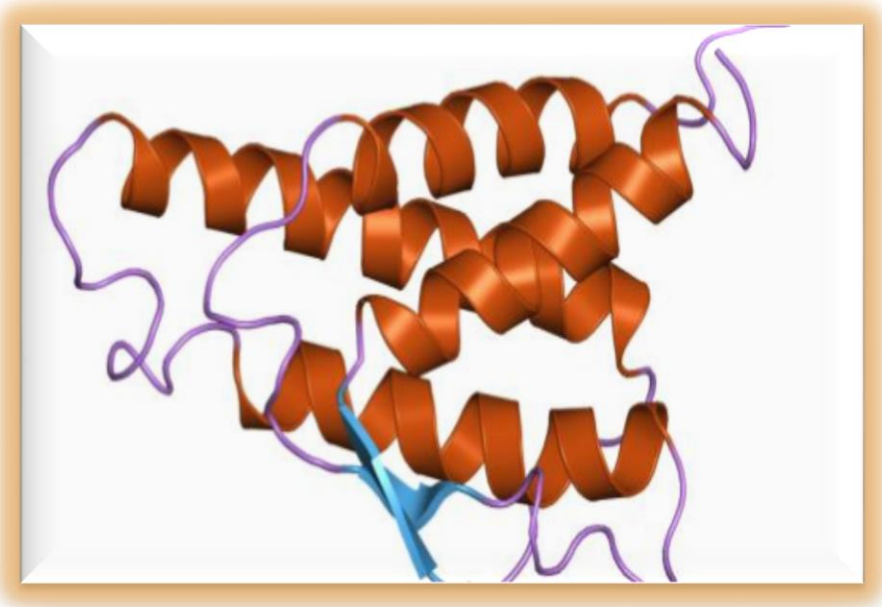


Figure 2.3: Crystallographic structure of IL-4.

2.1.12.3. Tumour Necrosis Factor- α (TNF- α): TNF (formerly known as TNF-α) is an inflammatory protein that plays a key role in the innate immune response [175] (Figure 2.4). TNF is largely generated by macrophages in response to antigens, and it activates inflammatory pathways via the two receptors TNFR1 and TNFR2 [176]. It belongs to a class of type II transmembrane proteins called the tumor necrosis factor superfamily, which also includes cytokines. Many inflammatory disorders are caused by excessive TNF production, and anti-TNF treatments are frequently used to treat these conditions. TNF plays such a crucial role in innate immunity and homeostasis, inhibiting it might raise the risk of infection and create new "paradoxical" autoimmunity. In the models of myocarditis and arthritis, nicotine was reported to dose-dependently suppress the release of inflammatory TNF-α and other inflammatory cytokines [177,178]. Nicotine reduces leukocyte accumulation and TNF-α in the rat stomach through nAChR. At a dosage of 2 mg/kg, nicotine decreased the mRNA expression of inflammatory markers in the liver of male Sprague-Dawley rats fed a high-fat diet, including PPAR-γ, TNF-α, and IL-6. Furthermore, nicotine reduced ER stress, which alleviated liver steatosis and hepatic damage.

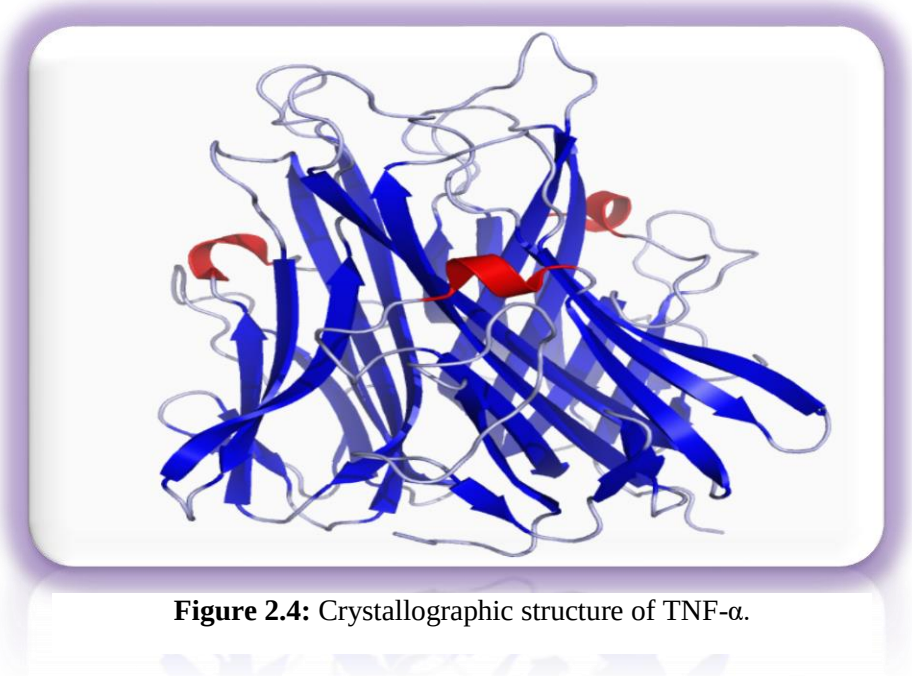


Figure 2.4: Crystallographic structure of TNF-α.

2.1.12.4. Interferon- γ (IFN-γ): IFN-γ is a soluble, dimerized cytokine and the sole type II interferon (Figure 2.5). It was also demonstrated to be generated in human lymphocytes or in the peritoneal lymphocytes of tuberculin-sensitized mice [179]. Interferon gamma regulates the immunological response of its target cell through cell signaling. The JAK-STAT signaling pathway is one of the primary signaling pathways triggered by type II IFN. IFNG contributes significantly to both innate and adaptive immunity. Type II IFN is largely produced by CD4+ T helper 1 (Th1) cells, natural killer (NK) cells, and CD8+ cytotoxic T cells. Cytokines influence type II IFN expression; both upregulate and downregulate [180]. It can enhance inflammation, antiviral or antibacterial activity, cell proliferation, and differentiation by activating signaling pathways in cells such as macrophages, B cells, and CD8+ cytotoxic T cells. Type II IFN is encoded by a distinct chromosomal location, interacts to distinct receptors, and differs serologically from interferon type 1. Because type II IFN can stop tumor growth, it has contributed to the development of cancer immunotherapy therapies. IFNG possesses antiviral, immunoregulatory, and antitumor activities [181]. It changes transcription in up to 30 genes, resulting in a range of physiological and cellular reactions. In humans, higher levels of IFN gamma have been linked to an increased risk of miscarriage. An elevated level of IFN-γ was found in the gingival tissue of chronic periodontitis patients [182]. A similar observation was also made among nicotine smokers [183-185]. Dendritic cells also showed a decreased level of IFN-γ [186,187]. Research conducted in vivo revealed that offspring of smokers had lower levels of interferon gamma (IFN-γ) than children of non-smokers, confirming the depressing effects of cigarette smoking on this hormone.

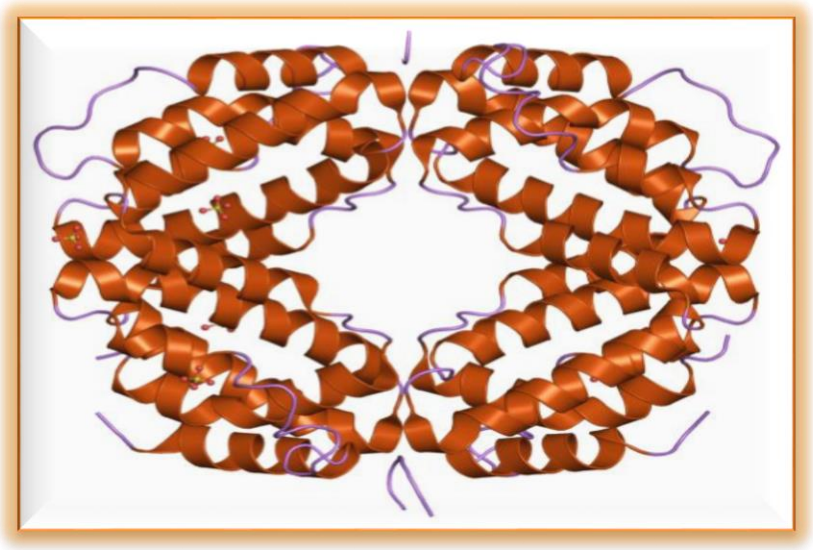


Figure 2.5: Crystallographic structure of IFN-γ.

2.1.12.5. BCL-2 and BAX: The BAX gene in humans encodes the apoptosis regulator BAX, often referred to as bcl-2-like protein 4. BAX is part of the Bcl-2 gene family [188]. Members of the BCL2 family participate in a wide range of cellular processes as pro- or anti-apoptotic regulators that can form hetero- or homodimers. This protein forms a heterodimer with BCL2 and acts as an apoptotic activator. This protein has been shown to interact with and open the mitochondrial voltage dependent anion channel (VDAC), resulting in membrane potential loss and release of cytochrome c [189]. The tumor suppressor P53 regulates the expression of this gene, which has been demonstrated to be involved in P53-mediated apoptosis. Smokers had higher levels of BAX and lower levels of Bcl-2 [190], whereas the reverse thing was recorded in breast cancer cell line [191]. Nicotine raised the ratio of BAX and BCL-2 in human renal proximal tubular epithelial cells [192]. Increased BAX expression in the smoker group was observed by Shaik et al., [193]. In schizophrenia, a neurodegenerative disorder, an aberrant ratio of pro- and anti-apoptotic factors is seen. BCL-2 is found on the outer membrane of mitochondria and plays a vital function in

supporting cellular survival by blocking the actions of pro-apoptotic proteins. The pro-apoptotic proteins in the BCL-2 family, including BAX and BCL-2, typically work on the mitochondrial membrane to enhance permeability and release of cytochrome c and ROS, which are crucial signals in the apoptosis cascade. BH3-only proteins activate these pro-apoptotic proteins, which are then blocked by the activity of BCL-2 and its relative BCL-Xl. The ELISA test demonstrates that nicotine exposure reduces the concentration of anti-apoptotic BCL-2 molecules while increasing the concentration of pro-apoptotic BAX molecules.

2.1.12.6. Estrogen and Progesterone

Estrogen and progesterone are steroid hormones formed by the enzymatic alteration of cholesterol. In women, estrogen and progesterone are produced mostly in the ovary, but also in the adrenal gland and other sites, including adipose tissue. Estrogen and progesterone levels are low throughout infancy, but rise rapidly around puberty due to pulsatile gonadotropin release from the pituitary gland [194]. Non-pregnant women produce the two most physiologically active estrogens, estrone and estradiol, whereas pregnant women create considerable amounts of estriol [195]. Estradiol circulates in larger amounts and has more biological potency than estrone [196,194]. Sex steroid hormones, such as estrogens and progesterone, play key roles in mammary gland growth, with excretion rates rising by about 100-fold [197-199]. Studies demonstrate that greater progesterone levels reduce women's inclination to smoke, estrogen levels alter women's subjective experience of smoking, and concurrent reductions or rises in these hormones are connected with increased smoking. Nicotine smoking decreases the activity of the aromatase enzyme, which catalyzes the conversion of androgens into estrogens. As a result, nicotine lowers the circulating estrogen levels, causing women to enter menopause earlier [200]. Nicotine alters the function of the endocrine system and affects the release of female hormones which are very

important agents for the protection of women's health [201]. Deficiency of estrogens results in irregular periods, infertility, bone weakness, hot flashes, depression, and urinary tract infections [202]. Similarly, a deficiency of progesterone affects the menstrual cycle, pregnancy, and embryogenesis of humans and other species. Nicotine decreases estrogen levels in female rats [200]. Smoking may diminish or eliminate the effectiveness of orally delivered estrogens [203]. According to Ruan and Mueck (2015), smoking cigarettes decreased the effectiveness of both endogenous and exogenous estrogen [204]. Wistar rats' blood levels of progesterone and estradiol have been shown to decrease due to nicotine use [205-207]. The ratio of progesterone to estrogen is inversely correlated with the desire to smoke [208].

2.1.13. Effect of Nicotine on the Female Population

Tobacco smoking is the leading cause of premature death and illness, and it poses additional dangers for some groups. Risks for women of reproductive age (15-45 years) include lower fertility; for pregnant women, smoking increases the likelihood of problems, miscarriage, and early delivery; and post-partum is connected with poor baby health outcomes. Female smokers have poorer long-term health outcomes than male smokers because some smoke during pregnancy, and the negative consequences of cigarette smoking during pregnancy are far greater than those of cigarette smoking during non-pregnant times. There are around 250 million female smokers worldwide, with the bulk of them living in affluent nations. Previous research revealing that female smokers experience worse reductions in lung function than male smokers [209]. Women who smoke are more likely to develop cervical cancer, osteoporosis, cardiovascular disease, atherosclerosis, and type 2 diabetes, as well as lung cancer, early menopause, preterm delivery, irregular fetal development, low birth weight, miscarriage, and fetal death. Women who smoke before and throughout pregnancy have a higher risk of preterm delivery, poor fetal development,

low birth weight, miscarriage, and fetal mortality. Cigarette smoking has a significant impact on human fertility. Smoking is both pro-androgenic and anti-estrogenic, which means it reduces estrogen levels in women. Nicotine significantly reduces 17 β-estradiol and progesterone levels in female rats fed a protein-restricted diet. Nicotine reduces 17 β-estradiol levels in female rats' serum due to its anti-estrogenic action on hepatic estrogen metabolism [210]. It reduces progesterone levels via boosting PGF-2α, which promotes autolysis, and VEGF mRNA expression. Curcumin and nano-curcumin supplementation increases 17β-estradiol and progesterone levels in rats on protein-restricted diets, counteracting nicotine's anti-estrogenic impact. This increase is due to curcumin, which decreases microvascular endothelial cell angiogenesis by inhibiting COX-2 expression. Women were more vulnerable to smoking-related mortality and morbidity than males because their immune systems were weaker. Women are more likely than males to get some types of cancer as a result of smoking. Previous research found that female smokers have a higher risk of colorectal cancer than male smokers [211]. The risk of breast cancer is higher among smoker women who have a family history of breast cancer and began smoking during adolescence or pre menarche than among nonsmokers [212]. Women who smoke are 25% more likely to get cardiovascular disorders than males [213]. Women smokers experience more overactive bladder symptoms and incontinence than males [214]. Women gain more weight than men after they quit smoking. According to Perkins et al. [215] women experienced a greater degree of yearning to smoke than men did when they were abstinent and in a bad mood. A greater risk of asthma was seen among smoking women regardless of body weight, however a higher rate of asthma was found among normal weight and underweight smoker males [216]

2.1.14. Nicotine and Malnutrition

Protein Energy Malnutrition (PEM) continues to be a major public health concern in developing countries, impacting people of all ages, especially infants, young children, pregnant and lactating women, and low-income communities. According to the Food and Agriculture Organization of the United Nations (FAO), 17.3% (26.5 million) of Bangladesh's total population was malnourished between 2010 and 2012. The negative health and economic repercussions of tobacco smoking are mixed with the worldwide concerns of addressing poverty and hunger. Low dietary protein levels reduce toxicity, disposition, and biosynthetic activity. Protein Energy Malnutrition (PEM) is a condition characterized by low protein and energy consumption, which can cause health problems [217]. Though it is widely recognized that nicotine causes numerous health issues under normal dietary condition, the toxic effects of nicotine, particularly on protein-restricted diets, are still a subject of worry. Decreased nutritional status of smokers is linked to the amount of nicotine in cigarettes; the more cigarettes taken; the more nicotine enters the body. This may slow the rate of metabolism and reduce appetite, affecting a person's BMI [218]. Other research show that in India, people who smoked had a BMI 30% lower than nonsmokers, and 99 studies found that males who smoked every day had a 3% lower BMI than men who did not smoke [219].

Several research found a link between smoking and food consumption, demonstrating that smoking can decrease hunger, reduce appetite, and weight. People frequently feel a reduction in appetite. In 1988, John Morley [220] defined this decline as 'aging anorexia'. As individuals age, 15% to 30% are predicted to develop aging anorexia, which is most typically encountered by women in nursing homes and the elderly who are hospitalized [221]. Nutritional deficiencies and weight loss in the elderly have serious effects associated with poor health and an increased risk of

death [222]. Malnutrition in smokers is connected with the nicotine impact, which affects cholinergic nicotinic receptors in the autonomic ganglia and brain. Following nicotine and associated receptors increases the consumption of numerous neurotransmitters. This mechanism causes systemic catecholamine release, which has the potential role to increase. Nicotine influences energy balance and food consumption by changing the activity of neurons carrying orexigenic and anorexigenic peptides in the brain. The hypothalamus is a crucial brain region that maintains energy balance through the activity of these neuropeptides. Nicotine may change the balance between these two sets of peptides, resulting in decreased food intake and weight reduction. Nicotine regulates energy balance via nicotinic acetylcholine receptors (nAChRs). These receptors are widely expressed throughout the central and peripheral nervous systems, but are especially well-positioned in the hypothalamus to influence the expression, secretion, or function of neuropeptides that regulate appetite and food intake, modulating energy homeostasis and feeding behavior. Nicotine has also been shown to alter the levels of certain peptides in the periphery by acting on nAChRs found in taste, visceral, and nociceptive vagal afferent pathways, which also play a functional role in nicotine's ability to influence food intake [223,224]. Nicotine has been demonstrated to influence several energy-related activities via altering the activity of AMP-activated protein kinase (AMPK). AMPK integrates hormonal and nutritive signals in peripheral organs and the hypothalamus, regulating energy balance [225]. Nicotine has been shown in rats to downregulate AMPK activity in the hypothalamus, which results in a decrease in food intake and BAT activation, as well as an increase in lipid oxidation. Furthermore, nicotine has been shown to stimulate the effects of adrenaline on the stomach muscles, hence suppressing appetite [226]. Other studies have also found that nicotine has an influence on hunger [227], despite claims that smokers have a lower body weight than nonsmokers. These findings were

consistent with prior research that found smokers, even long-term smokers, had lower body weight than nonsmokers [228]. Metabolism, as well as epinephrine, dopamine, norepinephrine, and serotonin expenditure, all of which impact the satiety system, causing a reduction in appetite and resulting in malnutrition [228]. The higher a person consumes cigarettes will have the risk of reducing the nutritional status value. Weight and height are determinants of nutritional status, as weight in the elderly usually tends to decline due to reduced muscle mass [230]. Several studies have shown that nicotine reduces body weight by increasing energy expenditure and decreasing food intake [231,232], and that these effects are due to nicotine's modulatory effect on both metabolic processes and reward circuits [233,234].

2.2. Curcumin

Curcumin, also known as diferuloylmethane, is an active component in the golden spice turmeric (Curcuma longa) and in Curcuma xanthorrhiza oil. Turmeric, the powdered rhizome of Curcuma longa, is widely used as a spice in curries and mustards, is often responsible for their distinguishing color, and contributes significantly to their flavor due to the presence of its oleoresins and essential oil. Turmeric, a member of the ginger family (Zingiberaceae), is widely used to treat diseases in traditional Chinese and Indian medicine. Curcuma has a long history of therapeutic usage (235,236], and it is made up of about 120 different species. Curcuma longa L. (Curcuma; Turmeric) is the most well-known of the Curcuma species; it is a cultivated plant growing in warm climates around the world [237]. According to a new study by Grand View Research, Inc., the global curcumin market size was worth over USD 46.6 million in 2016. North America was the largest regional market in 2016, while India was one of the largest curcumin manufacturers. Multitude research endeavors has demonstrated that curcumin serves as a remarkably effective antimicrobial agent, exhibiting activity against a range of chronic diseases,

encompassing various form of cancer, diabetes, obesity, cardiovascular, pulmonary, neurological and autoimmune diseases. Moreover, this chemical has been proven to be complementary with other nutraceuticals including resveratrol, piperine, catechins, quercetin and genistein. Over 100 clinical trials with curcumin have been done to date, demonstrating its safety, tolerability, and effectiveness against numerous chronic conditions in people. Curcumin is a highly pleiotropic chemical with a wide range of targets and methods of action, including changes in enzyme activity, growth factor receptors, cofactors, and other compounds.

2.2.1. The Overview of Curcumin

Curcumin, the principal active ingredient in turmeric, has been extensively researched over the last few decades for its potential medicinal benefits, particularly its anti-inflammatory, antioxidant, and anticancer capabilities. The active ingredient presents in turmeric which makes a magical spice that is curcumin. It is also known as “wonder drug of life” [238].

2.2.1.1. Chemistry of Curcumin

Curcumin's chemical structure is based on a diferuloylmethane backbone, (crystalline yellow-orange colour) and it is classed as a curcuminoid, a class of chemicals found in turmeric. Curcumin's IUPAC name is (1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (Figure 2.6), with the chemical formula $C_{21}H_{20}O_6$ and a molecular weight of 368.39 g/mol, and melting point of 183°C. The composition comprises three chemical components: two aromatic ring systems featuring o-methoxy phenolic groups, along with a seven-carbon linker derived from an, β-unsaturated β-diketone moiety. From a chemical perspective, it demonstrates keto-enol tautomerism, wherein the keto form is the dominant species in neutral and acidic environments, while the more stable enol form prevails in the solid state and in alkaline solutions [239]. There

exist two further compounds identified as curcumin, specifically curcumin II [demethoxycurcumin, 1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione] and curcumin III [bisdemethoxycurcumin, 1,7-bis(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione] [240]. This chemical structure of curcumin makes it soluble in methanol, ethanol, acetic acid, alkali, ketone, dimethyl sulfoxide (DMSO), chloroform, and acetone but insoluble in water at neutral and acidic pH [241]. Curcumin can degrade into dehydrocurcumin (an oxidized form) when exposed to light or heat, indicating its chemical instability. Curcumin is sensitive to alkaline pH, causing structural alterations that culminate in the disintegration of its conjugated system. The curcumin structure was first proposed by Polish scientists in 1910 [242].

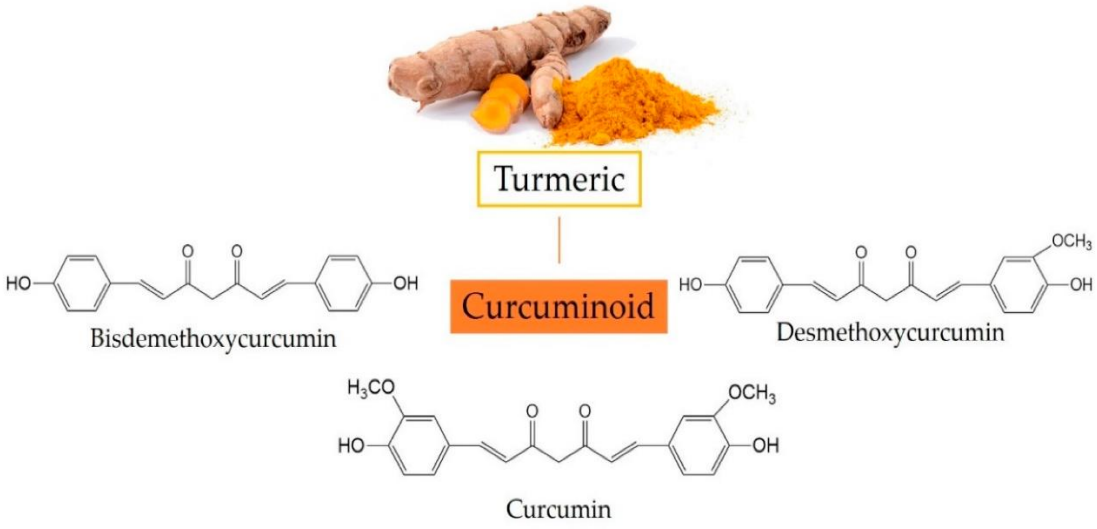


Figure 2.6: Chemical structure of curcumin.

2.2.1.2. Biological Properties of Curcumin

Curcuminoids, the bioactive molecule derived from Curcuma longa, has received great scientific attention due to its broad biological properties. Curcumin, which was first investigated

for its medicinal potential in the 1930s, has subsequently been proven to interact with a diverse spectrum of proteins, metals, growth factors, enzymes, and other important biomolecules. Making it an effective chemical for treating a wide range of disorders. These interactions help explain its wide range of biological properties, including anti-inflammatory, antioxidant, anticancer, and neuroprotective effects [243-248]. Different biological actions of curcumin are shorted in Figure 2.7.

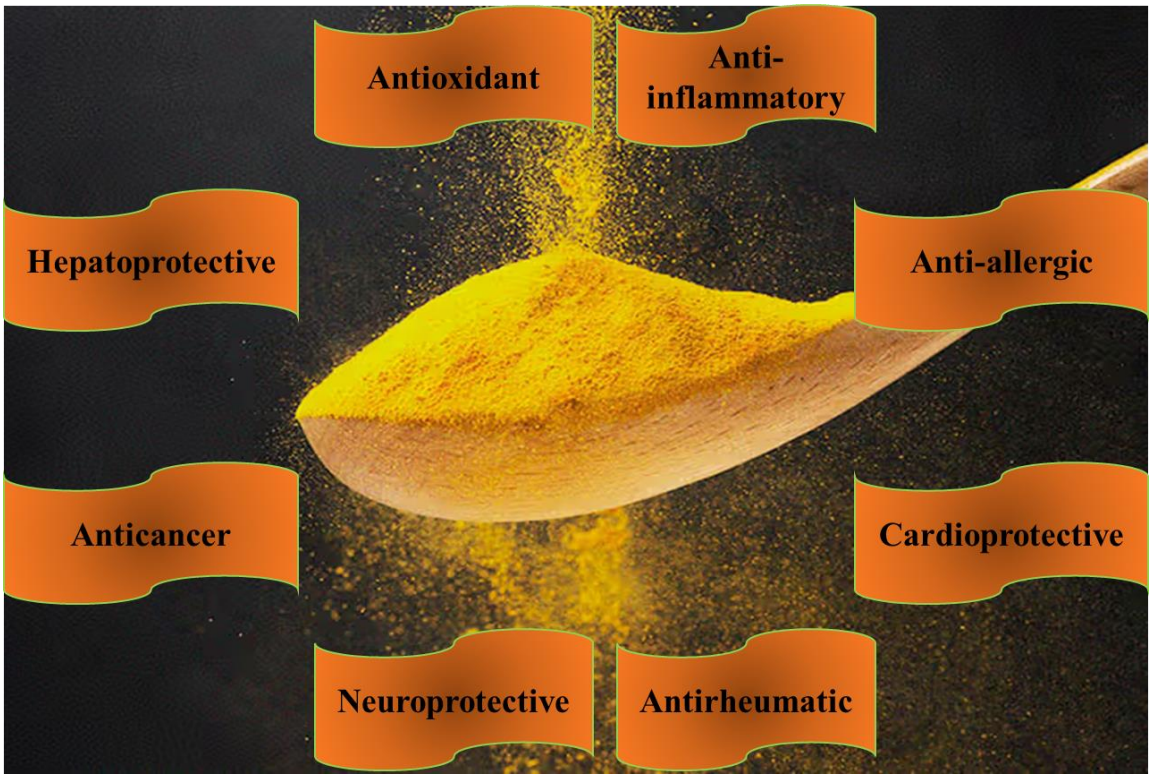


Figure 2.7: Schematic representation of biological activities of curcumin.

2.2.1.3. Pharmacological Property of Curcumin

Numerous advantageous pharmacological attributes have been attributed to the Curcuma species. These include anti-inflammatory, antidiabetic, antiproliferative, anticancer, hypocholesterolemic, antihepatotoxic, antithrombotic, anti-diarrheal, carminative, diuretic, hypotensive, antirheumatic, antimicrobial, antiviral, larvicidal, antioxidant, insecticidal, antivenomous, and antityrosinase effects, among various others [249-252]. Approximately 31 species of Curcuma have been examined, with the most significant and extensively researched being turmeric (C. longa) and zedoary.

2.2.1.4. Anti-Oxidant Actions

Several investigations have demonstrated that curcumin has a high ability to scavenge superoxide radicals, hydrogen peroxide, and nitric oxide (NO) from activated macrophages [253,254], decreasing iron complexes and preventing lipid peroxidation [255]. Curcumin may demonstrate its pharmacological and therapeutic properties primarily through these effects. The anti-oxidant properties of curcumin and its derivatives demethyoxycurcumin, bisdemethoxycurcumin, and diacetylcurcumin were examined by Unnikrishnan and Rao [256]. They discovered that these compounds, with the exception of diacetylcurcumin, which has little effect on the inhibition of nitrite-induced oxidation of Hb, can shield hemoglobin (Hb) from oxidation at concentrations as low as 0.08 mM. Curcumin has also been demonstrated to suppress macrophage NO synthase activity [257]. Additionally, it has been demonstrated by Bonte et al. [258] that curcumin shields human keratinocytes from xanthine oxidase damage by virtue of its antioxidant properties. Curcumin therapy has also been shown to reduce oxidative stress and lipid peroxidation brought on by nicotine [259]. It has been demonstrated that curcumin protects brain

glial cells and renal cells from oxidative damage. Curcumin is also known to increase the activity of other antioxidants, including glutathione peroxidase, catalase, and superoxide dismutase (SOD) [260]. Curcumin can also inhibit enzymes that generate ROS, such as lipoxygenase/cyclooxygenase and xanthine hydrogenase/oxidase [261]. Curcumin's lipophilic nature makes it an effective scavenger of peroxyl radicals, acting as a chain-breaking antioxidant [262].

2.2.1.5. Anti-Inflammatory Activity

Curcumin has a high potential for treating a variety of inflammatory disorders [263-266] Curcumin has been shown to: (i) inhibit pro-inflammatory transcription factors (NF-kB and AP-1); (ii) reduce the proinflammatory cytokines TNFa, IL-1b, IL-2, IL-6, IL-8, MIP-1a, MCP-1, CRP, and PGE2; (iii) down-regulate enzymes such as 5-lipoxygenase and COX-2 and -5; and (iv) inhibit the mitogen activated protein kinases (MAPK) and pathways involved in nitric oxide synthase (NOS) enzyme synthesis [267-270] Through intricate interactions with cytokines, lipid mediators, and proteolytic enzymes, curcumin regulates a variety of inflammatory mediators both in vitro and in vivo [271]. Curcumin was found to be more efficient than hydrocortisone in inhibiting the generation of leukotrienes in rat peritoneal polymorphonuclear neutrophils. Curcumin suppresses the growth of vascular smooth muscle cells and blood mononuclear cells, and have clinical application in transplant atherosclerosis as demonstrated by Huang et al. Pro-inflammatory transcription factors such as AP-1 and NF-kB are inhibited by curcumin [272]. It reduces the synthesis of proinflammatory cytokines, such as CRP, IL-2, IL-6, and TNF-alpha. Additionally, it inhibits the pathways of mitogen-activated protein kinases and inhibits enzymes such as COX-2, -5, and 5-lipoxygenase [263, 267-270].

2.2.1.6. Neuroprotective Effect

Chronic inflammation known as neuroinflammation causes alterations in neuronal metabolism, which ultimately lead to neuronal death. The activation of astrocytes and microglia increases neuronal death in neuroinflammatory conditions. The latter are in charge of releasing proinflammatory cytokines including IL-1 and TNFa. Several previous research confirmed, curcumin's antioxidant, anti-inflammatory, and anti-protein aggregating properties have made it a promising therapeutic agent for a number of neurological conditions, including multiple sclerosis, dementia, AD, PD, and Huntington's disease (HD) [273-277]. Curcumin prevents activated microglia and astrocytes from producing prostaglandins and inflammatory cytokines [143-264]. In microglial and astrocyte cells, it also reduces the synthesis of TNFa, IL-1b, monocyte chemoattractant protein (MCP-1), macrophage inflammatory protein (MIP-1b), and IL-8 [278]. Through a variety of mechanisms, including as antioxidant, anti-inflammatory, and anti-proliferative ones, curcumin has also demonstrated neuroprotective effects in multiple sclerosis, an autoimmune inflammatory illness that primarily affects women and young people through demyelinating lesions. Curcumin was also able to modulate several molecular targets, such as transcriptional factors (NF-kB, Nrf2, AP-1, STAT-1,-3,-4), enzymes (COX-2, iNOS, OH-1, LOX, XO), inflammatory cytokines (chemokine ligand, interleukin, TNFa), proteins (caspase-3,-9, Bcl-2, Prostaglandin, CRP, myosin light chain), protein kinases (AK, JNK, JAK, MAPK), growth factors and receptors (TLRs, chemokine receptor, TGF-a, TGF-ß) [279]. Curcumin is neuroprotective in multiple animal models and has great potential for the prevention or treatment of age-related dementia arising from AD or cardiovascular disease, Parkinson’s disease, other diseases of aging, and aspects of aging itself.

Curcumin has at least ten neuroprotective effects and it can apparently act at nanomolar or even picomolar doses are shown in Figure 2.8.

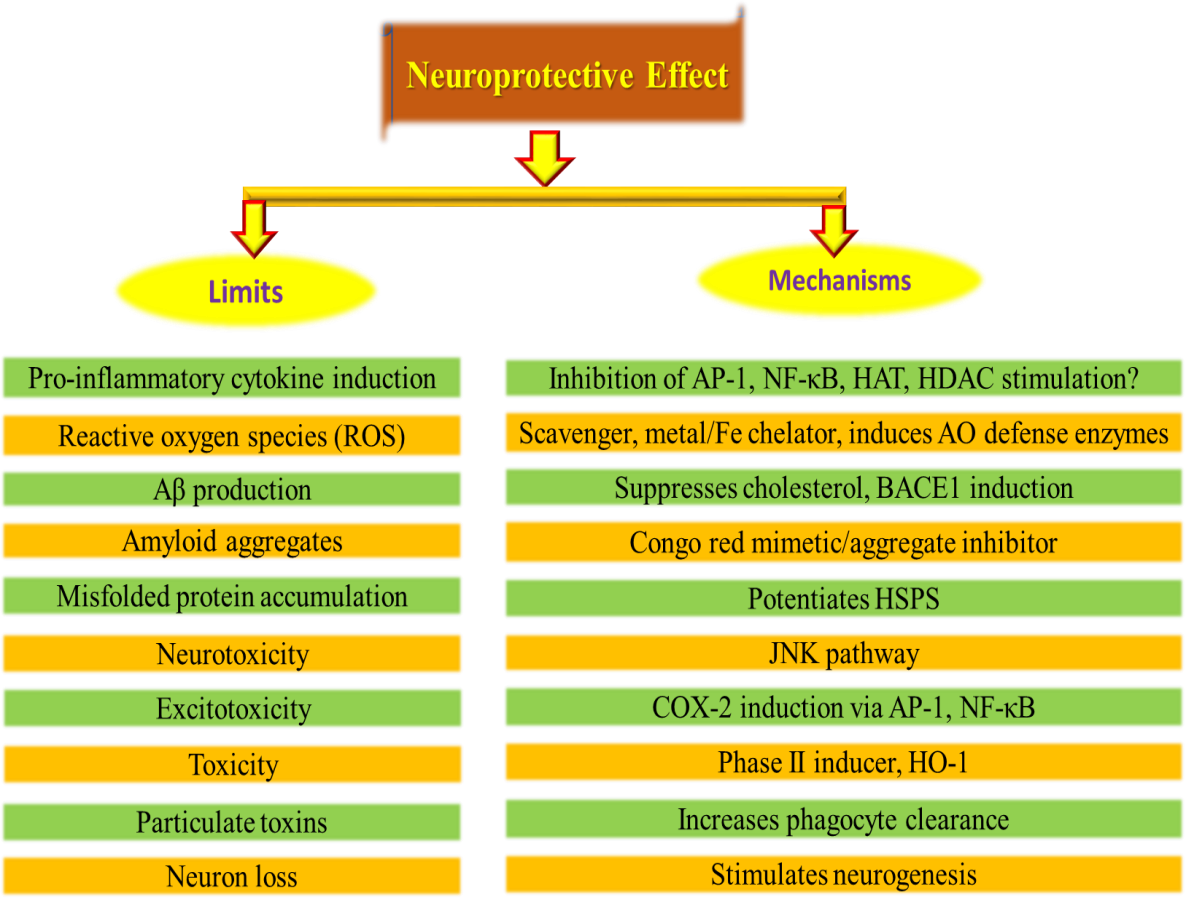


Figure 2.8: Neuroprotective effects of curcumin.

2.2.1.7. Anticancer Effect

Curcumin has great potential in treating a variety of cancers, including breast, colorectal, colon, liver, lung cancers and leukemia [280]. Curcumin, a strong anti-inflammatory and anti-oxidant substance, may help prevent cancer by inhibiting tumor growth in many phases. It also

suppresses proliferation and promotes apoptosis in a variety of cell types, including human bladder cancer cells, and arrests cancer cells in the S and G2/M cell cycle phases. Curcumin has been found to inhibit carcinogenesis through two mechanisms: angiogenesis and cancer cell proliferation. It also prevents cancer cell spread and promotes apoptosis. Curcumin has been demonstrated to have antiangiogenic effect by blocking angiogenic factor stimulators such as VEGF and basic fibroblast growth factor and downregulate VEGF expression via NF-kB and AP-1 regulation, hence reducing IL-8 expression [281]. Astinfeshan et al. discovered that curcumin inhibits angiogenesis by modulating VEFGR and the PI3K/Akt signaling pathway [282]. Furthermore, curcumin has been demonstrated to downregulate MMP-2 and MMP-9 while increasing the tissue inhibitor metalloproteinase-1, ensuring extracellular matrix stability and coherence [281]. Curcumin can also induce apoptosis in cancer cells through a p53-dependent pathway. p53 is known to be one of the most important tumor suppressor proteins, affecting cell proliferation apoptosis and DNA damage [283]. Several studies have revealed an interplay between p53 and cancer related miRNAs [284,285]. Ye et al. (2015) also showed that the curcumin proapoptotic effect depends on miR-192-5p and miR-215, which activates the p53 in non-small cell lung cancer. Many studies have demonstrated that it can inhibit the NF-kB and AP-1 transcriptional factor families [286,287]. Marquardt et al. [288] investigated the effect of curcumin in curcumin-sensitive, liver cancer cell lines. Curcumin inhibited the expression of PCNA, pPI3K, and NF-kB in lung cancer cells [287]. Curcumin has been shown to suppress STAT expression and decrease the expression levels of STAT-3-regulated cyclin D1, BCL-2, and BCL-xL in pancreatic cancer cells [289]. Shanmugam et al. (2015) shown that curcumin might reduce IL-6 mediated STAT-3 phosphorylation and STAT-3 nuclear translocation in multiple myeloma [290]. Curcumin works on various molecular targets, either downregulating or upregulating them, as illustrated in Figure 2.9.

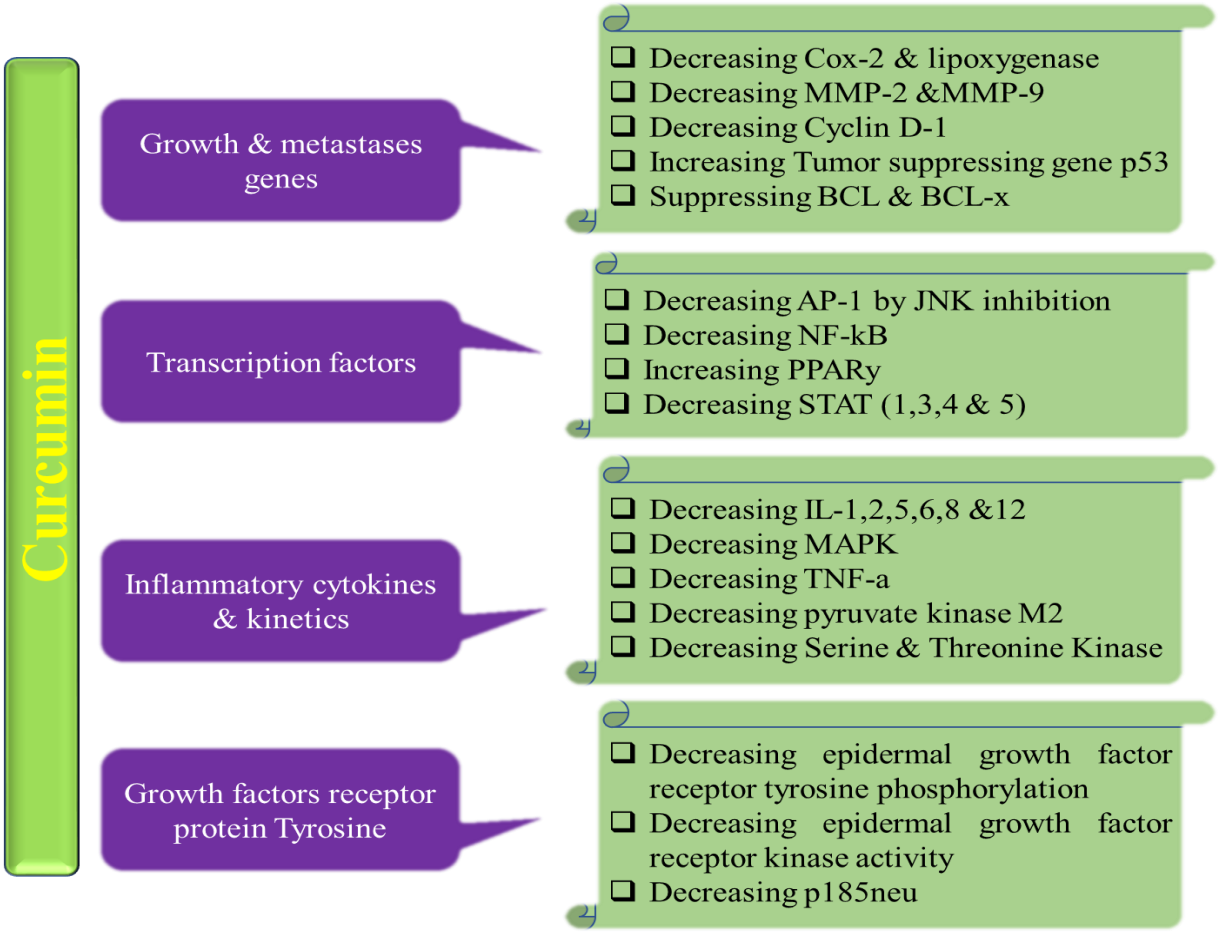


Figure 2.9: Curcumin molecular targets in cancer cells.

2.2.1.8. Therapeutic Properties of Curcumin

2.2.1.8.1. Anti-Viral Effect: Curcumin exhibits moderate to high inhibitory action on a number of viruses, including type I HIV [291] and human simple virus-2 [292], according to a number of in vitro and in vivo investigations. It has been demonstrated that curcumin effectively inhibits the production of P24 antigen in cells infected with HIV-1 either acutely or chronically. It also inhibits the enzymatic reactions linked to HIK 1 intergase, in contrast to other viral (HIV-1 reverse transcriptase) and cellular (RNA polymerase II) nucleic acid processing enzymes. Curcumin failed

to stop HIV-1 from multiplying in MT-4 cells that were acutely infected [293]. Two curcumin analogues, rosmarinic acid and dicaffeolymethan, have been demonstrated to have stronger antiviral activity than curcumin [294].

2.2.1.8.2. Effect on Immunity: It has been established that curcumin alters immunity. In rat heterotropic heart transplant models, curcumin enhances the immunosuppressive effects of cyclosporine [295]. Curcumin treatment increases mucosal CD4 (+) T and B cells, indicating that it alters lymphocyte-mediated immune responses [296]. The impact of curcumin on mitogen/antigen-induced splenic lymphocyte proliferation, cytotoxic T lymphocyte induction, lymphokine-activated killer cells, and T lymphocyte and macrophage cytokine production was investigated by Gao et al [297]. Curcumin was shown to permanently impede the generation of these immune responses. It was suggested that by blocking NF-κ B target genes involved in the production of certain immune responses, the chemical may have suppressed these immunological functions.

2.2.1.8.3. Effect on Cystic Fibrosis (CF): Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene cause cystic fibrosis (CF), the most prevalent genetic illness in the white population. Curcumin has been shown to be able to rectify the mutational abnormalities of cystic fibrosis in mice, most likely by inhibiting sarco (endo) plasmic reticulum Ca^{+2}-ATPase [298]. This encouraging discovery was later confirmed by several employees [299]. A few studies corroborated this conclusion in animals, but others were unable to replicate it in human and other animal models.

2.2.1.8.4. Anti Diabetic Effect: The administration of curcumin or turmeric to alloxan-induced diabetic rats led to a significant decrease in blood sugar, hemoglobin, and glycosylated hemoglobin

levels [282]. Curcumin also reduced the oxidative stress experienced by diabetic rats. Reduced TBARS levels and increased glutathione peroxidase activities were observed [300]. The enzyme sorbitol dehydrogenase, which converts sorbitol to fructose, was also reduced. Nishiyama et al. found that curcumin has an antidiabetic effect in mice and proposed a molecular mechanism involving peroximose proliferator-activated receptor (PPAR)-gamma activation [301]. The primary constituents of turmeric, namely curcuminoids and sesquiterpenoids, have the potential to lower hyperglycemia through either an additive or synergistic effect.

2.2.1.8.5. Antibacterial and Anti-Fungal, Anti-Parasitic Actions: Curcumin-derived bioconjugates were found to be more effective than curcumin against numerous common bacterial and fungal species [302]. The heightened efficacy of these bioconjugates, as compared to curcumin, may be due to enhanced cellular uptake or diminished metabolic degradation of the bioconjugates, resulting in a substantial concentration build-up within infected cells. Many studies proved that curcumin bioconjugates have the potential to be effective antibacterial/antifungal medicines [303]. Curcumin's leishmanicidal properties have also been confirmed in vitro [304]. Curcumin has recently been found to be effective in malaria treatment [305]. Curcumin was given orally to mice infected with the malaria parasite (Plasmodium berghei), resulting in an 80-90% reduction in blood parasitaemia and significantly enhancing the survival rate of the mice. Numerous constituents of turmeric, such as curcumin, demethoxycurcumin, bisdemethoxycurcumin, and cyclocurcumin, have demonstrated nematocidal properties [306].

2.2.1.8.6. Cardioprotective Effect: Cardiovascular (CV) illnesses are regarded as a global health threat, with significant morbidity and mortality rates. Curcumin has been found to be helpful for protecting against cardiovascular disease [307,308]. As previously stated, curcumin cardiovascular advantages are primarily associated with its preventive impact on conditions such as

atherosclerosis, cardiac hypertrophy, heart failure, aortic aneurysm, stroke, myocardial infarction, and cardiovascular issues related to diabetes [309]. Curcumin is a natural p300 histone acetyltransferase (HAT) inhibitor. Sunagawa et al. [310] and stimulates Nrf2 and induces HO-1, which are responsible for cytoprotective and anti-inflammatory actions against oxidative stress. Monfoulet et al. [311] discovered that curcumin can improve endothelial function and reduce TNFα-induced monocyte adhesion in endothelial cells by inhibiting NF-kB. According to Yao et al. [312], curcumin prevents CV disorders by lowering the expression of the angiotensin II type 1 receptor (AT1R) and effectively reduces the AT1R gene promoter's binding capacity with specificity protein 1 (SP1). Curcumin may reduce chronic heart failure by raising p38MAPK, JNK, and ASK1 [313-323], as well as having the capacity to lessen the pro-fibrotic effects in idiopathic pulmonary fibrosis [324-326]. Curcumin function on different types of cardiovascular diseases which is illustrated below in Figure 2.10.

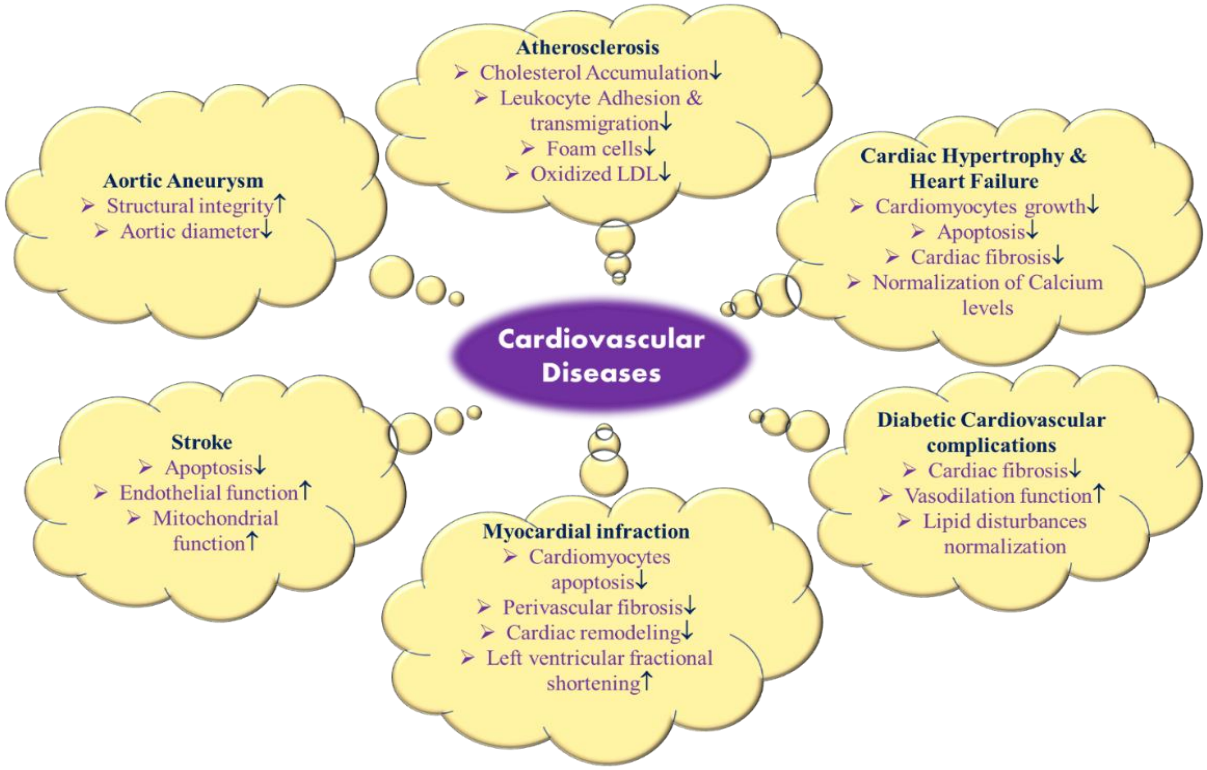


Figure 2.10: Curcumin action on cardiovascular diseases.

2.2.1.8.7. Hepatoprotective Effect: Numerous agents, including alcohol, drugs, environmental pollutants, parasites, and certain dietary elements, can lead to both acute and chronic liver injury. This may manifest as liver fibrosis, non-alcoholic steatohepatitis, non-alcoholic liver disease, and potentially progress to cirrhosis. Curcumin has been widely researched for its hepatoprotective properties [327,328]. Oxidative stress is exacerbated by oxidant and antioxidant imbalances as well as elevated reactive oxygen species (ROS) in chronic liver disease. It causes ischemia, necrosis, and apoptosis and impairs the liver's fibrogenic response. It causes progressive liver damage and altered gene expression [329]. Curcumin treatment (200 mg/kg) in Sprague-Dawley rats raised hepatic glutathione levels while decreasing lipid peroxidase and activities of ALT (alanine transaminase) / AST (aspartate aminotransferase) activity [330]. Curcumin has the potential to safeguard against liver disorders associated with oxidative stress by reducing the levels of ALT, AST, and alkaline phosphatase, while simultaneously increasing the levels of GST, GR, GPx, SOD, and CAT. Additionally, it may decrease nitric oxide levels and inhibit the production of reactive oxygen species [331]. Research indicates that curcumin treatment in Sprague Dawley rats suffering from streptozotocin-induced diabetes resulted in a reduction of TNF-alpha, IL-1 beta, MAPK, and apoptosis signal-regulating kinase 1 (ASK1) levels in liver tissues [332]. Curcumin was reported to cure nicotine-induced liver damage by reducing alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum nitric oxide and alkaline phosphatase (ALP) levels [333]. Curcumin ameliorates carbon tetrachloride (CCl4)-induced liver damage. Curcumin inhibits the TGF-β1/Smad signaling pathway and CTGF expression [334]. By scavenging free radicals, promoting the production of antioxidant enzymes, and blocking NF-kB and transient receptor potential melastatin 2 (TRPM2) channels, curcumin treatment reduced mitochondrial dysfunction [335]. Curcumin can help treat cadmium-induced liver oxidative

damage [336]. It has also been demonstrated that curcumin can attenuate liver fibrosis and cirrhosis [337].

2.2.1.8.8. Anti-Lipidemic Effects of Curcumin: Triglycerides and low-density lipoprotein cholesterol (LDL-C) were both markedly decreased by curcumin [338,339]. Curcumin administration effectively decreased hyperlipidaemia, which was characterized by elevated cholesterol, free fatty acids, and triglycerides [340-342]. Curcumin improved cardiovascular disease outcomes by regulating LDL-C, VLDL-C, IDL-C, total cholesterol, and HDL-C [343]. This natural component was shown to be effective in treating nicotine-induced hyperlipidaemia in Wistar rats.

2.2.1.9. Curcumin's Pharmacokinetics

Curcumin, while recognized for its diverse health benefits and established safety profile, faces limitations in its in vivo effectiveness primarily due to suboptimal pharmacokinetic properties, particularly its low bioavailability [344,345]. Its short half-life, restricted tissue distribution, low free serum concentrations, and apparent quick metabolism and excretion are some of the reasons [346]. Metabolism of curcumin mainly occur in liver and colon and small amount remains in tissues [344,345]. Curcumin is mostly eliminated by feces following PO treatment. Curcumin and its metabolites (glucuronide and sulfate derivatives) are excreted in the urine at extremely low levels, independent of the oral intake. Another study proven that rats exclusively excreted curcumin in their bile following IV and IP treatment [344].

2.2.1.10. Antigenotoxicity Properties of Curcumin

Curcumin, a polyphenolic molecule found in turmeric, has been proven to have antigenotoxic properties, preventing genetic damage and mutations. Curcumin has been proven to protect DNA from ROS and other genotoxic substances. Curcumin considerably reduces the frequency of micro nucleated polychromatic erythrocytes in mice exposed to gamma radiation. Curcumin may potentially improve DNA repair by boosting the production of DNA repair enzymes. Curcumin reduced cisplatin-induced chromosomal and DNA damage, as measured by the comet assay and the micronucleus assay. Previous research has found that curcumin protects against copper-induced genotoxicity in BalbC mice [347]. Curcumin supplementation inhibited the radiation-induced increase in micronuclei production [348]. Curcumin also has an antigenotoxic impact by inhibiting micronuclei induction by chromium trioxide [349]. According to Banerjee et al., curcumin reduces nicotine-induced DNA damage in blood cells [350].

2.2.1.11. Curcumin's Effect on the Reproductive System

Curcumin, a polyphenol produced from the spice turmeric, has been shown to have a variety of impacts on the reproductive system in both men and women. Curcumin may protect eggs from oxidative stress, thereby increasing fertility. Curcumin may impact estrogen levels, which might disrupt menstrual periods and fertility. Curcumin has been proven to have anti-inflammatory and antioxidant properties during pregnancy, which may improve fetal growth and reduce pregnancy problems. Curcumin may also help with breastfeeding, since it has been demonstrated to boost milk production and raise antioxidant levels in breast milk. Sadoughi (2016) demonstrated that curcumin increases estrogen and progesterone levels in alloxan-induced diabetic rats while still maintaining a healthy reproductive system [351]. According to Shah and Shrivastava (2021),

curcumin can cure letrozole-induced polycystic ovarian syndrome, which is a mix of endocrine metabolic problems and several forms of inflammation [352].

2.2.2. Drawbacks of Curcumin

Although curcumin is usually regarded as safe and well-tolerated, prolonged usage or large dosages may have negative consequences. Abdominal discomfort, diarrhea, vomiting, and nausea might result from high dosages (>2 g/day). High dosages may raise the chance of developing kidney stones. Curcumin may impair iron absorption, which might exacerbate iron insufficiency. Curcumin caused liver damage by increasing the production of reactive oxygen species (ROS) and pro-inflammatory cytokines IL-6, as well as decreasing the levels of the antioxidant enzyme SOD and the detoxifying enzyme GST. Curcumin has been shown to produce DNA damage both in vitro and in vivo, perhaps due to pro-oxidant actions. According to recent research, high dosages of curcumin impact DNA damage and inactivate P53 by interacting with thiol groups on cysteine residues [353]. Higher dosages of curcumin impair enzyme activity and may cause deleterious consequences [354]. Curcumin intake of up to 8 g/day for three months by healthy human participants shown no harm [355]. However, the same dosage is responsible for stomach discomfort in pancreatic cancer patients. It might be because curcumin inhibits prostaglandin production and COX [356].

2.2.2.1. Limitations of Curcumin

The pharmacokinetics of a substance are determined by its rate of gastrointestinal absorption, metabolism, and bioavailability. Several studies have found that curcumin is poorly absorbed after oral intake [357,358]. Curcumin has relatively limited bioavailability in many tissues and blood after intake. Dei and Ghidoni (2019) found a very low curcumin content (0.051µg/mL) in human

plasma after treatment with 2 g/kg of curcumin [358]. Curcumin has a half-life of around 4-6 hrs, requiring regular dosage and susceptible to deterioration under light, heat, and moisture, which reduces its efficacy. Due to its poor stability curcumin is metabolized and excreted rapidly. After receiving 2 g/kg of curcumin, a very low quantity of curcumin (0.051µg/mL) was found in human plasma. Similarly, after giving mice 1 g/kg of curcumin, the estimated plasma concentration of curcumin was 0.22 µg/mL [356]. In an alkaline media (pH > 7), curcumin breaks down in 30 minutes to provide trans-6-(40-hydroxy-30-methoxyphenyl)-2, ferulic acid, vanillin, and feruloyl methane [359]. Curcumin is quickly metabolized within the liver. Curcumin is converted into water-soluble compounds and eliminated via urine. According to Sharifi-Rad et al. (2020), around 90% of curcumin is excreted with the face [355]. Numerous strategies have been proposed to improve curcumin's cellular permeability, boost its bioavailability, raise its plasma concentration, and slow down its metabolism [360-362]. Curcumin is mainly water-insoluble and poorly bioavailable molecule [363-365].

2.3. Nanocurcumin

Nanocurcumin is a nanosized version of curcumin. The nano-formulation improves curcumin's bioavailability and solubility, allowing the body to absorb and utilize it more effectively. The synthesis of nanocurcumin solves the issues related with its administration, bioavailability, high rate of metabolism, and rapid expulsion, hence increasing its bioavailability and efficacy. The nanoparticle size improves curcumin's solubility in water, making it simpler to include into diverse items, such as supplements and functional foods. Nanocarriers can mix curcumin with other substances, increasing its medicinal potential.

2.3.1. Techniques

Through effective delivery methods, including nanoencapsulation, researchers have tried to improve curcumin's pharmacological and biological activities and get around some of its disadvantages. Nanocurcumin may be synthesized using a variety of methods [366], each having unique benefits and uses.

Different techniques are:

- Ultrasonication
- Fessi method
- Emulsion polymerization method
- Single emulsion method
- Green Synthesis
- Supercritical Fluid Technology
- Ionic gelation method
- Thin film hydration method
- Coacervation techniques
- Spray drying method Single emulsion method
- Electrospinning
- Antisolvent precipitation method
- Solid dispersion method
- Nanoprecipitation method
- Microemulsion
- Ultrasonic Dispersion

2.3.1.1. Different Curcumin Nano Formulations and Their Therapeutic Role

During last two decades many curcumin nano formulations have been prepared to overcome problems associated with its delivery and medicinal activities. The role of different curcumin nano formulations is described below**.**

2.3.1.1.1. Liposomes: Liposomes are spherical vesicles that closely mimic cell membranes in that they are made up of one or more phospholipid bilayers around aqueous units. It boosts curcumin's impact by dispersing it throughout the aqueous media and solubilizing it in the phospholipidic

bilayer [367]. Liposomes offer numerous benefits, including excellent biocompatibility and biodegradability, enhanced stability, low toxicity, improved solubility, targeted delivery to specific cells, controlled distribution, flexibility, and straightforward preparation [368]. Liposomal medicines often lung, accumulate in the liver, spleen, bone marrow, and other tissues and organs. This helps to increase the drug's therapeutic index and reduces negative effects. Extensive research revealed that liposomal curcumin was the best vehicle for treating various cancer types. Lung cancer, colorectal cancer, renal ischemia, melanoma, and malaria enhanced anticancer efficacy, superior bioactivity, increased antimalarial and antimelanoma effects, and increased encapsulation efficiency [369].

2.3.1.1.2. Polymers: Another popular and efficient drug delivery method for curcumin is polymers. It can increase curcumin's oral bioavailability and solubility. Colorectal cancer and wound healing shown improved anticancer action, enhanced cellular absorption, robust wound healing, prolonged blood circulation, tumor growth reduction, and more growth inhibition in cancer cells compared to free curcumin [370-372].

2.3.1.1.3. Gold nanoparticles: Gold nanoparticles have distinct physical and chemical characteristics, as well as diverse surface functions. It offers a versatile substrate for medication administration (curcumin). Prostate and colorectal cancer cells exhibited enhanced antioxidant activity, extended blood circulation, improved solubility and stability, increased biocompatibility, and notable anticancer efficacy [373,374].

2.3.1.1.4. Magnetic Nanoparticles: Magnetic nanoparticles are utilized in numerous applications, such as the delivery of medicinal compounds (like curcumin), hyperthermia treatment, and enhanced imaging quality. Cancer and inflammatory cells enhanced cellular uptake,

strengthened curcumin targeting abilities, facilitated magnetic resonance imaging, provided effective anti-inflammatory protection, regulated curcumin delivery, exhibited high biocompatibility, and demonstrated anticancer properties [375,376].

2.3.1.1.5. Solid Lipid Nanoparticles (SLNPs): Solid lipid nanoparticles (SLNs) possess a lipid core matrix capable of solubilizing various drugs, such as curcumin, with the stability of this lipid core being maintained by emulsifiers. Typically, SLNs exhibit a spherical shape. Allergy, colitis, cerebral ischemia, and breast cancer are conditions that benefit from improved blood circulation, which leads to increased anti-inflammatory effects, targeted and enhanced drug delivery to the brain, and greater efficacy in cancer treatment [377].

2.3.1.1.6. Conjugates: Conjugates are the complexes created when two or more molecules are joined together, particularly via covalent bonds. The conjugation of curcumin with small molecules and hydrophilic polymers improves its solubility and enhances its oral bioavailability. Fibroblasts, breast cancer, and amyloid fragments contribute to enhanced solubility, stability, and bioavailability, demonstrating significant anti-cancer efficacy, improved stability and bioavailability, as well as anti-amyloid properties [378].

2.3.1.1.7. Cyclodextrins: Cyclodextrins are oligosaccharides characterized by a bucket-like structure, recognized for their ability to enhance solubility and provide stabilization. It has the potential to dissolve curcumin within a lipophilic environment, while the hydrophilic exterior facilitates the dispersion of the formulation. This is relevant to conditions such as bowel disease, as well as various types of cancer including lung, pancreatic, breast, colorectal, and prostate cancers. The bioavailability and solubility were improved, along with enhanced anti proliferative,

anticancer, and anti-inflammatory properties, which were formulated and delivered as eye drops [379,380].

2.3.1.1.8. Solid Dispersions: Solid dispersions are characterized by the presence of one or more active ingredients within a specific matrix. This approach can enhance the bioavailability of hydrophilic pharmaceuticals, including curcumin. Benefits include effects on breast tumors, reduction of rat-paw edema, and promotion of wound healing, along with prolonged efficacy, anti-tumor and anti-metastatic properties, increased stability, enhanced bioavailability, anti-inflammatory and antibacterial effects, as well as improved healing of vaginal wounds [381].

2.3.1.1.9. Micelles: Micelles, ranging from 20 to 100 nanometers in size, are typically colloidal dispersions made up of amphiphilic molecules. They enhance the solubilization and targeted delivery of curcumin. This approach has been shown to improve the bioavailability and solubility of curcumin in lung tumors and colorectal cancer, leading to increased lifespan, customized drug delivery, high chemical stability, and enhanced antitumor and anticancer efficacy.

2.3.1.1.10. Nanospheres: Nanospheres are solid matrix particles in which the primary component (drug) is combined, whereas microcapsules include an interior core and an outside polymeric shell. Candida albicans, Staphylococcus aureus, and Escherichia coli are some of the bacteria. Breast cancer, melanoma, and Alzheimer's exhibited significant antibacterial and anticancer properties, efficient target delivery, and anti-amyloid impact [382,383].

2.3.1.1.11. Nanogels: A nanogel is a hydrogel-based nanoparticle that is created by carefully controlling the physical or chemical cross-linking of polymers. Nanogels' cross-linked structure provides a solid foundation for medication release and storage. It is a potential method for preparing and delivering active medications, such as curcumin, to cells for sustained action,

enhancing stability, and avoiding drug immunogenicity. Skin cancer cells, colorectal cancer, and pancreatic cancer improved anticancer activity, prolonged circulation, improved bioavailability, and targeted and regulated drug release [384,385].

2.3.1.1.12. Nanodisk: Nanodisks are disk-shaped bilayers that self-assemble and are stabilized by apolipoprotein. The solubility and targeted release of curcumin are improved. There is an enhancement in biological activity against Mantle Cell Lymphoma, promoting apoptosis and exhibiting anticancer properties. [386].

2.3.2. Application of Nanocurcumin

There are many therapeutic role of nanocurcumin which are shown in Figure 2.11.

2.3.2.1. Anti-inflammatory: Curcumin is a potential anti-inflammatory drug that works by inhibiting enzyme activity, cytokine synthesis, and transcription factor activation. Curcumin-loaded solid lipid nanoparticles (C-SLNs) were developed to enhance their effectiveness in a rat model of ovalbumin-induced asthma characterized by allergic reactions [387]. Experimental results showed that C-SLNs decreased airway hyper responsiveness and inflammatory cell infiltration. Additionally, C-SLNs predominantly suppressed the synthesis of T-helper-2-type cytokines, specifically interleukin-4 and interleukin-13, in Broncho alveolar lavage fluid. Nanocurcumin demonstrates efficacy against two esophageal adenocarcinoma (EAC) cell lines, OE33 and OE19. It enhances the sensitivity of EAC cells to cytotoxicity induced by T cells while simultaneously reducing pro-inflammatory signals originating from T cells [388]. Loss of NF-κβ activation reduces COX-2 and iNOS expression, preventing inflammation and carcinogenesis. The research indicated that curcumin-loaded PLGA nanoparticles (CUR-NP) diminish pro-inflammatory mediators in mammary tissues impacted by Staphylococcus aureus through the

enhancement of NF-κβ signaling. Furthermore, CUR-NP seems to serve as a more effective alternative to native curcumin in the treatment of murine mastitis [389]. A recent study indicates that the anti-inflammatory properties of curcumin liposomal formulations (CurLIP) in reaction to 2-hydroxyethyl methacrylate (HEMA) treatment in human dental pulp stem cells have enhanced the quality of dental care, resulting in a notable impact on the human community [390]. Nanocurcumin inhibits the synthesis of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β, which are critical in the inflammatory response. Nanocurcumin has been investigated for its effects on rheumatoid arthritis and inflammatory bowel disease (IBD). Research suggests possible advantages in neuroinflammation-related illnesses such as Alzheimer's disease. When paired with normal COVID-19 therapy, nanocurcumin has a powerful anti-inflammatory impact, aiding in the recovery from the acute inflammatory phase of the illness in hospitalized patients with mild-to-moderate disease severity. Nanocurcumin showed a positive impact in the treatment of central neurological disorder by reducing the expression of pro-inflammatory cytokines and raising the production of anti-inflammatory cytokines [391].

2.3.2.2. Anticancer Activity: Curcumin serves as a promising therapeutic agent for various types of cancers, including those of the lung, breast, prostate, myeloma, colorectal, pancreas, liver, and melanoma. Its efficacy is attributed to its ability to promote apoptosis, inhibit the proliferation of cancer cells, and impede the progression of the cell cycle [392]. Curcumin has been shown to inhibit cancer cell metastasis. Curcumin prevents cancer cells from attacking normal tissue by inhibiting matrix metalloproteinase activity, which regulates the process. Curcumin inhibits the production of the gene’s cyclin D1, BCL-2, BCL-xL, and c-myc, which are implicated in tumor development, proliferation, and apoptosis. Inhibiting nuclear factor-kappa (NF-κB) plays a crucial role in cancer development and progression. Curcumin has been shown to suppress NF-κB activity,

thereby promoting the expression of genes associated with cell proliferation (such as cyclin D1 and c myc), invasion (including matrix metalloproteinases), and resistance to apoptosis. Basniwal et al. conducted a study on the anticancer properties of curcumin nanoparticles, utilizing lung (A549), liver (HepG2), and skin (A431) cancer cell lines. It was demonstrated that curcumin nanoparticles exhibited a markedly enhanced effect on cancer cells in aqueous conditions compared to natural curcumin [393]. Triple-negative breast cancer (TNBC) represents one of the most important histological subtypes of breast tumors known for its metastatic properties. Research indicates that exogenous p53 and dendrosomal nanocurcumin collaborate effectively to suppress the proliferation of TNBC cells [394]. Research has demonstrated that anticancer drug combinations can enhance the effectiveness of chemotherapy-induced tumor growth suppression and death in ovarian cancer cell lines. Curcumin has been shown to have a multifunctional anticancer mechanism in primary epithelial ovarian cancer cells that are resistant to platinum and multidrug-resistance. Curcumin's usefulness is limited due to its poor bioavailability. Curcumin formulation as nanoparticles might increase absorption and efficacy [395]. A prior investigation indicated that both curcumin and nanocurcumin can enhance the anticancer efficacy of cisplatin by decreasing the volume and weight of ovarian tumors. No significant differences were observed in the effectiveness of curcumin compared to nanocurcumin regarding ovarian morphological outcomes. Nanocurcumin demonstrated superior results in comparison to curcumin regarding Ki-67 expressions, which are recognized prognostic markers and indicators of chemotherapy efficacy in ovarian cancer. The application of nanocurcumin has been evidenced to enhance its plasma concentrations, with the area under the curve (AUC) increasing by as much as 20 times.

2.3.2.3. Antioxidant Effects: Many researches have demonstrated curcumin's antioxidant action in biological models. There is a wealth of scientific data supporting curcumin's capacity to trap

free radicals such as reactive nitrogen and oxygen species in live cells via a variety of mechanisms, hence demonstrating antioxidant activity [396]. Curcumin has been shown to both boost the effectiveness of enzymes linked to free radical scavenging activity and to have inhibitory effects on the enzymes that generate free radicals [397]. In Wistar rats, curcumin nanocrystals prevented cardiovascular toxicity by lowering lipid peroxidation and enhancing the activity of detoxification and antioxidant enzymes [398]. The impact of curcumin and nano-curcumin on the oxidant and antioxidant mechanisms within liver mitochondria was examined using a rat model subjected to aluminum phosphide (AIP) toxicity. Nanocurcumin was shown to increase oxidative stress variables while protecting the liver from the harmful effects of AlP by scavenging free radicals and regulating the oxidative state [399].

2.3.2.4. Antigenotoxic Effect: According to the literature, nanocurcumin's antigenotoxic properties outperform those of free curcumin due to its higher solubility and bioavailability. In rats, nanocurcumin showed better effectiveness in tartazine-induced genotoxicity as determined by the comet assay [400]. It was demonstrated how nanocurcumin can protect against hyperthyroidism by reducing oxidative stress, inflammation, and DNA damage in rats [401]. When it comes to testicular genotoxicity caused by cyclophosphamide, curcumin nano formulation performs better than natural curcumin [402]. According to Yadav et al., nanocurcumin repaired DNA damage caused by co-exposure to fluoride and arsenic [403]. Nanocurcumin also restored chromosome damage caused by Iodine-131 in peripheral lymphocytes [404].

2.3.2.5. Nephroprotective Effect: It was discovered that the concentration of nanocurcumin in the renal tissues was greater than that of native curcumin. Compared to native curcumin, the nanocurcumin's higher bioavailability demonstrated superior renoprotective efficacy. By raising serum albumin levels and lowering blood urea nitrogen and creatinine levels, nanocurcumin

protects the kidneys from cisplatin-induced nephrotoxicity. Nanocurcumin was used to correct the histological aspects of kidney tissues that were altered by cisplatin [405]. By lowering blood urea and creatinine levels, nanocurcumin's superior solubility and delivery ability provide a greater renoprotective impact than curcumin against streptozotocin-induced diabetes [406]. Nanocurcumin enhances kidney function by inhibiting MMP-9 expression and reducing fibrosis area in rat kidneys with unilateral ureter blockage. El-Desoky et al. found that nanocurcumin reduced tartrazine-induced elevations in blood creatinine, urea, and uric acid, as well as alterations in the histology of kidney tissues [407].

2.3.2.6. Hepatoprotective Effect: Nanocurcumin has higher effectiveness and bioavailability than native curcumin. This increased effectiveness has several implications in medicine. For example, nanocurcumin has higher bioavailability and anti-oxidant activity in the mouse brain than curcumin. Nanocurcumin decreases oxidative stress signs as well as growing levels of AST and ALP. Nanocurcumin has a stronger hepatoprotective impact against acrylamide-induced stress than native curcumin [408]. Nanocurcumin reduces the hepatocarcinogenic impact of diethyl nitrosamine by lowering the toxicity of heterocyclic aromatic amines. According to Kheiripour et al. [409], nanocurcumin is more effective than natural curcumin at inhibiting paraquat-induced hepatotoxicity via regulating oxidative stress and gene expression in the Nrf2 signaling pathway. Paraquat distorts the usual configuration of liver cells and central veins, and nanocurcumin exposure aids in their rearranging. According to Mohammed et al., 2020, nanocurcumin can be used to treat diethyl nitrosamine-induced hepatic carcinoma by increasing serum albumin, glutathione peroxidase (GPx), glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) levels while decreasing malondialdehyde (MDA) levels [410].

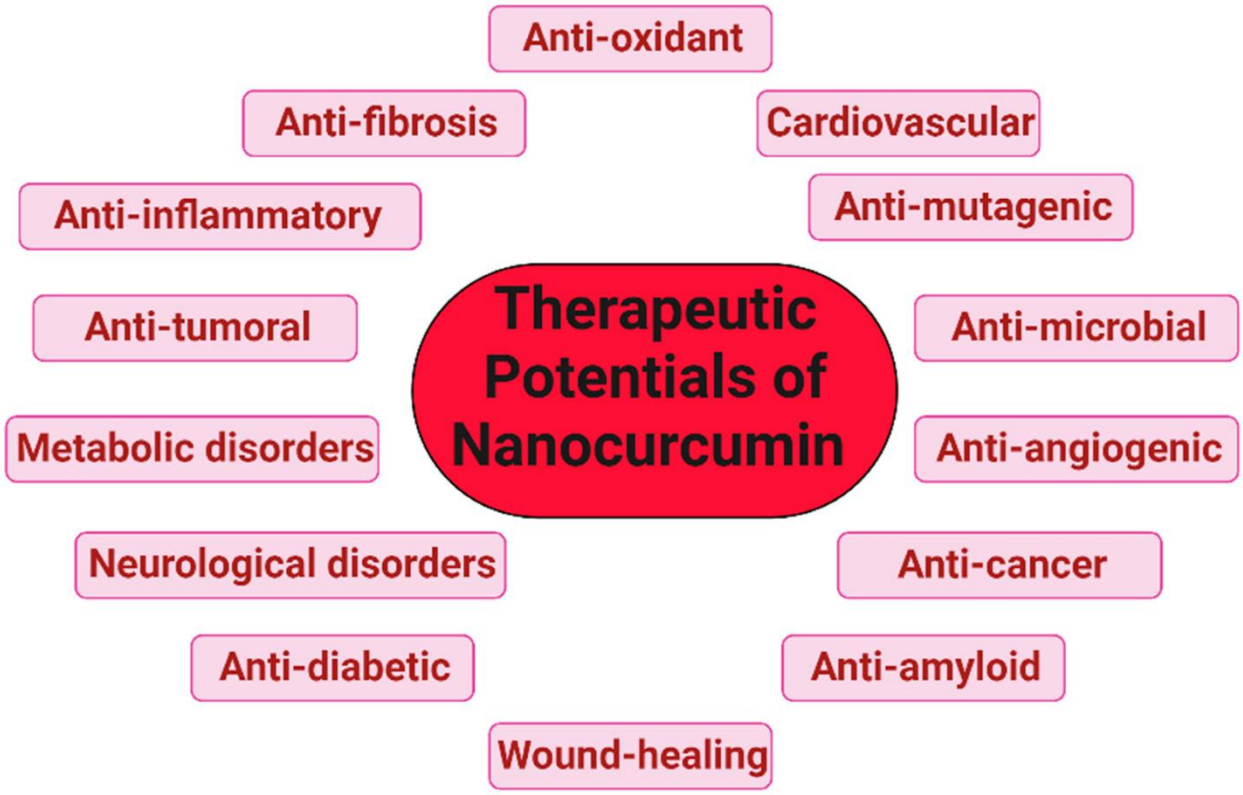


Figure 2.11: Different therapeutic effect of nanocurcumin.

2.3.3. Comparative Characteristics and Efficacy of Nanocurcumin and Curcumin as a Drug

Nanocurcumin's physical qualities are determined by both its chemical makeup and physical attributes. The transformation of regular curcumin into the nano form is largely influenced by its physical and chemical characteristics. Nanocurcumin is more effective than native curcumin due to essential physicochemical features such as particle size, surface area, surface charge, and hydrophobicity. Research has indicated that these characteristics may enhance solubility rates and increase oral bioavailability, which encompasses a favorable pharmacokinetic profile and active

targeting capabilities [411]. The characteristics of curcumin change with variations in particle size at the nanoscale. Research has shown that decreasing the particle size markedly improves the efficacy of nanocurcumin, rendering it more effective than its native form. Generally, nanoparticles ranging from 10 to 100 nm have been utilized in numerous therapeutic applications and clinical investigations [412]. Because of its higher surface area, nanocurcumin is regarded as an optimal therapeutic candidate when compared to standard curcumin. Nanocurcumin reaches organs that curcumin cannot normally access. It was discovered that nanocurcumin may have a better intracellular absorption capacity than regular curcumin. This characteristic is especially useful for targeting intracellular microorganisms in infectious disorders. Nanocurcumin has been shown to have higher systemic absorption in plasma and tissues than free curcumin [413]. Ma et al. have reported that nanocurcumin enhances in vivo bioavailability and tissue distribution, resulting in a 60-fold increase in biological half-life compared to traditional curcumin therapy in rat models [414]. Dende et al. discovered that nanocurcumin had higher bioavailability than natural curcumin and inhibits degenerative alterations in cerebral malaria tests. An oral dosage of 5 mg PLGA-curcumin with 350 µg of curcumin increased curcumin content in brain tissues thrice compared to 5 mg of native curcumin [415]. Curcumin nano formulation has also been shown to improve its circulation, retention, and mean residence duration inside the body [416]. Surface area is also an important characteristic of nanoparticles. Materials containing nanoparticles have a comparatively greater surface area, which enhances the rate of breakdown and water solubility, resulting in enhanced medication bioavailability. Nonetheless, a big surface area increases a drug's reaction to a specific molecular target and boosts its pharmacological efficacy [417]. Due to their increased surface area, drugs administered through nanoparticles will have enhanced exposure to the particle surface, facilitating swift drug release. Additionally, the substantial surface area of

nanoparticles sets them apart, rendering them highly suitable for a wide range of applications. The Brunauer-Emmett-Teller (BET) theorem is the simplest and most effective approach for determining the surface area of nanoparticle materials. Mohanty and Sahoo (2010) report pharmacological activity.

The significance of surface charges in curcumin nanoparticles has been recognized. Typically, the electric potential of nanoparticles is determined by their surface charge, which is entirely influenced by their chemical composition. Muller and Keck discovered that negative and positive zeta potentials inhibit nanoparticles from aggregating. Thus, nanoparticles are exceedingly stable in suspension. Curcumin tends to form aggregates and is susceptible to opsonization because of its poor solubility in water. In contrast, nanocurcumin completely dissolves in aqueous environments without forming aggregates, attributed to the presence of zeta potential. [418]. The positive charge acquired on the surface of nanoparticles is always regarded excellent since it may penetrate deep into cell membranes and absorb at a higher rate than negatively charged particles. Nanoparticles possessing a slight positive charge enhance their ability to be internalized, whereas an increased positive charge can lead to cellular toxicity [419]. Conversely, the negative charge does not penetrate the cell wall; instead, it serves to inhibit degradation under certain conditions and improves the stability of particles in circulation. Additionally, a correlation between surface charge and the antibacterial efficacy of nanocurcumin was identified by No et al. No et al. [420] found that positively charged curcumin nanoparticles had superior antibacterial action against Listeria monocytogenes.

Many biological activities rely on surface hydrophobicity, including protein adsorption and denaturation [421], immune cell activation, contact with biological membranes or cellular absorption, and increased toxicity [422]. Previous research demonstrated that the hydrophobicity

of nanomaterials has a direct impact on nanocarrier stability and biodistribution [423]. As a result, it is a key component under control in drug delivery systems. Curcumin's hydrophobic nature makes it difficult for it to penetrate the cell membrane and attach to the fatty acyl chains of membrane lipids through hydrophobic interactions and hydrogen bonds. As a result, there is relatively little curcumin in the cytoplasm. Curcumin nano formulations overcome these challenges and improved its bioavailability, showing potential as a medication delivery strategy. The choice of carrier system and preparation technique utilized to create nanodrugs have a significant impact on their loading and entrapment efficiency. Both have a significant influence on the quantity and degree of medication release from the carrier and are essential to drug delivery. Entrapment efficiency refers to the percentage of the drug that is successfully adsorbed or encapsulated within the nanoparticles, while loading efficiency pertains to the ratio of the drug to the carrier system [424].

REFERENCES

1. Mahmoudzadeh L, Froushani SMA, Ajami M, Mahmoudzadeh M. Effect of Nicotine on Immune System Function. Adv. Pharm. Bull. 2023; 13: 69-78.

2. Fagerström K. Nicotine: Pharmacology, Toxicity and Therapeutic use. J. Smok. Cessat. 2014; 9:53-59.

3. Tharappe JC, Cholewa J, Espandiari P, Spear BT, Gairola CG, Glauert HP. Effects of cigarette smoke on the activation of oxidative stress-related transcription factors in female a/j mouse lung. J. Toxicol. Environ. Health Sci. 2010; 73: 1288–1297.

4. Sansone L, Milani F, Fabrizi R, Belli M, Cristina M, Zagà V, Iure AD, Cicconi L, Stefano Bonassi S, Russo P. Nicotine: From Discovery to Biological Effects. Int. J. Mol. Sci. 2023; 26: 14570.

5. Global Burden of Disease. Institute of Health Metrics; 2019. IHME,accessed 17 July 2023.

6. World Health Organisation 2011. WHO report on the global tobacco epidemic Avenue Appia, CH-1211 Geneva 27, Switzerland. www. Who.int/tobacco.

7. Balfour DJK, Dani JA. Historical and Current Perspective on Tobacco use and Nicotine Addiction. Trends Neurosci. 2011; 34:383–392.

8. Rock VJ, Malarcher A, Kahende JW, Asman K, Husten C, Caraballo R. Cigarette smoking among adults--United States, 2006. Centers for Disease Control and Prevention. 2007; 56:1157-61.

9. Wadgave U, Nagesh L. Nicotine Replacement Therapy: An Overview. Int J Health Sci (Qassim). 2016; 10(3):425-35.

10. Mishra A, Chaturvedi P, Datta S, Sinukumar S, Joshi P, Garg A. Harmful effects of nicotine. Indian J Med Paediatr Oncol. 2015; 36: 24-31.

11. Chang CM, Corey CG, Rostron BL, Apelberg BJ. Systematic review of cigar smoking and all cause and smoking related mortality. BMC Public Health. 2015; 15: 390.

12. Jukema JB, Bagnasco DE, Jukema RA. Waterpipe smoking: not necessarily less hazardous than cigarette smoking: possible consequences for (cardiovascular) disease. Neth Heart J. 2014; 22: 91–99.

13. Qasim H, Alarabi AB, Alzoubi KH, Karim ZA, Alshbool FZ, Khasawneh FT. The effects of hookah/waterpipe smoking on general health and the cardiovascular system. Environ Health Prev. Med. 2019; 24: 58.

14. Clarke E, Thompson K, Weaver S, Thompson J, O'Connell G. Snus: a compelling harm reduction alternative to cigarettes. Harm. Reduct. J. 2019; 16: 62.

15. Warnakulasuriya S, Straif K. Carcinogenicity of smokeless tobacco: Evidence from studies in humans & experimental animals. Indian J Med Res. 2018; 148(6):681-686.

16. Gupta B, Kumar A, Chaturvedi P. Bidi smoking and its impact on health. J. Epidemiol. Community Health. 2010; 64: 207-212.

17. Brown CJ, Cheng JM. Electronic cigarettes: Product characterisation and design considerations.Tob. Control. 2014; 2(2): 4–10.

18. Narake SS, Gupta PC. Nasal use of snuff. Indian J Cancer. 2014; 51(1):288.

19. Bhatta DN., et al. "Respiratory effects of kretek smoking." Tobacco Control. 2020; 29: 253-259.

20. Sharma A, et al. "Oral health consequences of smoking kretek." Journal of Dental Research, 2021; 100: 45-52.

21. Henningfield JE, Neal LB. Dissolvable Tobacco Products: A Public Health Perspective. Tob. Control.2013; 22: 212–219.

22. Kumar S, et al. "Health risks associated with dissolvable tobacco products." Tobacco Control. 2014; 23: 162-167.

23. Qasim H, Alarabi AB, Alzoubi KH, Karim ZA, Alshbool FZ, Khasawneh FT. The effects of hookah/waterpipe smoking on general health and the cardiovascular system. Environ Health Prev Med. 2019; 24(1):58.

24. Gupta PC, and Samira A. Bidi Smoking and public health. MoHFW.2016.

25. Malik VS, Matthias BS, Frank BH. Intake of Sugar-Sweetened Beverages and Weight Gain: A Systematic Review. AJCN. 2006; 84 (2):274–288.

26. Paula M, Petersen PE. Diet, Nutrition and the Prevention of Dental Diseases.Public Health Nutr.2004; 1:201–226.

27. Gupta PC, Samira A. Bidi Smoking and public health. MoHFW. 2014.

28. National Institute of Dental and Craniofacial Research (NIDCR). "Oral Health and Tobacco Use." Fact Sheet. Available at: https://www.nidcr.nih.gov/research/data-statistics/tobacco-use.

29. Niaz K, Maqbool F, Khan F, Bahadar H, Hassan FI, Abdollahi M. Smokeless tobacco (paan and gutkha) consumption, prevalence, and contribution to oral cancer. Epidemiol Health. 2017; 9:2017009.

30. Banerjee SC, Ostroff JS, Bari S, D'Agostino TA, Khera M, Acharya S, et al. Gutka and Tambaku paan use among South Asian immigrants: a focus group study. J Immigr Minor Health. 2014; 16: 531-539.

31. Stratton K, Shetty P, Wallace R, Bondurant, S. Institute of Medicine: Clearing the smoke: Assessing the science base for tobacco harm reduction. Washington, DC: National Academy Press. 2001.

32. Armstrong DW, Wang X, Ercal N. Enantiomeric composition of nicotine in smokeless tobacco, medicinal products and commercial reagents. In Chirality: Special issue: Proceedings from the Eighth International Symposium on Chiral Discrimination. 1998; 10: 587–591.

33. Willits CO, Swaim ML, Connelly JA, Brice BA. Spectrophotometric determination of nicotine. Anal. Chem. 1950; 22: 430–433.

34. Hegde VH, Ambili. Comparison of Nicotine Concentration and PH of Commercially Available Smokeless Tobacco Products. J. Oral. Res. Rev.2017; 9:21-24.

35. Murphy SE. Biochemistry of Nicotine Metabolism and Its Relevance to Lung Cancer. J Biol. Chem. 2021; 296: 100722.

36. Benowitz NL, Jacob P 3rd, Fong I, Gupta S. Nicotine metabolic profile in man: comparison of cigarette smoking and transdermal nicotine. J. Pharmacol. Exp. Ther. 1994; 268: 296–303.

37. Perez-Paramo YX, Chen G, Ashmore JH, Watson CJW, Nasrin S, Adams-Haduch J, Wang R, Gao YT, Koh WP, Yuan JM, Lazarus P. Nicotine-N'-Oxidation by Flavin Monooxygenase Enzymes. Cancer Epidemiol. Biomarkers Prev. 2019; 28: 311-320.

38. Yildiz D. Nicotine, its metabolism and an overview of its biological effects. Toxicon. 2004; 619-632.

39. Hukkanen J, Jacob P 3rd, Benowitz NL. Metabolism and disposition kinetics of nicotine. Pharmacol Rev. 2005; 57:79–115.

40. Catassi A, Servent D, Paleari L, Cesario A, Russo P. Multiple Roles of Nicotine on Cell Proliferation and Inhibition of Apoptosis: Implications on Lung Carcinogenesis. Mutat. Res. 2008; 659: 221-231.

41. Breese CR, Marks MJ, Logel J, Adams CE, Sullivan B, Collins AC, Leonard S. Effect of smoking history on [3H] nicotine binding in human postmortem brain. J. Pharmacol. Exp.Ther. 1997; 282: 7–13.

42. Perry DC, Davila-Garcia MI, Stockmeier CA, Kellar KJ. Increased nicotinic receptors in brains from smokers: membrane binding and autoradiography studies. J. Pharmacol. Exp. Ther. 1999; 289:1545–1552.

43. Aoki Y, Ikeda T, Tani N, Shida A, Oritani S, Ishikawa T. Evaluation of the Distribution of Nicotine Intravenous Injection: An Adult Autopsy Case Report with a Review of Literature. Int. J. Legal Med. 2020; 134: 243-249.

44. Lindell G, Lunell E, Graffner H. Transdermally administered nicotine accumulates in gastric juice. Eur. J Clin. Pharmacol. 1996; 51: 315–318.

45. Dahlstrom A, Lundell B, Curvall M, Thapper L. Nicotine and cotinine concentrations in the nursing mother and her infant. Acta. Paediatr. Scand. 1990; 79:142–147.

46. Dempsey DA, Benowitz NL. Risks and benefits of nicotine to aid smoking cessation in pregnancy. Drug Saf. 2001; 24: 277–322.

47. Rose JE, Behm FM, Westman EC, Coleman RE. Arterial Nicotine Kinetics during Cigarette Smoking and Intravenous Nicotine Administration: Implications for Addiction. Drug Alcohol Depend. 1999; 56: 99-107.

48. Gourlay SG, Benowitz NL. Arteriovenous Differences in Plasma Concentration of Nicotine and Catecholamines and Related Cardiovascular Effects after Smoking, Nicotine Nasal Spray, and Intravenous Nicotine. Clin. Pharmacol. Ther. 1997; 62: 453-463.

49. Henningfield JE, Keenan RM. Nicotine Delivery Kinetics and Abuse Liability. J Consult Clin Psychol. 1993; 61:743-750.

50. Lunell E, Molander L, Ekberg K, Wahren J. Site of Nicotine Absorption from a Vapour Inhaler–Comparison with Cigarette Smoking. Eur. J. Clin. Pharmacol. 2000; 55:737-741.

51. Perlman HH, Dannenberg AM, Bokolof N. The excretion of nicotine in breast milk and urine from cigarette smoking its effect on location and the nursling. J. Am. Med. Assoc. 1992; 120: 1003–1009.

52. Balabanova S, Buhler G, Schneider E, Boschek HJ, Schneitler H. Nicotine excretion by the apocrine and eccrine sweat in smokers and passive smokers. Hautarzt. 1992; 43: 73–76.

53. Seaton MJ, Kyerematen GA, Vesell ES. Rates of excretion of cotinine, nicotine glucuronide, and 3-hydroxycotinine glucuronide in rat bile. Drug Metab. Dispos. 1993; 21: 927–932.

54. Luck W, Nau H. Nicotine and cotinine concentrations in serum and urine of infants exposed via passive smoking or milk from smoking mothers. J. Pediatr. 1985; 107:816–820.

55. Benowitz NL, Jacob P. Nicotine renal excretion rate influences nicotine intake during cigarette smoking.J. Pharmacol. Exp. Ther. 1985; 234: 153–155.

56. Becket AH, Rowland M, Triggs EJ. Significance of smoking in investigation of urinary excretion rates of amines in man. Nature. 1965; 207: 200–201.

57. Gotti C, Zoli M, Clementi F. Brain nicotinic acetylcholine receptors: native subtypes and their relevance. Trends Pharmacol. Sci. 2006; 27: 482–91.

58. Picciotto MR, Zoli M, Rimondini R, L´ena C, Marubio M. Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine. Nature. 1998; 391: 173–77.

59. Maskos U, Molles BE, Pons S, Besson M, Guiard BP. Nicotine reinforcement and cognition restored by targeted expression of nicotinic receptors. Nature. 2005; 436:103–107.

60. Tapper AR, McKinney SL, Nashmi R, Schwarz J, Deshpande P. Nicotine activation of alpha4∗ receptors: sufficient for reward, tolerance, and sensitization. Science. 2004; 306:1029–1032.

61. Aberger K, Chitravanshi VC, Sapru HN. Cardiovascular responses to microinjections of nicotine into the caudal ventrolateral medulla of the rat. Brain Res. 2001; 892:138–146.

62. Levin ED, Bettegowda C, Blosser J, Gordon J. AR-R17779, and alpha7 nicotinic agonist, improves learning and memory in rats. Behav. Pharmacol. 1999; 10:675–80.

63. Haj´os M, Hurst RS, Hoffmann WE, Krause M, Wall TM. The selective alpha7 nicotinic acetylcholine receptor agonist PNU-282987 [N-[(3R)-1-Azabicyclo[2.2.2]oct-3-yl]-4-chlorobenzamide hydrochloride] enhances GABAergic synaptic activity in brain slices and restores auditory gating deficits in anesthetized rats. J. Pharmacol. Exp. Ther. 2005; 312:1213–1222.

64. Salas R, Orr-Urtreger A, Broide RS, Beaudet A, Paylor R, De Biasi M. The nicotinic acetylcholine receptor subunit alpha 5 mediates short-term effects of nicotine in vivo. Mol. Pharmacol. 2003; 63:1059–1066.

65. Carstens E, Carstens MI. Sensory Effects of Nicotine and Tobacco. Nicotine Tob. Res. 2022; 24: 306-315.

66. D'Souza MS, Markou A. Neuronal Mechanisms Underlying Development of Nicotine Dependence: Implications for Novel Smoking-Cessation Treatments. Addict Sci. Clin. Pract. 2011; 6: 4-16.

67. Mameli-Engvall M, Evrard A, Pons S, Maskos U, Svensson TH, Changeux JP, Faure P. Hierarchical control of dopamine neuron-firing patterns by nicotinic receptors. Neuron. 2006; 50: 911-21.

68. Picciotto MR, Higley MJ, Mineur YS. Acetylcholine as a neuromodulator: cholinergic signalling shapes nervous system function and behavior. Neuron. 2012; 76: 116-29.

69. Wills L, Kenny PJ. Addiction-related neuroadaptations following chronic nicotine exposure. J. Neurochem. 2021; 157: 1652-1673.

70. Salas R, Pieri F, De Biasi M. Decreased signs of nicotine withdrawal in mice null for the β4 nicotinic acetylcholine receptor subunit. J. Neurosci. 2004; 24: 10035–10039.

71. Salas R, Sturm R, Boulter J, De Biasi, M. Nicotinic receptors in the habenulo-interpeduncular system are necessary for nicotine withdrawal in mice. J. Neurosci. 2009; 29: 3014–3018.

72. Hughes JR, Hatsukami D. Signs and symptoms of tobacco withdrawal. Arch. Gen. Psychiatry. 1986; 43:289–294.

73. Hughes JR. Clinical significance of tobacco withdrawal. Nicotine Tob. Res. 2006; 8:153–156.

74. Koob GF, LeMoal M. Drug abuse: Hedonic homeostatic dysregulation. Science. 1997; 278: 52–58.

75. Hopkins JM, Evans HJ. Cigarette smoke-induced DNA damage and lung cancer risks. Nature. 1980; 283: 388–390.

76. Jones NJ, Kadhim MA, Hoskins PL, Parry JM, Waters R. 32P- postlabelling analysis and micronuclei induction in primary Chinese hamster lung cells exposed to tobacco particulate matter. Carcinogenesis. 1991; 12:1507–1514.

77. Bishun NP, Lloyd N, Raven RW, Williams DC. The in vitro and in vivo cytogenetic effects of nicotine. Acta. Biol. Acad. Sci. Hung. 1972; 23:175–180.

78. Arabi M. Nicotinic infertility: assessing DNA and plasma membrane integrity of human spermatozoa. Andrologia. 2004; 36:305–310.

79. Argentin G, Cicchetti R. Genotoxic and antiapoptotic effect of nicotine on human gingival fibroblasts. Toxicol. Sci. 2004; 79:75-81.

80. Dalberto D, Nicolau CC, Garcia ALH, Nordin AP, Grivicich I, Silva JD. Cytotoxic and genotoxic evaluation of cotinine using human neuroblastoma cells (SH-SY5Y). Genet. Mol. Biol. 2020; 43: 20190123.

81. Sobkowiak R, Musidlak J, Lesicki A. In vitro genoprotective and genotoxic effect of nicotine on human leukocytes evaluated by the comet assay. Drug Chem Toxicol. 2014; 37:322-328.

82. Mohanapriya S, Maheswaran T, Ganapathy N, Yoithapprabhunath TR, Dineshshankar J, Ilayaraja V, Vinodhini RS, Devi R. Evaluation of DNA damage in tobacco associated human buccal cells using comet assay. Med Pharm Rep. 2021; 94: 214-219.

83. Sudheer AR, Muthukumaran S, Kalpana C, Srinivasan M, Menon VP. Protective changes in cultured rat peripheral blood lymphocytes: a comparison with N-acetylcysteine. Toxicol. In Vitro. 2007; 21:576-585.

84. Cordelli E, Bignami M, Pacchierotti F. Comet assay: a versatile but complex tool in genotoxicity testing. Toxicol. Res. 2021; 10:68-78.

85. Yang YS, Su YF, Yang HW, Lee YH, Chou JI, Ueng KC. Lipid-lowering effects of curcumin in patients with metabolic syndrome: a randomized, double blind, placebo-controlled trial. Phytother. Res. 2014; 28:1770-1777.

86. Sharifi-Rad J, Rayess YE, Rizk AA, Sadaka C, Zgheib R, Zam W, Sestito S, Rapposelli S, Neffe-Skocińska K, Zielińska D, Salehi B, Setzer WN, Dosoky NS, Taheri Y, El Beyrouthy M, Martorell M, Ostrander EA, Suleria HAR, Cho WC, Maroyi A, Martins N. Turmeric and Its Major Compound Curcumin on Health: Bioactive Effects and Safety Profiles for Food, Pharmaceutical, Biotechnological and Medicinal Applications. Front. Pharmacol. 2020; 11:01021.

87. Mansour SN, Mosaferi M, Babaie J, Mohammadpoorasl A, Dehghanzadeh R,Nikniaz L ,Miri M.Interplay of arsenic exposure and cigarette smoking on oxidative DNA damage in healthy males. Environ. Sci. Eur. 2024; 10: 1186.

88. Tiwari RK, Sharma V, Pandey RK, Shukla SS. Nicotine Addiction: Neurobiology and Mechanism. J. Pharmacopunct. 2020; 23:1–7.

89. Benowitz NL, Warner KE, Myers, ML, Hatsukami D, Ph.D., Berman ML, Vallone D, Cohen JE. How the FDA can improve public health — Helping people stop smoking. N. Engl. J. Med. 2023; 388:1540-1542.

90. Huang SH, Weng KP, Huang SM. The effects of maternal smoking exposure during pregnancy on postnatal outcomes: a cross sectional study. J. Chin. Med. Assoc. 2017; 80:796-802.

91. Benowitz NL, Helen GS, Liakoni E. Clinical Pharmacology of Electronic Nicotine Delivery Systems (ENDS): Implications for Benefits and Risks in the Promotion of the Combusted Tobacco Endgame. J. Clin. Pharmacol. 2021; 2: 18-36.

92. Zhang W, Lin H, Zou M, Yuan Q, Huang Z, Pan X, Zhang W. Nicotine in Inflammatory Diseases: Anti-Inflammatory and Pro-Inflammatory Effects. Front. Immunol. 2022; 13: 826889.

93. Mehranfard N, Ghasemi M, Rajabian A, Ansari L. Protective potential of naringenin and its Nano formulations in redox mechanisms of injury and disease. Heliyon. 2023; 9: 22820.

94. Cha SR, Jang J, Park SM, Ryu SM, Cho SE, Yang SR. Cigarette Smoke-Induced Respiratory Response: Insights into Cellular Processes and Biomarkers. Antioxidants. 2023; 12: 1210.

95. Zheng CM, Lee YH, Chiu IJ, Chiu YJ, Sung LC, Hsu YH, Chiu HW. Nicotine Causes Nephrotoxicity through the Induction of NLRP6 Inflammasome and Alpha7 Nicotinic Acetylcholine Receptor. Toxics 2020; 8: 86-92.
96. Lang SM, Schiffl H. Smoking status, cadmium, and chronic kidney disease. Ren. Replace. Ther. 2024; 10: 17.

97. Wang X, Su S. The hidden impact: the rate of nicotine metabolism and kidney health. Front. Endocrinol. 2024; 15: 1424068.

98. Chen Z, Ma Y. Effects of nicotine on liver health and implications for metabolic disease. Hepatol. 2022; 52: 1234–1245.

99. Zhang Y, Li X, Chen W, Huang Z, Zhou P. Impact of nicotine on oxidative stress and mitochondrial dysfunction in liver tissues. J. Hepatol. 2023; 48: 342-356.

100. Kim J, Lee S. Role of nicotine in exacerbating inflammation and liver damage through upregulation of pro-inflammatory cytokines. J. Hepatol. 2022; 45: 123-135.

101. Gupta A, Sharma P, Mehta V, Singh R. Impact of smoking on fibrosis progression in chronic hepatitis C patients. J. Hepatol. 2022; 76: 456-467.

102. Johnson D, Martinez L, Patel R, Wang T. Nicotine and carboxyhemoglobin levels in relation to secondary polycythemia. J. Hematol. Res. 2023; 58: 275-289.

103. Kumar P, Singh A, Gupta R, Verma S. Cardiovascular risks associated with smoking-induced polycythemia. Cardiovasc. Med. 2023; 47: 89-102.

104. Singh R, Singh S. Histopathological changes in rat liver following chronic nicotine exposure. J. Exp. Biol. Med. 2019; 44: 215-223.

105. Iranloye BO, Bolarinwa AF. Effect of nicotine administration on the histology of the liver in male rats. Afr. J. Med. Sci. 2009; 38: 149-154.

106. Marzouk MA, El-Sheikh M, Salem A. Long-term nicotine exposure induces liver damage: A histopathological study. Int. J. Hepatol. 2002; 12: 311-320.

107. Amer MS, Mahmoud HR. Nicotine-induced liver injury in rats: A histological and biochemical study. Egypt. J. Pathol. 2014; 34: 45-54.

108. Syed AM, Shangloo S. Effects of nicotine on liver architecture and function in a rat model. Journal of Clinical and Experimental Hepatology. J. Clin. Exp. Hepatol. 2020; 10: 123-131.

109. Wannamethee SG, Shaper AG. Cigarette smoking and its association with liver enzymes: Increased gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP) levels and an inverse relationship with aspartate aminotransferase (AST). Eur. J. Clin. Nutr. 2010; 64: 1190-1197.

110. Rutledge SM, Asgharpour A. The effect of nicotine on liver enzymes among HCV-positive patients: A focus on alanine aminotransferase (ALT). J. Hematol. Res. 2020; 52: 789-797.

111. Wang Y, Zhu H, Li X. Nicotine-induced elevation of ALT levels in hepatitis C virus-positive patients. Hepatol. Int. 2002; 16: 115-123.

112. Abdul-Razaq H, Ahmed Z. Effect of smoking on liver enzymes and bilirubin levels: A comparative study. Clin. Biochem. 2013; 25: 245-25.

113. Khaitan T, Gupta S, Mehta R. Evaluation of liver enzyme alterations in chronic smokers: A clinical study. Ind. J. Clin. Biochem. 2019; 34: 197-203.

114. Setyawati M, Anggraeni A. The impact of smoking on liver enzyme activity and bilirubin levels. J. Med. Sci. 2018; 38: 123-129.

115. Alsalhen K, Abdalsalam A. The effect of chronic smoking on liver function biomarkers. Int. J. Hepatol. 2014; 12: 56-62.

116. Jain S, Jaimes EA. The effects of nicotine on protein levels in liver tissue: A biochemical analysis. Hepat Res. 2013; 21: 111-118.

117. Alsalhen K, Abdalsalam A. The impact of nicotine on total protein levels in liver and its relation to hepatic function. Int. J. Hepat. Std. 2014; 12: 56-62.

118. Wang H, Li X, Zhang Y, Liu M. Nicotine treatment in rats caused considerable follicular depletion and elevated rates of follicular atresia, reducing reproductive capacity and hastening ovarian aging. Reprod. Biol. Endocrinol. 2023; 41: 345-356.

119. Li X, Chen Y. Hormone production and regulation in the ovary are disrupted by nicotine, affecting the hypothalamic-pituitary-ovarian (HPO) axis, resulting in altered sex hormone levels that may impact fertility and menstrual cycle regularity. Endocrinol. Reprod. Med. 2022; 29: 204-214.

120. Wannamethee SG, Shaper AG. Nicotine-induced oxidative stress harms ovarian cells, lowering viability and resulting in a depleted ovarian reserve, which can impair fertility and reproductive health. Eur. J. Clin. Nutr. 2010; 64: 1190-1197.

121. Zhao J, Zhang L, Liu T, Wang Y. Nicotine-induced oxidative stress harms ovarian cells, lowers viability, and depletes ovarian reserve, impairing fertility and reproductive health. Reprod. Toxicol. 2023; 95: 123-134.

122. Anna L, Garcia M, Thompson R. The effects of nicotine on cellular function: Implications for cardiovascular disease and cancer development. J. Cell. Biochem. 1987; 56: 145-152.

123. Heusch G, Maneckjee R. Nicotine and its role in cardiovascular disease and lung cancer. Cardiovasc. Res. 1998; 39: 505-512.

124. Ahmed S, Khan M, Rahman M. Nicotine and its impact on lipid profiles: Elevated triglycerides, reduced HDL, and increased LDL in human and animal models, contributing to atherosclerosis risk. J. Lipid Res. 2023; 64: 145-158.

125. Singh A, Sharma P, Gupta R. Nicotine and its effects on lipid profiles: A significant increase in VLDL cholesterol levels. J. Clin. Lipidol. 2016; 10: 312-320.

126. Zhao L, Wang Q. Nicotine-induced oxidative stress and its role in endothelial dysfunction and nitric oxide synthesis impairment: Implications for cardiovascular disease. Cardiovasc. Res. 2022; 118: 847-860.

127. Pre J, Smith R, Patel K. Increased lipid peroxidation levels in smokers: A biochemical study. J. Environ. Health. 1989; 51: 235-240.

128. Ashakumary L, Vijayammal P. Effects of nicotine on lipid peroxidation and antioxidant enzyme activity in rat tissues Indian J. Exp. Biol. 1996; 34: 573-576.

129. Liu H, Tan Y. Nicotine accelerates lipid accumulation within arterial walls, creating plaque deposits and increasing the risk of coronary artery disease and other cardiovascular complications. J. Cardiovasc. Dis. Res. 2023; 58: 35-45.

130. American Heart Association (AHA). 2023.

131. Benowitz NL. Nicotine addiction. N Engl J Med. 2010; 362:2295–2303.

132. Stanfill SB, Hecht SS, Joerger AC, Gonzalez PJ, Maia LB, Rivas MG. From cultivation to cancer: formation of N-nitrosamines and other carcinogens in smokeless tobacco and their mutagenic implications. Crit. Rev. Toxicol. 2023; 53:658-701.

133. Wells SI, Aronow BJ, Wise TM, William SS, Couge JA, Howley PM. Transcriptome signature of irreversible senescence in human papillomavirus-positive cervical cancer cells. Proceedings of the National Academy of Sciences of the United States of America. 2003; 100: 7093-7098.

134. Dehn D, Torkko KC, Shroyer KR. Human papillomavirus testing and molecular markers of cervical dysplasia and carcinoma. Cancer. 2007; 111: 1-14.

135. Chakrabarti O, Krishna S. Molecular interactions of "high risk" human papillomaviruses E6 and E7 oncoproteins: implications for tumour progression. J. Biosci. 2003; 28:337-48.

136. Castellsagué X, Diaz M, Sanjosé SD, Muñoz N, Herrero R, Franceschi S, Peeling RW, Ashley R, Smith JS, Snijders PJF, Meijer CJLM, Bosch FX, Plummer M, Moreno VP, Ruiz Alonso de, Chichareon SC, J Ngelangel, Eluf-Neto Roló A, Caceres E, Santos C, Chaouki N, Gueddari BEl, Hammouda D, Rajkumar T. Worldwide human papillomavirus etiology of cervical adenocarcinoma and its cofactors: implications for screening and prevention. Journal of the National Cancer Institute, J. Nat. Cancer Ins. 2006; 98:303-15.

137. Jayachandra S, Selvaraj R, Agnihotram G. Determination of serum total antioxidant capacity in male smokers and non-smokers. Nat. J Physiol. Pharm. Pharmacol. 2017; 7:591.

138. Azzi A. Molecular mechanism of α-tocopherol action. Free Radic. Biol Med. 2007; 43:16–21.

139. Erel O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin. Biochem. 2004; 37:277–285.

140. Gajowiak A, Ziemiński M. Nicotine-induced oxidative stress and its link to cardiovascular disease and cancer. J. Toxicol. Environ. Health. 2021; 84: 345-358.

141. Masella R, Di Benedetto R, Varì R, Sileo F. The role of lactate dehydrogenase (LDH) as a marker of cell damage and its relationship with stroke, heart attack, and other tissue injuries. Free Radic. Biol. Med. 2005; 39: 1187-1195.

142. Chelikhani HS, Ahmadi R, Ghaffari M. The role of catalase (CAT) in protecting cells from oxidative stress: Conversion of hydrogen peroxide into water and oxygen. Cell Biochem. Biophys. 2004; 42: 123-131.

143. Yang Y, Zhang X, Liu Q. Catalase localization in peroxisomes and mitochondria and its implications for cellular hydrogen peroxide detoxification. J. Cell. Physiol. 2014; 229: 1840-1849.

144. Maier CM, Chan SH. Superoxide dismutase (SOD) and its role in the antioxidant defense mechanism: Catalysis of superoxide transformation to oxygen and hydrogen peroxide. J. Cell. Biochem. 2002; 82: 84-92.

145. Bhabak KP, Mugesh G. Functional mimics of glutathione peroxidase: bioinspired synthetic antioxidants. Acc. Chem. Res. J. 2010; 43:1408-1419.

146. Oyeyipo IP, Raji Y, Bolarinwa AF. Nicotine alters serum antioxidant profile in male albino rats. N Am J Med Sci. 2014; 6:168-71.

147. Aspera-Werz RH, Ehnert S, Heid D, Zhu S, Chen T, Braun B, Sreekumar V, Arnscheidt C, Nussler AK. Nicotine and Cotinine Inhibit Catalase and Glutathione Reductase Activity Contributing to the Impaired Osteogenesis of SCP-1 Cells Exposed to Cigarette Smoke. Oxid Med. Cell Longev. 2018; 2018:3172480.

148. Raddam QN, Moafaq MZ, Mostafa AA, Nahla KA. Smoking Effects on Blood Antioxidants Level: Lactate Dehydrogenase, Catalase, Superoxide Dismutase and Glutathione Peroxidase in University Students. J. Clin. Exp. Pathol. 2017; 7: 331.

149. Agarwal P, Bagewadi A, Keluskar V, Vinuth DP. Superoxide dismutase, glutathione peroxidase, and catalase antioxidant enzymes in chronic tobacco smokers and chewers: A case-control study. Indian J. Dent. Res. 2019; 30:219-225.

150. Delijewski M, Wrześniok D, Otręba M, Beberok A, Rok J, Buszman E. Nicotine impact on melanogenesis and antioxidant defense system in HEMn-DP melanocytes. Mol Cell Biochem. 2014; 395:109-16.

151. Sezer Z, Yilmaz TE, Gungor ZB, Kalay F, Guzel E. Effects of vitamin E onnicotine-induced lipid peroxidation in rat granulosa cells: Folliculogenesis Reprod. Biol. 2020; 20: 63-74.

152. Chattopadhyay K, Chattopadhyay BD. Effect of nicotine on lipid profile, peroxidation & antioxidant enzymes in female rats with restricted dietary protein. Indian J. Med. Res. 2008; 127:571-576.

153. Ben Saad A, Rjeibi I, Alimi H, Ncib S, Bouhamda T, Zouari N. Protective effects of Mentha spicata against nicotine-induced toxicity in liver and erythrocytes of Wistar rats. Appl. Physiol. Nutr. Metab. 2018; 43:77-83.

154. Khademi F, Totonchi H, Mohammadi N, Zare R, Zal F. Nicotine-Induced Oxidative Stress in Human Primary Endometrial Cells. Int. J. Toxicol. 2019; 38:202-208.

155. Sokolowska M, Quesniaux VFJ, Akdis CA, Chung KF, Ryffel B, Togbe D. Acute Respiratory Barrier Disruption by Ozone Exposure in Mice. Front. Immunol. 2019; 10:2169.

156. Durazzo TC, Mattsson N, Weiner MW. Alzheimer's disease Neuroimaging Initiative. Smoking and increased Alzheimer's disease risk: A review of potential mechanisms. Alzheimers Dement. 2014; 10: 122-245.

157. Shein M, Jeschke G. Comparison of Free Radical Levels in the Aerosol from Conventional Cigarettes, Electronic Cigarettes, and Heat-Not-Burn Tobacco Products. Chem. Res. Toxicol. 2019; 32:1289-1298.

158. Bergström J. Tobacco smoking and chronic destructive periodontal disease. Odontology. 2004; 92:1-8.

159. Zhang W, Lin H, Zou M, Yuan Q, Huang Z, Pan X, Zhang W. Nicotine in Inflammatory Diseases: Anti-Inflammatory and Pro-Inflammatory Effects. Front Immunol. 2022; 13:826889.

160. Adeyemi DH, Oyeyipo IP, Akanbi KA, Oluwole T. Nicotine alters progesterone and estradiol levels during the first trimester of pregnancy in Wistar rats. JBRA Assist. Reprod. 2018; 22:78-81.

161. Hirano T, Nakajima K, Hibi M. Signaling mechanisms through gp130: a model of the cytokine system. Cytokine Growth Factor Rev. 1997; 8:241–52.

162. Hirano T, Ishihara K, Hibi M. Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors. Oncogene. 2000; 19:2548–56.

163. Ung TT, Nguyen TT, Lian S, Li S, Xia Y, Kim NH, Jung YD. Nicotine stimulates IL-6 expression by activating the AP-1 and STAT-3 pathways in human endothelial EA. hy926 cells. J Cell. Biochem. 2019; 120: 5531-5541.

164. Carpagnano GE, Kharitonov SA, Foschino-Barbaro MP, Resta O, Gramiccioni E, Barnes PJ. Eur. Res. J. 2003; 21: 589-593.

165. Jamil A, Rashid A, Naveed AK, Asim M. Effect of smoking on interleukin-6 and correlation between IL-6 and serum amyloid A-low density lipoprotein in smokers. J Postgrad Med Inst. 2017; 31:336-8.

166. Al-tameemi S, Hameed N, Gomes K, Abid H. Cigarette smoking increases plasma levels of IL-6 and TNF-α. Baghdad Journal of Biochemistry and Applied Biological Sciences. 2022; 3:60-68.

167. Gadani SP, Cronk JC, Norris GT, Kipnis J. "IL-4 in the brain: a cytokine to remember". J. Immun. 2012; 189: 4213–4219.

168. Zhu J. T helper 2 (Th2) cell differentiation, type 2 innate lymphoid cell (ILC2) development and regulation of interleukin-4 (IL-4) and IL-13 production. Cytokine. 2015; 75: 14–24.

169. Wu S, Zhou Y, Liu S, Zhang H, Luo H, Zuo X, Li T. Regulatory effect of nicotine on the differentiation of Th1, Th2 and Th17 lymphocyte subsets in patients with rheumatoid arthritis. Eur. J. Pharmacol. 2018; 831:38-45.

170. Abed AK, Hassen WA, Salman MA. Evaluation of the effect of cigarette smoking on interleukin-4. Int. J. Psychosol. Rehabilitation. 2020:24:2386-2392.

171. Elisia I, Lam V, Cho B, Hay M, Li MY, Yeung M, Bu L, Jia W, Norton N, Lam S, Krystal G. The effect of smoking on chronic inflammation, immune function and blood cell composition. Sci. Rep. 2020; 10:19480.

172. Byron KA, Varigos GA, Wootton AM. IL-4 production is increased in cigarette smokers. Clin. Exp. Immunol. 1994; 95:333-6.

173. Merghani TH, Saeed A, Alawad A. Changes in plasma IL4, TNFá and CRP in response to regular passive smoking at home among healthy school children in Khartoum, Sudan. Afr. Health Sci. 2012; 12:41-47.

174. Olsson P, Skogstrand K, Nilsson A, Turesson C, Jacobsson LTH, Theander E, Houen G, Mandl T. Smoking, disease characteristics and serum cytokine levels in patients with primary Sjögren's syndrome. Rheumatol Int. 2018; 38:1503-1510.

175. Bradley J.R. TNF-Mediated inflammatory disease. J. Pathol. 2008; 214:149–160.

176. Gough P, Myles IA. Tumor Necrosis Factor Receptors: Pleiotropic Signaling Complexes and Their Differential Effects. Frontiers in Immunology.2020; 11: 585880.

177. Li X, Zhang Y, Wang Q. Nicotine suppresses the release of inflammatory TNF-α and cytokines in a dose-dependent manner in myocarditis models. J. Inflamm. Res. 2015; 8: 123-132.

178. Li-Sha J, Chen H, Zhao Y. Anti-inflammatory effects of nicotine on cytokine release in arthritis models: Dose-dependent suppression of TNF-α. Int. Immunopharmacol. 2015; 25: 345-355.

179. Gray PW, Goeddel DV. Structure of the human immune interferon gene. Nature. 1982; 298: 859–863.

180. Castro F, Cardoso AP, Gonçalves RM, Serre K, Oliveira MJ. Interferon-Gamma at the Crossroads of Tumor Immune Surveillance or Evasion. Front. Immunol. 2018; 9: 847.

181. Bhat MY, Solanki HS, Advani J, Khan AA, Keshava Prasad TS, Gowda H. Comprehensive network map of interferon gamma signaling. J. Cell Comm. Signal. 2018; 12: 745–751.

182. César-Neto JB, Duarte PM, de Oliveira MC, Casati MZ, Tambeli CH, Parada CA, Sallum EA, Nociti FH Jr. Smoking modulates interferon-gamma expression in the gingival tissue of patients with chronic periodontitis. Eur. J. Oral Sci. 2006; 114: 403-408.

183. Lee G, Jung K-H, Shin D, Lee C, Kim W, Lee S, Kim J, Bae H. Cigarette Smoking Triggers Colitis by IFN-γ+ CD4+ T Cells. Front. Immunol. 2017; 8:1344.

184. Rahimi S, Khosravi A, Aazami S, et al. Effect of smoking on cyanide, IL-2 and IFN-γ levels in saliva of smokers and nonsmokers. Polish Ann Med 2018; 25: 203-206.

185. Singh KP, Lawyer G, Muthumalage T, Maremanda KP, Khan NA, McDonough SR, Ye D, McIntosh S, Rahman I. Systemic biomarkers in electronic cigarette users: implications for noninvasive assessment of vaping associated pulmonary injuries. ERJ Open Res. 2019; 5:00182-2019.

186. Tao X, Li H, Xing Y, Liu F, Hu Y, Tao H, Mu M; Pang G, Zhang R. Nicotine Protects Dendritic Cells from Apoptosis and Support DCs-dependent CD4+ T-cell Priming in vitro. Indian J. Pharm. Sci. 2019; 81: 1000- 1010.

187. Raymond WD, Hamdorf M, Furfaro M, Eilertsen GO, Nossent JC. Smoking associates with increased BAFF and decreased interferon-γ levels in patients with systemic lupus erythematosus. Lupus Sci. Med. 2021; 8: 000537.

188. Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell. 1993; 74: 609–619.

189. Sedlak TW, Oltvai ZN, Yang E, Wang K, Boise LH, Thompson CB, et al. Multiple Bcl-2 family members demonstrate selective dimerizations with Bax. Proceedings of the National Academy of Sciences of the United States of America. 1995; 92: 7834–7838.

190. Biswas S, Manna K, Das U, Khan A, Pradhan A, Sengupta A, Bose S, Ghosh S, Dey S. Smokeless tobacco consumption impedes metabolic, cellular, apoptotic and systemic stress pattern: A study on Government employees in Kolkata, India Sci. Rep. 2015;5:18284.

191. Aali N, Motalleb G. The effect of nicotine on the expressions of the α7 nicotinic receptor gene and Bax and Bcl-2 proteins in the mammary gland epithelial-7 breast cancer cell line and its relationship to drug resistance. Cell Mol. Biol. Lett. 2015; 20:948-64.

192. Kim CS, Choi JS, Joo SY, Bae EH, Ma SK, Lee J, Kim SW. Nicotine-Induced Apoptosis in Human Renal Proximal Tubular Epithelial Cells. PLoS One. 2016; 11: 0152591.

193. Shaik FB, Nagajothi G, Swarnalatha K, Kumar CV, Dhania KN, Kumar CS, Maddu N. Possible Association of Smokeless Tobacco Dependent Impairment in the Erythrocytes and Platelets Membranes of Human Male Volunteers: An Observation. Asian Pac. J. Cancer Prev. 2019; 20:2167-2176.

194. Gillies, G. E., & McArthur, S. Estrogen actions in the brain and the basis for differential action in men and women: A case for sex-specific medicines. Pharmacol. Rev, 2010; 62, 155–198.

195. Torrealday S, Kodaman P, Pal L. Understanding the role of estradiol, estrone, and estriol in female reproductive health. Fertil Steril. 2000; 93: 505–513.

196. Blaustein JD. Neuroendocrine regulation of reproductive behavior and physiology. In J. D. Blaustein. 2008; 1-32.

197. Kuijper EAM, Ket JCF, Caanen MR, Lambalk CB. Reproductive hormone concentrations in pregnancy and post-partum: A systematic review. Reprod. Biomed. Online. 2013; 27: 33–63.

198. Norman AW, Henry HL. (2022). Hormones (4th Ed.). Academic Press.

199. Tucker HA. Hormones, mammary growth, and lactation: A 41-year perspective. J. Dairy Sci. 2000; 83: 874–884.

200. d'Adesky ND, de Rivero Vaccari JP, Bhattacharya P, Schatz M, Perez-Pinzon MA, Bramlett HM, Raval AP. Nicotine Alters Estrogen Receptor-Beta- Regulated Inflammasome Activity and Exacerbates Ischemic Brain Damage in Female Rats. Int. J. Mol. Sci. 2018; 19: 1330.

201. Maiti M, Chattopadhyay K, Verma M, Chattopadhyay B. Curcumin protects against nicotine-induced stress during protein malnutrition in female rat through immunomodulation with cellular amelioration. Mol. Biol. Rep. 2015; 42:1623-37.

202. Jandikova H, Duskova M, Starka L. The Influence of Smoking and Cessation on the Human Reproductive Hormonal Balance. Physiol. Res. 2017; 66: 323 - 331.

203. Mueck AO, Seeger H. Smoking, estradiol metabolism and hormone replacement therapy. Curr. Med. Chem. Cardiovasc Hematol Agents. 2005; 3:45-54.

204. Ruan X, Mueck AO. Impact of smoking on estrogenic efficacy. Climacteric. 2015; 18: 38-46.

205. Adeyemi DH, Oyeyipo IP, Akanbi KA, Oluwole T. Nicotine alters progesterone and estradiol levels during the first trimester of pregnancy in Wistar rats. JBRA Assist Reprod. 2018; 22:78-81.

206. Ahmadi R, Lotfizadeh M J, Heidari F, Mafi M. Comparing the effects of cigarette and waterpipe smoke on serum levels of LH, FSH, estradiol and progesterone in female rats. Feyz. 2013; 17: 232-238.

207. Khalid A, Qureshi HJ, Nazir S, Lone KP. Effect of Second-Hand Smoke Exposure on Serum Estradiol and Progesterone Levels in women having First Trimester Spontaneous Abortions. Pak J Med Health Sci. 2017; 11:163-167.

208. Pang RD, Liautaud MM, Kirkpatrick MG, Huh J, Monterosso J, Leventhal AM. Ovarian Hormones and Transdermal Nicotine Administration Independently and Synergistically

Suppress Tobacco Withdrawal Symptoms and Smoking Reinstatement in the Human Laboratory. Neuropsychopharmacol. 2018; 43:828-837.

209. Xu X, Weiss ST, Rijcken B, Schouten JP. Smoking, changes in smoking habits, and rate of decline in FEV1: New insights into gender differences. American Journal of Respiratory and Critical Care Medicine, 1994; 149: 1209–1217.

210. Barbieri RL. The effects of cigarette smoking on reproduction. "Hum. Reprod. 2001; 6: 121–127.

211. Gram IT, Park SY, Wilkens LR, Haiman CA, Le Marchand L. Smoking-related risks of colorectal cancer by anatomical subsite and sex. Am J Epidemiol. 2020; 189:543–553.

212. Jones ME, Schoemaker MJ, Wright LB, Ashworth A, Swerdlow AJ. Smoking and risk of breast cancer in the Generations Study cohort. Breast Cancer Res. 2017; 19:118.

213. Gallucci G, Tartarone A, Lerose R, Lalinga AV, Capobianco AM. Cardiovascular risk of smoking and benefits of smoking cessation. J Thorac Dis. 2020; 12:3866–3876.

214. Kawahara T, Ito H, Yao M, Uemura H. Impact of smoking habit on overactive bladder symptoms and incontinence in women. Int J Urol. 2020; 27:1078-86.

215. Perkins KA, Karelitz JL, Giedgowd GE, Conklin CA. Negative mood effects on craving to smoke in women versus men. Addict Behav. 2013; 38:1527-1531.

216. Chen Y, Mai X-M. Smoking and asthma in men and women with normal weight, overweight, and obesity. J Asthma. 2011; 48:490-494.

217. Food and Agriculture Organization of the United Nations, International Fund for Agricultural Development, World Food Programme. Food and Agriculture Organization of the United Nations; 2015.

218. Chiolero A, Faeh D, Paccaud F, et al. Consequences of smoking for body weight, body fat distribution, and insulin resistance. Am J Clin Nutr. 2008; 87:801-809.

219. Chhabra P, Chhabra SK. Effect of smoking on body mass index: a community-based study. Natl J Community Med. 2011; 2:325-30.

220. Morley JE, Silver AJ. Anorexia in the elderly. Neurobiol Aging. 1988;9:9-16.

221. Malafarina V, Uriz-Otano F, Gil-Guerrero L, et al. The anorexia of ageing: physiopathology, prevalence, associated comorbidity and mortality. A systematic review. Maturitas. 2013;74:293-302.

222. Pilgrim A, Robinson S, Sayer AA, et al. An overview of appetite decline in older people. Nurs Older People. 2015;27:29-35.

223. Boucher Y, Simons C, et al. Activation of brain stem neurons by irritant chemical stimulation of the throat assessed by c-fos immunohistochemistry. Experimental Brain Research. 2003;148(2):211–218.

224. Mao D, Yasuda RP, et al. Heterogeneity of Nicotinic Cholinergic Receptors in Rat Superior Cervical and Nodose Ganglia. Mol Pharmacol. 2006;70(5):1693–1699.

225. Kahn BB, Alquier T, et al. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. Cell Metab. 2005;1(1):15–25.

226. Oktavianis O. Efek pemberian asap rokok terhadap kehamilan tikus putih (Rattus norvegicus). Universitas Andalas; 2011.

227. Young-Hwan J, David AT, Lorna WR. Nicotinic Receptor-Mediated Effects on Appetite and Food Intake. J Neurobiol. 2002; 53:618-32.

228. Khademolhosseini F, Mehrabani D, Zare N, et al. Prevalence of dyspepsia and its correlation with demographic factors and lifestyle in Shiraz, southern Iran. Middle East J Dig Dis. 2010;2:24-28.

229. Ilfandari A, Ervina A. Hubungan Perilaku Merokok Dengan Indeks Masa Tubuh Remaja Putra. E-Jurnal Obstretika. 2015;3:1-15.

230. Kusumaratna RK, Hidayat A. Body Mass Index and Quality of Life among the Elderly. Universa Med. 2009;28:34-41.

231. Hofstetter A, Schutz Y, et al. Increased 24-Hour Energy Expenditure in Cigarette Smokers. New England Journal of Medicine. 1986;314(2):79–82.

232. Perkins KA. Metabolic effects of cigarette smoking. Journal of Applied Physiology. 1992;72(2):401–409.

233. Blendy JA, Strasser A, et al. Reduced nicotine reward in obesity: cross-comparison in human and mouse. Psychopharmacology (Berl) 2005;180(2):306–315.

234. Porter C. Quantification of UCP1 function in human brown adipose tissue. Adipocyte. 2017:1–8.

235. Akarchariya N, Sirilun S, Julsrigival J, Chansakaowa, S. Chemical profiling and antimicrobial activity of essential oil from Curcuma aeruginosa Roxb., Curcuma glans K. Larsen & J. Mood and Curcuma cf. xanthorrhiza Roxb. collected in Thailand. Asian Pacific J. Trop. Biomed. 2017; 7, 881–885.

236. Dosoky NS, Setzer WN. Chemical Composition and Biological Activities of Essential Oils of Curcuma Species Nutrients 2018; 10: 1–42.

237. Wu D. (2015). The Zingiberaceae Resource of China (Wuhan, China: Huazhon University of Science and Technology Press).

238. Verma RK, Kumari P, Maurya RK, Kumar V, Verma RB, Singh RK. Medicinal properties of turmeric (Curcuma longa L.): A review. Int J Chem Stud. 2018; 6:1354-1357.

239. Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB. Bioavailability of Curcumin: Problems and Promises. Mol. Pharmaceut. 2017. 4: 807–818.

240. Buckingham, J. (2018). Dictionary of Natural Products on DVD. (Chapman & Hall/CRC)

241. Sohn SI, Priya A, Balasubramaniam B, Muthuramalingam P, Sivasankar C, Selvaraj A, Valliammai A, Jothi R, Pandian S. Biomedical Applications and Bioavailability of Curcumin-An Updated Overview. Pharmaceutics. 2021; 13:2102.

242. Miłobędzka J, Kostanecki V, Lampe V. Zur Kenntnis des Curcumins. Berichte Der Deutschen Chem. Gesellschaft. 1910; 43, 2163–2170.

243. Menon VP, Sudheer AR. Antioxidant and anti-inflammatory properties of curcumin. Adv Exp Med Biol. 2007; 595:105-25.

244. Wilken R, Veena MS, Wang MB, Srivatsan ES. Curcumin: A review of anticancer properties and therapeutic activity in head and neck squamous cell carcinoma. Mol Cancer. 2011; 10:12.

245. Den Hartogh DJ, Gabriel A, Tsiani E. Antidiabetic Properties of Curcumin II: Evidence from In Vivo Studies. Nutrients. 2019;12: 58.

246. Hashish EA, Elgaml SA. Hepatoprotective and Nephroprotective Effect of Curcumin Against Copper Toxicity in Rats. Indian J Clin Biochem. 2016; 31:270-277.

247. Prasad S, Tyagi AK. Curcumin and its analogues: a potential natural compound against HIV infection and AIDS. Food Funct. 2015; 6:3412-9.

248. Sharifi-Rad J, Rayess YE, Rizk AA, Sadaka C, Zgheib R, Zam W, Sestito S, Rapposelli S, Neffe-Skocińska K, Zielińska D, Salehi B, Setzer WN, Dosoky NS, Taheri Y, El Beyrouthy M, Martorell M, Ostrander EA, Suleria HAR,Cho WC, Maroyi A, Martins N. Turmeric and Its Major Compound Curcumin on Health: Bioactive Effects and Safety Profiles for Food, Pharmaceutical, Biotechnological and Medicinal Applications. Front Pharmacol. 2020; 11:01021.

249. Wilson B, Abraham G, Manju V. Antioxidant, antibacterial and antitumor activity of Curcuma longa. J. Med. Plant Res. 2005; 4: 56-64.

250. Reanmongkol W, Itharat A, Bouking P. Anti-inflammatory and analgesic activities of extracts from Curcuma longa. J. Ethnopharmacol. 2006; 103: 485-490.

251. Lin CC, Chen HH, Lin JM. Curcumin induces apoptosis and cell cycle arrest in human breast cancer cells. Planta Med. 2010; 76: 737-743.

252. Angel GR, Menon SR, Jose JK. Pharmacological activities of Curcuma species. Phytother. Res. 2014; 28: 540-551.

253. Joe B, Vijaykumar M, Lokesh BR. Biological properties of curcumin-Cellular and molecular mechanisms of action. Crit. Rev. Food Sci. Nutr. 2004; 44: 97-111.

254. Das KC, Das CK. Curcumin (diferuloylmethane), a singlet oxygen quencher. Biochem. Biophys. Res. Commun. 2002; 295, 62-66.

255. Tilak JC, Banerjee M, Mohan H, Devasagaym TPA. Antioxidant availability of turmeric in relation to its medicinal and culinary uses. Phytother. Res. 2004; 18: 798-804.

256. Unnikrishnan MK, Rao MN. Inhibition of nitrite induced oxidation of hemoglobin by curcuminoids. Die Pharmazie. 1995; 507, 490-492.

257. Brouet I, Ohshima H. Curcumin, an anti-tumour promoter and anti-inflammatory agent, inhibits induction of nitric oxide synthase in activated macrophages. Biochem. Biophys. Res. Commun. 1995; 206, 533-540.

258. Bonte F, Noel-Hudson MS, Wepierre J, Meybeck A. Protective effect of curcuminoids on epidermal skin cells under free oxygen radical stress. Planta Med. 1997; 63: 265-266.

259. Kalpana C, Menon VP. Modulatory effects of curcumin on lipid peroxidation and antioxidant status during nicotine-induced toxicity. Pol. J. Pharmacol. 2004; 56: 581-586.

260. Reddy AC, Lokesh BR. Effect of dietary turmeric (Curcuma longa) on iron-induced lipid peroxidation in the rat liver. Food Chem. Toxicol. 1994; 32: 279-283.

261. Lin YG, Kunnumakkara AB, Nair A, Merritt WM, Han LY, Armaiz-Pena GN, Kamat AA, Spannuth WA, Gershenson DM, Lutgendorf SK, Aggarwal BB, Sood AK. Curcumin inhibits tumour growth and angiogenesis in ovarian carcinoma by targeting the nuclear factor-kappaB pathway. Clin Cancer Res. 2007; 13:3423-30.

262. Priyadarsini KI, Maity DK, Naik GH, Kumar MS, Unnikrishnan MK, Satav JG, Mohan H. Role of phenolic O-H and methylene hydrogen on the freeradical reactions and antioxidant activity of curcumin. Free Radic BiolMed. 2003;35: 475-84.

263. Aggarwal BB, Sung B. Pharmacological basis for the role of curcumin in chronic diseases: an age-old spice with modern targets. Trends Pharmacol. Sci. 2009; 30:85-94.

264. Cianciulli A, Calvello R, Porro C, Trotta T, Salvatore R, Panaro MA. PI3k/Akt signalling pathway plays a crucial role in the anti-inflammatory effects of curcumin in LPS-activated microglia. Int Immunopharmacol. 2016; 36:282-290.

265. Edwards RL, Luis PB, Varuzza PV, Joseph AI, Presley SH, Chaturvedi R, Schneider C. The anti-inflammatory activity of curcumin is mediated by its oxidative metabolites. J Biol Chem. 2017; 292:21243-21252.

266. Dai W, Wang H, Fang J, Zhu Y, Zhou J, Wang X, Zhou Y, Zhou M. Curcumin provides neuroprotection in model of traumatic brain injury via the Nrf2-ARE signalling pathway. Brain Res Bull. 2018; 140:65-71.

267. Panahi Y, Rahimnia AR, Sharafi M, Alishiri G, Saburi A, Sahebkar A. Curcuminoid treatment for knee osteoarthritis: a randomized doubleblind placebo-controlled trial. Phytother Res. 2014; 28:1625-31.

268. Panahi Y, Saadat A, Beiraghdar F, Sahebkar A. Adjuvant therapy with bioavailability-boosted curcuminoids suppresses systemic inflammation and improves quality of life in patients with solid tumors: a randomized double-blind placebo-controlled trial. Phytother Res. 2014b; 28:1461-7.

269. He Y, Yue Y, Zheng X, Zhang K, Chen S, Du Z. Curcumin, inflammation, and chronic diseases: how are they linked? Molecules. 2015; 20: 9183-213.

270. Machova UL, Karova K, Ruzicka J, Kloudova A, Shannon C, Dubisova J, Murali R, Kubinova S, Sykova E, Jhanwar-Uniyal M, Jendelova P. The Anti-Inflammatory Compound Curcumin Enhances Locomotor and Sensory Recovery after Spinal Cord Injury in Rats by Immunomodulation. Int J Mol Sci. 2015; 17:49.

271. Joe B, Vijaykumar M, Lokesh BR. (2004) Biological properties of curcumin-Cellular and molecular mechanisms of action. Critical Revues of Food and Science Nutrition, 2004; 44, 97-111.

272. Huang MT, Ma W, Yen P, Xie J-G, Han J, Frenkel K, Grunberger D, Conney AH. Inhibitory effects of topical application of low doses of curcumin on 12-O-tetradecanoyl-phorbol-13-acetate-induced tumor promotion and oxidized DNA bases in mouse epidermis. Carcinog. 1997; 18: 83-88.

273. Ye J, Zhang, Y. Curcumin protects against intracellular amyloid toxicity in rat primary neurons. Int. J. Clin. Exp. Med. 2012; 5: 44–49.

274. Wu J, Li Q, Wang X, Yu S, Li L, Wu X, et al. Neuroprotection by curcumin in ischemic brain injury involves the Akt/Nrf2 pathway. PloS One. 2013; 8: 59843.

275. Song S, Nie Q, Li Z, Du G. Curcumin improves neurofunctions of 6-OHDA-induced parkinsonian rats. Pathol. Res. Pract. 2016; 212: 247–251.

276. Teter B, Morihara T, Lim GP, Chu T, Jones MR, Zuo X. Curcumin restores innate immune Alzheimer's disease risk gene expression to ameliorate Alzheimer pathogenesis. Neurobiol. Dis. 2019; 127, 432–448.

277. Salehi B, Calina D, Docea AO, Koirala N, Aryal S, Lombardo D. Curcumin's Nanomedicine Formulations for Therapeutic Application in Neurological Diseases. J. Clin. Med. 2020; 9: 430.

278. Chen N, Geng Q, Zheng J, He S, Huo X, Sun X. Suppression of the TGF-beta/Smad signaling pathway and inhibition of hepatic stellate cell proliferation play a role in the hepatoprotective effects of curcumin against alcohol-induced hepatic fibrosis. Int. J. Mol. Med. 2014; 34, 1110–1116.

279. Qureshi M, Al-Suhaimi EA, Wahid F, Shehzad O, Shehzad A. Therapeutic potential of curcumin for multiple sclerosis. Neurol Sci. 2018; 39: 207–214.

280. Sikand K, Singh S, Kumar S. Curcumin as a therapeutic agent in cancer: Mechanisms and clinical potential. Cancer Res. Treat. 2022; 18: 245–259.

281. Yance DR Jr, Sagar SM. Targeting angiogenesis with integrative cancer therapies. Integr. Cancer Ther. 2006; 5, 9–29.

282. Astinfeshan M, Rasmi Y, Kheradmand F, Karimipour M, Rahbarghazi R, Aramwit P. Curcumin inhibits angiogenesis in endothelial cells using downregulation of the PI3K/Akt signaling pathway. Food Biosci. 2019; 29, 86– 93.

283. Kandoth C, Mclellan MD, Vandin F, Ye K, Niu B, Lu C. Mutational landscape and significance across 12 major cancer types. Nature 2013; 502: 333–339.

284. Hermeking H. p53 enters the microRNA world. Cancer Cell. 2007; 12, 414–418.

285. Ye M, Zhang J, Zhang J, Miao Q, Yao L, Zhang J. Curcumin promotes apoptosis by activating the p53-miR-192-5p/215-XIAP pathway in non-small cell lung cancer. Cancer Lett. 2015; 357: 196–205.

286. Mishra A, Kumar R, Tyagi A, Kohaar I, Hedau S, Bharti A. C. Curcumin modulates cellular AP-1, NF-kB, and HPV16 E6 proteins in oral cancer. E cancer medical science. 2015; 9: 525.

287. Man S, Zhang L, Cui J, Yang L, Ma L, Gao W. Curcumin enhances the anti-cancer effects of Paris Saponin II in lung cancer cells. Cell Prolif. 2018; 51: 12458.

288. Marquardt JU, Gomez-Quiroz L, Arreguin Camacho LO, Pinna F, Lee YH, Kitade M. Curcumin effectively inhibits oncogenic NF-kappaB signaling and restrains stemness features in liver cancer. J. Hepatol. 2015; 63: 661–669.

289. Rajitha B, Nagaraju GP. Curcumin and Genistein Role in Regulation of STAT-3 in Pancreatic Cancer," in Role of Transcription Factors in Gastrointestinal Malignancies. Eds. G. P. Nagaraju and P. V. Bramhachari (Singapore: Springer Singapore), 2017; 32: 427–435.

290. Shanmugam MK, Rane G, Kanchi MM, Arfuso F, Chinnathambi A, Zayed ME. The multifaceted role of curcumin in cancer prevention and treatment. Molecules 2015; 20: 2728–2769.

291. Jiang MC, Lin JK, Chen SS. Inhibition of HIV-1 Tat-mediated transactivation by quinacrine and chloroquine. Biochem. Biophys. Res. Commun. 1996; 226: 1-7.

292. Bourne KZ, Bourne N, Reising SF, Stanberry LR. Plant products as topical microbiocide candidates: assessment of in vitro and in vivo activity against herpes simplex virus type 2. Antivir. Res. 1999; 42: 219-226.

293. Artico M, Di Santo R, Costi R, Novellino E, Greco G, Massa S, Tramontano E, Marongiu ME, De Montis A, La Colla P. Geometrically and conformationally restrained cinnamoyl compounds as inhibitors of HIV-I intergrase. Journal of Medicinal Chemistry, 1998; 41: 3948-3960.

294. Mazumder A, Neamati N, Sunder S, Schulz J, Pertz H, Eich E, Pommier Y. Curcumin analogs with altered potencies against HIV-1 intergrase as probes for biochemical mechanisms of drug action. J. Med. Chem. 1997; 40: 3057-3063.

295. Chueh SC, Lai MK, Liu IS, Teng FC, Chen J. Curcumin enhances the immunosuppressive activity of cyclosporine in rat cardiac allografts and in mixed lymphocyte reactions. Transplant. Proc. 2003; 35: 1603-1605.

296. Churchill M, Chadburn A, Bilinski RT, Bertagnolli MM. Inhibition of intestinal tumors by curcumin is associated with changes in the intestinal immune cell profile. J. Surg. Res. 2000; 89: 169-175.

297. Gao X, Kuo J, Jiang H, Deeb D, Liu Y, Divine G, Chapman RA, Dulchavsky SA, Gautam SC. Immunomodulatory activity of curcumin. Biochem. Pharmacol. 2004; 68: 51-61.

298. Egan ME, Pearson M, Weiner SA, Rajendran V, Rubin, D, Glockner-Pagel J, Canny S, Du K, Lukacs GI, Caplan MJ. Curcumin, a major constituent of turmeric, corrects cystic fibrosis defects. Science. 2004; 304: 600-602.

299. Mall M, Kunzelmann K. Correction of the CF defect by curcumin: hypes and disappointments. Bioessays. 2005; 27: 9-13

300. Arun N, Nalini N. Efficacy of turmeric on blood sugar and polyol pathway in diabetic albino rats. Plant Foods Hum. Nutr. 2002; 57: 41-52.

301. Nishiyama T, Mae T, Kishida H, Tsukagawa M, Mimaki Y, Kuroda M, Sashida Y, Takahashi K, Kawada T, Nakagawa K, Kitahara M. Curcuminoids and sesquiterpenoids in turmeric

(Curcuma longa L.) suppress an increase in blood glucose level in type 2 diabetic kk-Ay mice. J. Agric. Food Chem. 2005; 53: 959-963.

302. Kim SH, Choi GJ, Lee HS. Fungicidal property of Curcuma longa L. rhizome-derived curcumin against phytopathogenic fungi in a greenhouse. J. Agric. Food Chem. 2003; 51: 1578-1581.

303. Mishra S, Narain U, Mishra K, Misra K. Design, development and synthesis of mixed bioconjugates of piperic acid-glycine, curcumin-glycine/alanine and curcumin-glycine-piperic acid and their antibacterial and antifungal properties. Bioorg. Med. Chem. 2005; 13: 1477-1486.

304. Koide T, Nose M, Ogihara Y, Yabu Y, Ohta N. Leishmanicidal effect of curcumin in vitro. Biol. Pharm. Bull. 2002; 25: 131-133.

305. Reddy RC, Vatsala PG, Keshamouni VG, Padmanaban G, Rangarajan PN. Curcumin for malaria therapy. Biochem. Biophys. Res. Commun. 2005; 326: 472-474.

306. Kiuchi F, Goto Y, Sugimoto N, Akao N, Kondo K, Tsuda Y. Nematocidal activity of turmeric: synergistic action of curcuminoids. Chem. Pharm. Bull. 1993; 41: 1640-1643.

307. Jiang S, Han J, Li T, Xin Z, Ma Z, Di W, et al. Curcumin as a potential protective compound against cardiac diseases. Pharmacol. Res. 2017; 119: 373–383.

308. Li H, Sureda A, Devkota HP, Pittala V, Barreca D, Silva AS. Curcumin, the golden spice in treating cardiovascular diseases. Biotechnol. Adv. 2019; 38: 107343.

309. Salehi B, Del Prado-Audelo ML, Cortés H, Leyva-Gómez G, Stojanović- Radić Z, Singh YD. Therapeutic Applications of Curcumin Nanomedicine Formulations in Cardiovascular Diseases. J. Clin. Med. 2018; 9: 746.

310. Sunagawa Y, Funamoto M, Sono S, Shimizu K, Shimizu S, Genpei M. Curcumin and its demethoxy derivatives possess p300 HAT inhibitory activity and suppress hypertrophic responses in cardiomyocytes. J. Pharmacol. Sci. 2018; 136: 212–217.

311. Monfoulet LE, Mercier S, Bayle D, Tamaian R, Barber-Chamoux N, Morand C. Curcumin modulates endothelial permeability and monocyte transendothelial migration by affecting endothelial cell dynamics. Free Radic. Biol. Med. 2017; 112: 109–120.

312. Yao Y, Wang W, Li M, Ren H, Chen C, Wang J. Curcumin Exerts its Anti-hypertensive Effect by Down-regulating the AT1 Receptor inVascular Smooth Muscle Cells. Sci. Rep. 2016; 6: 25579.

313. Goel A, Aggarwal BB. Curcumin, the golden spice from Indian saffron, is a chemosensitizer and radiosensitizer for tumors and chemoprotector and radioprotector for normal organs. Nutr. Cancer. 2010; 62: 919–930.

314. Shahani K, Swaminathan SK, Freeman D, Blum A, Ma L, Panyam, J. Injectable sustained release microparticles of curcumin: a new concept for cancer chemoprevention. Cancer Res. 2010; 70: 4443–4452.

315. Gou M, Men K, Shi H, Xiang M, Zhang J, Song J. Curcuminloaded biodegradable polymeric micelles for colon cancer therapy in vitro and in vivo. Nanoscale. 2011; 3: 1558–1567.

316. Wang S, Chen P, Zhang L, Yang C, Zhai G. Formulation and evaluation of microemulsion-based in situ ion-sensitive gelling systems for intranasal administration of curcumin. J. Drug Target. 2012; 20: 831–840.

317. Chang MT, Tsai TR, Lee CY, Wei YS, Chen YJ, Chen CR. Elevating bioavailability of curcumin via encapsulation with a novel formulation of artificial oil bodies. J. Agric. Food Chem. 2013; 61: 9666–9671.

318. Dovigo LN, Carmello JC, De Souza Costa CA, Vergani CE, Brunetti IL, Bagnato VS. Curcumin-mediated photodynamic inactivation of Candida albicans in a murine model of oral candidiasis. Med. Mycol. 2013; 51: 243–251.

319. Phillips J, Moore-Medlin T, Sonavane K, Ekshyyan O, Mclarty J, Nathan CA. Curcumin inhibits UV radiation-induced skin cancer in SKH-1 mice. Otolaryngol. Head Neck Surg. 2013; 148: 797–803.

320. Subhashini C, Kumari S, Kumar JP, Chawla R, Dash D, Singh M. Intranasal curcumin and its evaluation in murine model of asthma. Int. Immunopharmacol. 2013; 17: 733–743.

321. Sun J, Bi C, Chan HM, Sun S, Zhang Q, Zheng Y. Curcuminloaded solid lipid nanoparticles have prolonged in vitro antitumour activity, cellular uptake and improved in vivo bioavailability. Colloids Surf B. Biointerf. 2013; 111: 367–375.

322. Karlowicz-Bodalska K, Han S, Freier J, Smolenski M, Bodalska A. Curcuma Longa as Medicinal Herb in The Treatment of Diabet- Ic Complications. Acta Pol. Pharm. 2017; 74: 605–610.

323. Salehi B, Lopez-Jornet P, Pons-Fuster López E, Calina D, Sharifi-Rad M, Ramírez-Alarcón K. Plant-Derived Bioactives in Oral Mucosal Lesions: A Key Emphasis to Curcumin, Lycopene, Chamomile, Aloe vera, Green Tea and Coffee Properties. Biomolecules. 2019; 9: 106.

324. Smith MR, Gangireddy SR, Narala VR, Hogaboam CM, Standiford TJ, Christensen PJ. Curcumin inhibits fibrosis-related effects in IPF fibroblasts and in mice following bleomycin-induced lung injury. Am. J. Physiol. Lung Cell Mol. Physiol. 2010; 298: 616–625.

325. Sun Y, Dai M, Wang Y, Wang W, Sun Q, Yang GY. Neuroprotection and sensorimotor functional improvement by curcumin after intracerebral hemorrhage in mice. J. Neurotrauma. 2011; 28: 2513–2521.

326. Chowdhury R, Nimmanapalli R, Graham T, Reddy G. Curcumin attenuation of lipopolysaccharide induced cardiac hypertrophy in rodents. ISRN Inflammation 2013; 539305.

327. Rahmani S, Asgary S, Askari G, Keshvari M, Hatamipour M, Feizi A. Treatment of Non-alcoholic Fatty Liver Disease with Curcumin: A Randomized Placebo-controlled Trial. Phytother. Res. 2016; 30: 1540–1548.

328. Macías-Pérez JR, Vázquez-López BJ, Muñoz-Ortega MH, Aldaba-Muruato LR, Martínez-Hernández SL, Sánchez-Alemán E. Curcumin and a/b-Adrenergic Antagonists Cotreatment Reverse Liver Cirrhosis in Hamsters: Participation of Nrf-2 and NF-kB. J. Immunol. Res. 2019; 3019794–3019794.

329. Cichoż-Lach H, Michalak A. Oxidative stress as a crucial factor in liver diseases. World J Gastroenterol. 2014; 20:8082-8091.

330. Lee HY, Kim SW, Lee GH, Choi MK, Jung HW, Kim YJ. Turmeric extract and its active compound, curcumin, protect against chronic CCl4-induced liver damage by enhancing antioxidation. BMC Complement. Altern. Med. 2019; 16: 316.

331. Farzaei MH, Zobeiri M, Parvizi F, El-Senduny FF, Marmouzi I, Coy- Barrera E. Curcumin in Liver Diseases: A Systematic Review of the Cellular Mechanisms of Oxidative Stress and Clinical Perspective. Nutrients. 2018; 10: 855.

332. Afrin R, Arumugam S, Soetikno V, Thandavarayan RA, Pitchaimani V, Karuppagounder V. Curcumin ameliorates streptozotocininduced liver damage through modulation of

endoplasmic reticulum stressmediated apoptosis in diabetic rats. Free Radic. Res. 2015; 49: 279–289.

333. Salahshoor M, Mohamadian S, Kakabaraei S, Roshankhah S, Jalili C. Curcumin improves liver damage in male mice exposed to nicotine. J Tradit. Complement Med. 2015; 6:176-83.

334. Zhao Y, Ma X, Wang J, He X, Hu Y, Zhang P, Wang R, Li R, Gong M, Luo S, Xiao X. Curcumin protects against CCl4-induced liver fibrosis in rats by inhibiting HIF-1α through an ERK-dependent pathway. Molecules. 2014; 19:18767-80.

335. Granados-Castro LF, Rodriguez-Rangel DS, Fernandez-Rojas B, Leon-Contreras JC, Hernandez-Pando R, Medina-Campos ON. Curcumin prevents paracetamol-induced liver mitochondrial alterations. J. Pharm. Pharmacol. 2016; 68: 245–256.

336. Mohajeri M, Rezaee M, Sahebkar A. Cadmium-induced toxicity is rescued by curcumin: A review. Biofactors. 2017; 43:645-661.

337. Zhong W, Qian K, Xiong J, Ma K, Wang A, Zou Y. Curcumin alleviates lipopolysaccharide induced sepsis and liver failure by suppression of oxidative stress-related inflammation via PI3K/AKT and NF-kappaB related signaling. BioMed. Pharmacother. 2016; 83: 302–313.

338. Den Hartogh DJ, Gabriel A, Tsiani E. Antidiabetic Properties of Curcumin I: Evidence from In Vitro Studies. Nutrients. 2020; 12:118.

339. Pivari F, Mingione A, Brasacchio C, Soldati L. Curcumin and Type 2 Diabetes Mellitus: Prevention and Treatment. Nutrients. 2019; 11:1837.

340. Zingg JM, Hasan ST, Meydani M. Molecular mechanisms of hypolipidemic effects of curcumin. Biofactors. 2013; 39:101-21.

341. Jalali M, Mahmoodi M, Mosallanezhad Z, Jalali R, Imanieh MH, Moosavian SP. The effects of curcumin supplementation on liver function, metabolic profile and body composition in patients with non-alcoholic fatty liver disease: A systematic review and meta-analysis of randomized controlled trials. Complement Ther Med. 2020; 48:102283.

342. Baziar N, Parohan M. The effects of curcumin supplementation on body mass index, body weight, and waist circumference in patients with nonalcoholic fatty liver disease: A systematic review and dose-response meta-analysis of randomized controlled trials. Phytother Res. 2020; 34:464-474.

343. Rafiee S, Bagherniya M, Askari G, Sathyapalan T, Jamialahmadi T, Sahebkar A. The Effect of Curcumin in Improving Lipid Profile in Patients with Cardiovascular Risk Factors: A Systematic Review of Clinical Trials. Adv Exp Med Biol. 2021; 1291:165-177.

344. Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB. Bioavailability of Curcumin: Problems and Promises. Mol. Pharmaceut. 2017; 4: 807–818.

345. Prasad S, Tyagi AK, Aggarwal BB. Recent developments in delivery, bioavailability, absorption and metabolism of curcumin: the golden pigment from golden spice. Cancer Res. Treat. 2014; 46: 2–18.

346. Karlowicz-Bodalska K, Han S, Freier J, Smolenski M, Bodalska A. Curcuma Longa as Medicinal Herb in The Treatment of Diabet- Ic Complications. Acta Pol. Pharm. 2017; 74: 605–610.

347. Corona-Rivera A, Urbina-Cano P, Bobadilla-Morales L, Vargas-Lares Jde J, Ramirez-Herrera MA, Mendoza-Magaua ML, Troyo-Sanroman R, Diaz- Esquivel P, Corona-Rivera JR. Protective in vivo effect of curcumin on copper genotoxicity evaluated by comet and micronucleus assays. J Appl Genet. 2007; 48:389-96.

348. Jagetia GC. Antioxidant activity of curcumin protects against the radiationinduced micronuclei formation in cultured human peripheral blood lymphocytes exposed to various doses of γ-Radiation. Int J Radiat Biol. 2021; 97:485-493.

349. Prasad R, Kumar M, Trivedi SP. Antigenotoxic effect of turmeric powder extract curcumin against chromium trioxide induced genotoxicity in fish Channa punctatus. J Entomol Zool Stud. 2017; 5:89-94.

350. Banerjee S, Bandyopadhyaya G, Chattopadhyhay K, Chattopadhyhay BD. Amelioration of nicotine-induced damage of blood cells in protein malnourished female rats by curcumin. Int J. Pharmacol. 2010; 6:609- 620.

351. Sadoughi S. Investigation the Effect of Curcumin on the Hormones of Pituitary-Ovarian Axis in Alloxan-induced Diabetic Rats. J Ardabil Univ Med Sci. 2016; 16:441-451.

352. Shah MZUH, Shrivastava VK. Turmeric extract alleviates endocrine-metabolic disturbances in letrozole-induced PCOS by increasing adiponectin circulation: A comparison with Metformin. Metabol Open. 2021; 13:100160.

353. Jantawong C, Priprem A, Intuyod K, Pairojkul C, Pinlaor P, Waraasawapati S, Mongkon I, Chamgramol Y, Pinlaor S. Curcumin-loaded nanocomplexes: Acute and chronic toxicity studies in mice and hamsters. Toxicol Rep. 2021; 8:1346-1357.

354. Nelson KM, Dahlin JL, Bisson J, Graham J, Pauli GF, Walters MA. The Essential Medicinal Chemistry of Curcumin. J Med Chem. 2017; 60:1620-1637.

355. Sharifi-Rad J, Rayess YE, Rizk AA, Sadaka C, Zgheib R, Zam W, Sestito S, Rapposelli S, Neffe-Skocińska K, Zielińska D, Salehi B, Setzer WN, Dosoky NS, Taheri Y, El Beyrouthy M, Martorell M, Ostrander EA, Suleria HAR,Cho WC, Maroyi A, Martins N. Turmeric and Its Major Compound Curcumin on Health: Bioactive Effects and Safety Profiles for Food, Pharmaceutical, Biotechnological and Medicinal Applications. Front Pharmacol. 2020; 11:01021.

356. Hassanzadeh K, Buccarello L, Dragotto J, Mohammadi A, Corbo M, Feligioni M. Obstacles against the Marketing of Curcumin as a Drug. Int J Mol Sci. 2020; 21:6619.

357. Lopresti AL. The Problem of Curcumin and Its Bioavailability: Could Its Gastrointestinal Influence Contribute to Its Overall Health-Enhancing Effects? Adv Nutr. 2018; 9(1):41-50.

358. Dei Cas M, Ghidoni R. Dietary Curcumin: Correlation between Bioavailability and Health Potential. Nutrients. 2019; 11:2147.

359. Zheng B, McClements DJ. Formulation of More Efficacious Curcumin Delivery Systems Using Colloid Science: Enhanced Solubility, Stability, and Bioavailability. Molecules. 2020; 25:2791.

360. Ishida J, Ohtsu H, Tachibana Y, Nakanishi Y, Bastow KF, Nagai M, Wang HK, Itokawa H, Lee KH. Antitumor agents. Part 214: synthesis and evaluation of curcumin analogues as cytotoxic agents. Bioorg. Med. Chem. 2002; 10: 3481–3487.

361. Selvam C, Jachak SM, Thilagavathi R, Chakraborti AK. Design, synthesis, biological evaluation and molecular docking of curcumin analogues as antioxidant, cyclooxygenase inhibitory and anti-inflammatory agents. Bioorg Med Chem Lett. 2005; 15:1793-7.

362. Sun A, Shoji M, Lu YJ, Liotta DC, Snyder JP. Synthesis of EF24-tripeptide chloromethyl ketone: a novel curcumin-related anticancer drug delivery system. J Med Chem. 2006; 49:3153-8.

363. Priyadarsini KI. The Chemistry of Curcumin: From Extraction to Therapeutic Agent. Molecules. 2014; 19: 20091-20112.

364. Jamwal R. Bioavailable curcumin formulations: A review of pharmacokinetic studies in healthy volunteers. J Integr Med. 2018; 16:367-374.

365. Tabanelli R, Brogi S, Calderone V. Improving Curcumin Bioavailability: Current Strategies and Future Perspectives. Pharmaceutics. 2021; 13:1715.

366. Rajalakshmi N, Dhivya S. A Review on the preparation methods of Curcumin Nanoparticles. PharmaTutor. 2018; 6:6-10.

367. Faraji AH, Wipf P. Nanoparticles in cellular drug delivery. Bio. Medic. Chem. 2009; 17: 2950-2962.

368. Moballegh Nasery M, Abadi B, Poormoghadam D, Zarrabi A, Keyhanvar P, Khanbabaei H, Ashrafizadeh M, Mohammadinejad R, Tavakol S, Sethi G. Curcumin Delivery Mediated by Bio-Based Nanoparticles: A Review. Molecules. 2020; 25: 689.

369. Vetha BSS, Kim EM, Oh PS, Kim SH, Lim ST, Sohn MH, Jeong HJ. Curcumin Encapsulated Micellar Nanoplatform for Blue Light Emitting Diode Induced Apoptosis as a New Class of Cancer Therapy. Macromol. Res. 2019; 27: 1179-1184.

370. Moballegh Nasery M, Abadi B, Poormoghadam D, Zarrabi A, Keyhanvar P, Khanbabaei H, Ashrafizadeh M, Mohammadinejad R, Tavakol S, Sethi G. Curcumin Delivery Mediated by Bio-Based Nanoparticles: A Review. Molecules. 2020; 25: 689.

371. Li X, Chen S, Zhang B, Li M, Diao K, Zhang Z, Li J, Xu Y, Wang X, Chen H. In situ injectable nano-composite hydrogel composed of curcumin, N, O-carboxymethyl chitosan and oxidized alginate for wound healing application. Int. J. Pharm. 2012; 437: 110-119.

372. Anitha A, Sreeranganathan M, Chennazhi KP, Lakshmanan VK, Jaya kumar R. In vitro combinatorial anticancer effects of 5-fluorouracil and curcumin loaded N, O-carboxymethyl chitosan nanoparticles toward colon cancer and in vivo pharmacokinetic studies. Eur. J. Pharm. Biopharm. 2014; 88: 238-251.

373. Nambiar S, Osei E, Fleck A, Darko J, Mutsaers AJ, Wettig S. Synthesis of curcumin-functionalized gold nanoparticles and cytotoxicity studies in human prostate cancer cell line. Appl. Nanosci. 2018; 8: 347-357.

374. Elbialy NS, Abdelfatah EA, Khalil WA. Antitumor activity of curcumin-green synthesized gold nanoparticles: In vitro study. BioNanoScience. 2019; 9: 813-820.

375. Saikia C, Das MK, Ramteke A, Maji TK. Controlled re of curcumin from thiolated starch-coated iron oxide magnetic nanoparticles: An in vitro evaluation. Int. J. Polym. Mater. 2017; 66: 349-358.

376. Ayubi M, Karimi M, Abdpour S, Rostamizadeh K, Parsa M, Zamani A, Saedi, A. Magnetic nanoparticles decorated with PEGyla curcumin as dual targeted drug delivery: Synthesis, toxicity and bioco atibility study. Mater. Sci. Eng. C. 2019; 104: 109810.

377. Fathy Abd-Ellatef GE, Gazzano E, Chirio D, Hamed AR, Belisario DC, Zuddas C, Peira E, Rolando B, Kopecka J, Assem Said Mae M. Curcumin-Loaded Solid Lipid Nanoparticles Bypass P-Gl oprotein Mediated Doxorubicin Resistance in Triple Negative Breast Cancer Cells. Pharmaceutics. 2020; 12: 96.

378. Brahmkhatri VP, Sharma N, Sunanda P, D'souza A, Raghothama S, Atreya HS. Curcumin nanoconjugate inhibits aggregation of N-t erminal region (Aβ-16) of an amyloid beta peptide. New J. Chem. 2018; 42: 19881-19892.

379. Nabih Maria D, R Mishra, S, Wang L, Helmy Abd-Elgawad AE, Abd-Elazeem Soliman O, Salah El-Dahan M, M Jablonski M. Water-soluble complex of curcumin with cyclodextrins: enhanced physical properties for ocular drug delivery. Curr. Drug Deliv. 2017; 14: 875-886.

380. Guo S. "Encapsulation of curcumin into β-cyclodextrins inclusion: A re view", in: 2nd International Conference on Biofilms (ChinaBiofilms 2019), eds. Z.B. Xu, D.Q. Chen & J.Y. Liu: EDP Sciences), 1-4.

381. Silva IDS, Peron AP, Leimann FV, Bressan GN, Krum BN, Fachinetto R, Pinela J, Calhelha RC, Barreiro MF, Ferreira IC. In vitro and in vivo evaluation of enzymatic and antioxidant activity, cytotoxicity and genotoxicity of curcumin-loaded solid dispersions. Food Chem. Toxicol. 2019; 125: 29-33.

382. Masoule SF, Pourhajibagher M, Safari J, Khoobi M. Base-free green synthesis of copper (II) oxide nanoparticles using highly cross-link ed poly (curcumin) nanospheres: synergistically improved antimicrobial activity. Res. Chem. Intermed. 2019; 45: 4449-4462.

383. Kim JY, Lee YM, Kim DW, Min T, Lee SJ. Nanosphere Loaded with Curcumin Inhibits the Gastrointestinal Cell Death Signaling Pathway Induced by the Foodborne Pathogen Vibrio vulnificus. Cells. 2020; 9: 631.

384. Priya P, Raj RM, Vasanthakumar V, Raj V. Curcumin-loaded layer-by-layer folic acid and casein coated carboxymethyl cellulose/caseinnanogels for treatment of skin cancer. Arab. J. Chem. 2020; 13: 694-708.

385. Mangalathillam S, Rejinold NS, Nair A, Lakshmanan VK, Nair SV, Jayakumar R. Curcumin loaded chitin nanogels for skin cancer treatment via the transdermal route. Nanoscale 2012; 4: 239-250.

386. Subramani PA, Panati K, Narala VR. Curcumin nanotechnologies and its anticancer activity. Nutr. Cancer. 2017; 69: 381-393.

387. Wang XS, Williams LA, Krishnan S, Liao Z, Liu P, Mao L, Shi Q, Mobley GM, Woodruff JF, Cleeland CS. Serum sTNF-R1, IL-6, and the development of fatigue in patients with gastrointestinal cancer undergoing chemoradiation therapy. Brain Behav. Immun. 2012; 26:699-705.

388. Milano F, Mari L, van de Luijtgaarden W, Parikh K, Calpe S, Krishnadath KK. Nano-curcumin inhibits proliferation of esophageal adenocarcinoma cells and enhances the T cell mediated immune response. Front Oncol. 2013; 3:137.

389. Suresh S, Sankar P, Telang AG, Kesavan M, Sarkar SN. Nanocurcumin ameliorates Staphylococcus aureus-induced mastitis in mouse by suppressing NF-κB signaling and inflammation. Int. Immunopharmacol. 2018; 65: 408–412.

390. Sinjari B, Pizzicannella J, D'aurora M, Zappacosta R, Gatta V, Fontana A, Trubiani O, Diomede F. Curcumin/Liposome Nanotech nology as Delivery Platform for Anti-inflammatory Activities via NFkB/ ERK/pERK Pathway in Human Dental Pulp Treated With 2-HydroxyEthyl MethAcrylate (HEMA). Front. physiol. 2019; 10: 372.

391. Yavarpour-Bali H, Ghasemi-Kasman M, Pirzadeh M. Curcumin-loaded nanoparticles: a novel therapeutic strategy in treatment of central nervous system disorders. Int J Nanomedicine. 2019; 14:4449-4460.

392. Shishodia S, Sethi G, Aggarwal BB. Curcumin: getting back to the roots. Ann. N. Y. Acad. Sci. 2005; 1056: 206-217.

393. Basniwal RK, Khosla R, Jain N. Improving the anticancer activity of curcumin using nanocurcumin dispersion in water. Nutr. Cancer. 2014; 66: 1015-1022.

394. Baghi N, Bakhshinejad B, Keshavarz R, Babashah S, Sadeghizadeh, M. Dendrosomal nanocurcumin and exogenous p53 can act synergistically to elicit anticancer effects on breast cancer cells. Gene. 2018; 670: 55-62.

395. Karthikeyan A, Senthil N, Min T. Nanocurcumin: A Promising Candidate for Therapeutic Applications. Front Pharmacol. 2020; 11:487.

396. Rafiee S, Bagherniya M, Askari G, Sathyapalan T, Jamialahmadi T, Sahebkar A. The Effect of Curcumin in Improving Lipid Profile in Patients with Cardiovascular Risk Factors: A Systematic Review of Clinical Trials. Adv Exp Med Biol. 2021; 1291:165-177.

397. Hewlings S, Kalman D. Curcumin: a review of its' effects on human health. Foods. 2017; 6: 92.

398. Rajasekar A. Facile synthesis of curcumin nanocrystals and validation of its antioxidant activity against circulatory toxicity in Wistar rats. J. Nanosci. Nanotechnol. 2015; 15: 4119-4125.

399. Ranjbar A, Gholami L, Ghasemi H, Kheiripour N. Effects of nano-curcumin and curcumin on the oxidant and antioxidant system of the liver mitochondria in aluminum phosphide-induced experimental toxicity. Nanomed. J. 2020; 7: 58-64.

400. El-Desoky GE, Wabaidur SM, AlOthman ZA, Habila MA. Regulatory Role of Nano-Curcumin against Tartrazine-Induced Oxidative Stress, Apoptosis- Related Genes Expression, and Genotoxicity in Rats. Molecules. 2020; 25:5801.

401. Ali BH, Karaca T, Al Suleimani Y, Al Za'abi M, Al Kalbani J, Ashique M, Nemmar A. The effect of swimming exercise on adenine-induced kidney disease in rats, and thes influence of curcumin or lisinopril thereon. PLoS One. 2017; 12:0176316.

402. Poojary KK, Nayak G, Vasani A, Kumari S, Dcunha R, Kunhiraman JP, Gopalan, D, Rao RR, Mutalik S, Kalthur SG, Murari MS, Raghu SV, Adiga SK, Kalthur G. Curcumin nanocrystals attenuate cyclophosphamide-induced testicular toxicity in mice. Toxicology Appl Pharmacol. 2021; 433:115772.

403. Yadav A, Flora S, Kushwaha P. Nanocurcumin Prevents Oxidative Stress Induced Following Arsenic and Fluoride Co-exposure in Rats. Defence Life Science Journal. 2016; 1:69-77.

404. Farhadi M, Bakhshandeh M, Shafiei B, Mahmoudzadeh A, Hosseinimehr S J. The Radioprotective Effects of Nano-Curcumin Against Genotoxicity Induced by Iodine-131 in

Patients with Differentiated Thyroid Carcinoma (DTC) by Micronucleus Assay. Int J Cancer Manag. 2018; 11:14193.

405. Sandhiutami NMD, Arozal W, Louisa M, Rahmat D, Mandy T. Comparative Effect of Curcumin and Nanocurcumin on Nephroprotection at Cisplatininduced Rats. J Pharm Bioallied Sci. 2019; 11:567-573.

406. Jie Z, Chao M, Jun A, Wei S, LiFeng M. Effect of Curcumin on Diabetic Kidney Disease: A Systematic Review and Meta-Analysis of Randomized, Double-Blind, Placebo-Controlled Clinical Trials. Evid Based Complement Alternat Med. 2021; 2021:6109406.

407. El-Desoky GE, Wabaidur SM, AlOthman ZA, Habila MA. Evaluation of Nanocurcumin effects against Tartrazine-induced abnormalities in liver and kidney histology and other biochemical parameters. Food Sci. Nutr. 2022; 10:1344-1356.

408. Atia MM, Abdel-Tawab HS, Mostafa AM, Mobarak SA. Nanocurcumin and curcumin prevent N, N'-methylenebisacrylamide-induced liver damage and promotion of hepatic cancer cell growth. Sci Rep. 2022; 12:8319.

409. Kheiripour N, Plarak A, Heshmati A, Asl SS, Mehri F, Ebadollahi-Natanzi A, Ranjbar A, Hosseini A. Evaluation of the hepatoprotective effects of curcumin and nanocurcumin against paraquat-induced liver injury in rats: Modulation of oxidative stress and Nrf2 pathway. J. Biochem. Mol. Toxicol. 2021; 35: 22739.

410. Mohammed AM, Hussen DF, Rashad H, Hasheesh A. The Micronuclei Scoring as a Biomarker for Early Detection of Genotoxic Effect of Cigarette Smoking. Asian Pac J Cancer Prev. 2020; 21:87-92.

411. Biswas AK, Islam MR, Choudhury ZS, Mostafa A, Kadir MF. Nanotechnology based approaches in cancer therapeutics. Advances in Natural Sciences: J. Nanosci. Nanotechnol. 2014; 5: 04300.

412. Flora G, Gupta D, Tiwari A. Preventive efficacy of bulk and nanocurcumin against lead-induced oxidative stress in mice. Biol. Trace Elem. Res. 2013; 152:31-40.

413. Zou P, Zhang J, Xia Y, Kanchana K, Guo G, Chen W, Huang Y, Wang Z, Yang S, Liang G. ROS generation mediates the anti-cancer effects of WZ35 via activating JNK and ER stress apoptotic pathways in gastric cancer. Oncotarget. 2015; 6: 5860.

414. Ma Z, Shayeganpour A, Brocks DR, Lavasanifar A, Samuel J. High-performance liquid chromatography analysis of curcumin in rat plasma: application to pharmacokinetics of polymeric micellar formulation of curcumin. Biomed. Chromatogr. 2007; 21: 546-552.

415. Dende C, Meena J, Nagarajan P, Nagaraj VA, Panda AK, Padmanaban G. Nanocurcumin is superior to native curcumin in preventing degenerative changes in Experimental Cerebral Malaria. Sci Rep. 2017; 7:10062.

416. Mythri RB, Jagatha B, Pradhan N, Andersen J, Bharath MS. Mitochondrial complex I inhibition in Parkinson's disease: how can curcumin protect mitochondria? Antioxid. Redox Signal. 2007; 9: 399-408.

417. Mohanty C, Sahoo SK. The in vitro stability and in vivo pharmacokinetics of curcumin prepared as an aqueous nanoparticulate formulation. Biomaterials 2010; 31: 6597-6611.

418. Muller RH, Keck CM. Challenges and solutions for the delivery of biotech drugs–a review of drug nanocrystal technology and lipid nanoparticles. J. Biotech. 2004; 113:151-170.

419. Yallapu MM, Nagesh PKB, Jaggi M, Chauhan SC. Therapeutic applications of curcumin nanoformulations. The AAPS journal 2015; 17: 1341-1356.

420. No DS, Algburi A, Huynh P, Moret A, Ringard M, Comito N, Drider D, Takhistov P, Chikindas ML. Antimicrobial efficacy of curcumin nanoparticles against Listeria monocytogenes is mediated by surface charge. J Food Saf. 2017; 37:12353.

421. Gessner A, Waicz R, Lieske A, Paulke BR, Mäder K, Müller R. Nanoparticles with decreasing surface hydrophobicities: influence on plasma protein adsorption. Int. J. Pharm. 2000; 196: 245-249.

422. Chompoosor A, Saha K, Ghosh PS, Macarthy DJ, Miranda OR, Zhu ZJ, Arcaro KF, Rotello VM. The role of surface functiona lity on acute cytotoxicity, ROS generation 1287 and DNA damage by cationicgold nanoparticles. Small. 2010; 6: 2246-2249.

423. Jones MC, Jones SA, Riffo-Vasquez Y, Spina D, Hoffman E, Morgan A, Patel A, Page C, Forbes B, Dailey LA. Quantitative assessment of nanoparticle surface hydrophobicity and its influence on p ulmonary biocompatibility. J. Control. Release. 2014; 183: 94-104.

424. Prokop A, Davidson JM. Nanovehicular intracellular delivery syst ems. J. Pharm. Sci. 2008; 97: 3518-3590.

CHAPTER III

MATERIALS & METHODS

3. Materials and Methods

3.1 Nanocurcumin Synthesis and Characterization

3.1.1 Reagents

Curcumin: Curcumin was purchased from Sigma Chemicals Company, St. Louis

3.1.2 Methodology

Curcumin was dissolved in dichloromethane (DCM) to create a 0.018 M curcumin solution for nanocurcumin production. For 10 minutes, the solution was added to warm (500C) Milli-Q ultrapure water at a flow rate of 0.2 mL/min while being continuously stirred using an ultrasonic-pulse sonicator (Hielscher Ultrasonic Processor UP100H, Germany). It was then sterilized by autoclaving, freeze-dried at - 800C followed by lyophilization (Eyela-FDU- 2000, Japan). A dry orange coloured powder of nanocurcumin was obtained.

The laboratory-synthesized nanocurcumin's surface plasmon resonance was optically examined with a UV-Vis spectrophotometer (UV-3101PC, Shimadzu, Japan). A Field Emission Scanning Electron Microscope (FE-SEM, FEI INSPECT F50, Netherlands) was used to assess the size and morphologically characterize the nanocurcumin. The produced nanocurcumin was analyzed using Fourier Transform Infrared Spectroscopy (FTIR) (Perkin-Elmer FTIR-1600, USA) after mixing dried nanoparticle powder with KBr. Spectra were taken of 4 cm-1. FTIR was used to identify the presence of various functional groups. Using XRD, the produced nanocurcumin's crystalline nature was identified (Bruker AXS, Inc., Model D8,WI). To identify the crystalline phase, the sample was subjected to 40 kV voltage, 40 mA current, and a scanning rate of 0.028 min-1 over a range of 2Θ (5 to 40°).

3.2 Experiments

3.2.1 Animals and Treatments

Thirty female albino rats of the Wistar strain (Rattus norvegicus), weighing 140–150 g and aged 60–75 days, were procured from the Animal Housing Facility and maintained with protein restricted diet according to the guidelines of the Institutional Animal Ethics Committee of the Jadavpur University, Kolkata, India (Ref. No.: AEC/PHARM/1502/14/2015, Dated: 112 30/07/2015)24. The animals were in polypropylene cages in an air-conditioned room. The animals were fed a regular protein-restricted diet consisting of 5% casein, 83% carbohydrate, 7% fat, 4% salt mixture, and 1% vitamin mixture [1].

Thirty female albino rats equally divided into six groups of five rats in each and treated as below for 21 days:

Group-I: Served as Control group in which animals were received no treatment.

Group-II: Nicotine treated group in which animals were injected (subcutaneously) with the effective dose of nicotine (2.5 mg/kg body weight).

Group-III: Nicotine and curcumin treated group in which animals were injected (subcutaneously) with the effective dose of nicotine (2.5 mg/kg body weight) and received effective dose of curcumin (80 mg/kg body weight) orally.

Group-IV: Nicotine and nanocurcumin treated group in which animals were injected (subcutaneously) with the effective dose of nicotine (2.5 mg/kg body weight) and received effective dose of nanocurcumin (4 mg/kg body weight) orally.

Group -V: Nanocurcumin treated group in which animals were supplemented with effective dose of nanocurcumin (4 mg/kg body weight) orally for three weeks.

3.2.2 Administration of Nicotine

As a stock solution, 2.5 g of nicotine hydrogen tartrate salt was diluted in 1 mL of normal saline. It was diluted with normal saline to achieve a final concentration of 2.5 μg/μL. A syringe was filled with the required volume of nicotine solution based on the animals' body weights, and it was administered subcutaneously into the animals every day for 21 days.

3.2.3 Administration of Curcumin and Nanocurcumin

Curcumin and nanocurcumin were disseminated separately in sterile distilled water and supplied orally with gavages three hours after nicotine treatment in their respective groups of animals. After 21 days of treatment, the animals were starved for 12 hours and then sacrificed the next day after being given mild anesthesia. The selective dose for this experiment is nicotine (2.5 mg/kg bodyweights/day), curcumin (80 mg/kg bodyweights/day), and nanocurcumin (4 mg/kg bodyweights/day) for 21 consecutive days to the respective groups of animals as designed. Blood samples were drawn directly from the heart and preserved in a simple vial with no anticoagulant. Sera were separated and kept at -20°C for further analysis. To avoid auto-oxidation, vital organs such as the kidney, liver, and pancreas were dissected out and kept in vacuum desiccators at -20°C.

3.3 Biochemical Analysis

3.3.1. Estimation of Hepatic Enzymes

3.3.1.1. Acid Phosphatase Activity (ACP) in Serum

The method described by Bessey et al., 1946 [2] was used to measure the serum Acid Phosphatase level using a commercially available kit (Arkray Healthcare Pvt. Ltd, India).

3.3.1.1.1. Reagents

1. Acid buffer-substrate solution
2. NaOH (0.1N)

3.3.1.1.2. Procedure

Two dry sterile glass tubes were labeled as "sample" and "control", respectively. Each test tube was filled with 1ml of acid buffer-substrate solution and incubated for 5 minutes at 37°C. The tubes were then incubated for another 30 minutes at 37°C after serum (0.2ml) was added to the "sample". Each tube was filled with 4 ml of sodium hydroxide (0.1N); serum (0.2 ml) was added as a "control" and thoroughly mixed. After proper mixing, optical density measured at 405nm against in Spectrophotometer that recorded was the difference of the "Sample" and corresponding "Control".

3.3.1.2. Alkaline Phosphatase Activity in Serum

Using a commercially accessible kit (Arkray Healthcare PVT. Ltd., India), the serum alkaline phosphatase level was calculated using the methodology outlined by Kind and King, 1954 [3].

3.3.1.2.1. Reagents

1. Buffered substrate (pH 10)
2. Chromogen reagent
3. Phenol standard (10 mg %)

4. Distilled Water

3.3.1.2.2. Procedure

Four test tubes were taken for every serum sample. "Sample" was the label for one, "Control" for another, and "Blank" and "Standard" for the other two. 1.5 ml of distilled water and 0.5 ml of alkaline buffer–substrate solution were added to each tube, and the tubes were then allowed to equilibrate for 3 minutes at 37°C. 0.05 ml of serum was placed in the "sample," and 0.05 ml of phenol standard (10 mg; 0.05 ml) was placed in the "standard" tube. Both were then incubated for 15 minutes at 37°.Each tube was filled with 1 ml of the chromogen reagent. The "control" tube was filled with 0.05 ml of serum. Finally, the Spectrophotometer was used to measure the absorbance of Blank (B), Standard (S), Control (C), and Test (T) at 510 nm against purified water.

3.3.1.3. Aspartate Aminotransferase (AST) Or SGOT Assay

The AST level was determined as described by Reitman and Frankel (1957) [4] using a commercially available kit (Arkray Healthcare Pvt. Ltd., India).

3.3.1.3.1. Reagents

1. Alanine α Keto Glutarate substrate (pH-7.4)
2. DNPH (2,4- dinitrophenylhydrazine) colour reagent
3. NaOH solution (4N)
4. 6mM working pyruvate standard (150IU/L)
5. Deionized water

3.3.1.3.2. Procedure

In this procedure, four dry, sterile test tubes were taken and labeled as "Blank", "Standard", "Test", and "Control". Each test tube filled 0.25 ml of buffered aspartate-ketoglutarate solution (pH-7.4). Then serum sample of 0.05 ml was added into the tube marked as "Test". The vial labeled "Standard" was filled with 0.05 ml of the working pyruvate standard (6 mM) sample. After that, the tubes were incubated for 60 minutes at 37°C. Each tube was then filled with 0.25 ml of the 2, 4-DNPH color reagent. In the meantime, 0.05 ml of deionized water was put into the "Blank" tube. 0.05 ml serum sample was added to the "Control" tube. After being equilibrated, each tube was let to stand at room temperature (+15°C to +30°C) for 20 minutes. After the incubation period ended, each tube received 2.5 ml of sodium hydroxide (4N). Lastly, within 15 minutes, O.D. was measured in a spectrophotometer at 505 nm against purified water.

3.3.1.4. Alanine Aminotransferase (ALT) or SGPT Assay

Reitman and Frankel (1957) used a commercially available kit (Arkray Healthcare Pvt. Ltd., India) to assess the ALT level [4].

3.3.1.4.1. Reagents

1. Alanine α Keto Glutarate substrate (pH-7.4)
2. DNPH (2,4- dinitrophenylhydrazine) colour reagent
3. 8mM working pyruvate standard (IU/L)
4. Deionized water

3.3.1.4.2. Procedure

In this procedure, four dry, sterile test tubes were taken and labeled as "Blank", "Standard", "Test", and "Control". In each test tube, 0.25 mL of buffered Alanine α Keto Glutarate substrate (pH-7.4) was added. A serum sample of 0.05 ml was placed in the tube labeled "Test". Add 0.05 ml of the working pyruvate standard (8mM) sample to the "Standard" tube. After that, each tube was incubated for 30 minutes at 37°C. Meanwhile, 0.05 ml of deionized water was added to the tube labeled "Blank". The "Control" tubes were filled with 0.05 ml of serum sample. After being equilibrated, each tube was maintained at room temperature (+15°C to +30°C) for 20 minutes. Each tube was then filled with 2.5 ml of sodium hydroxide (4N). After evenly mixing each tube, the O.D. was determined within 15 minutes at 505 nm using purified water in a spectrophotometer.

3.3.2. Assessment of Renal Parameters

3.3.2.1. Urea Assay

The Diacetylmonoxide (DAM) technique, as outlined by Friedman and Young (2000), was used to estimate the concentration of urea in serum using a commercially available kit (Arkray Healthcare Pvt. Ltd., India) [5].

3.3.2.1.1. Reagents

1. Urea reagent
2. Diacetylmonoxime (DAM)
3. Working urea standard (30 mg%)

3.3.2.1.2. Procedure

Three test tubes, designated "Blank," "Test," and "Standard," were needed for the experiment. The urea reagent (2.5 ml) was added to each tube. The tube marked as “Test" was then filled with 0.01 ml of the serum sample. The "Standard" tube was then filled with 0.01 ml of working urea standard (30 mg%). After proper mixing, Diacetylmonoxime (DAM), 0.25 ml, was then added. The tubes were immersed in boiling water for 10 minutes, then cooled under running tap water for 5 minutes. Optical density was measured using a spectrophotometer at 525nm against "Blank".

3.3.2.2. Creatinine Assay

Creatinine in serum was estimated using the Alkaline Pictrate Method described by Moss et al., 1975, and a commercially available kit (Arkray Healthcare Pvt. Ltd., India) [6].

3.3.2.2.1. Reagents

1. Picric Acid
2. 0.75 (N) NaOH solution
3. Stock Creatinine Standard (150 mg%)

3.3.2.2.2. Procedure

0.5 ml of water and 3 ml of picric acid was added into the tube containing 0.5ml of serum samples, this helps in deproteinization. The tube was then immediately cooled by keeping it under running tap water after being in a boiling water bath for precisely one minute. It was then either filtered or centrifuged. After that, 2 ml of the filtrate was collected and placed in the "Test" tube. The "Standard" tube was filled with 0.5 ml of stock creatinine standard (150 mg%). 0.5ml of

double distilled water was added to the "Blank" tube. Picric acid (1.5ml) was added to the "Blank" and "Standard" tubes. Finally, stopping the reaction by 0.5 ml of sodium hydroxide (0.75N) was added to each tube. After thoroughly mixing each tube and incubating them for precisely 20 minutes at room temperature (+15°C to +30°C), the optical densities of "Blank," "Standard," and "Test" were measured spectrophotometrically at 520 nm against purified water.

3.3.2.3. Estimation of Blood Urea Nitrogen (BUN)

Using a commercially available kit (Arkray Healthcare Pvt. Ltd., India), the BUN in serum was estimated using the calculation method outlined by Wallker et al., 1990 [7].

3.3.2.2.1. Calculation

$$BUN = A \times 0.467$$

Here, A= Urea (mg/dl) calculated as above

3.3.3. Total Cholesterol Assay

Using a commercially available kit (Arkray Healthcare Pvt. Ltd., India), total cholesterol was calculated in accordance with Allain et al., 1974 [8].

3.3.3.1. Reagents

1. Cholesterol mono reagent
2. Cholesterol Standard

3.3.3.2. Procedure

For each serum sample, three test tubes (dry, sterile) were used. One used as a "Test" and another two were labeled as "Blank" and "Control". 1ml of enzyme reagent was added into all three tubes and the 10µl of cholesterol standard solution (200mg/dl) was added into the "Standard" tubes only. 10µl of serum sample was pipetted out and added into the tube marked as "Test". All the tubes were equilibrated properly and kept for incubation at 37°C for 5 minutes. Then the absorbance was measured against Reagent Blank at 505nm.

3.3.4. Estimation of High-Density Lipoprotein

Using a commercially available kit (Arkray Healthcare Pvt. Ltd., India), total cholesterol was calculated in accordance with Grundy's 1993 description [9].

3.3.4.1. Reagent

1. Cholesterol mono reagent
2. Precipitating Reagent
3. HDL- Cholesterol Standard

3.3.4.2. Procedure

To separate HDL-cholesterol (HDL-C), 200 µl of serum and 200 µl of precipitating reagent were placed in a dry test tube, incubated for 10 minutes at 15°C to 30°C, and then centrifuged at 2000 x g for 15 minutes. HDL was estimated using the supernatant. We took three dry glass tubes and labeled them "Sample," "Blank," and "Standard." Respectively. One ml of cholesterol mono reagent was taken in each tube. HDL- Cholesterol Standard (100 µl) was added in “Standard”, 100

µl sample in “Sample,” mixed well and incubated at 37°C for 10 mins. A 505 nm filter was used to capture the O.D. against "Blank."

3.3.5. Triglycerides Assay

A commercial kit (Arkray Healthcare Pvt. Ltd., India) was used to quantify serum triglycerides using the methodology outlined by Werner et al., 1981 [10].

3.3.5.1. Reagents

1. Triglyceride mono reagent
2. Triglyceride Standard solution

3.3.5.2. Procedure

Three tubes (dry, glass) were taken and marked as “Sample,” “Blank,” and “Standard,” respectively. Each tube was filled with 1 ml of the triglyceride mono reagent. 10 ml of triglyceride standard solution were placed in "Standard" and 10 ml of serum in "Sample." All tubes were then incubated for ten minutes at 37°C. O.D. was obtained against "Blank" using a 505 nm filter.

3.3.6. Estimation of Very Low-Density Lipoprotein (VLDL) Cholesterol

VLDLC was calculated by the formula described by Suchanda, 2008 [11].

$$VLDLC = Triglycerides\ (mg/dl)/\ 5$$

3.3.7. Estimation of Low-Density Lipoprotein (LDL)

LDL cholesterol was calculated by the using the formula described by Oliveira et al., 2013 [12].

$$LDL\ cholesterol = Total\ cholesterol\ (mg/dl) – Triglycerides\ (mg/dl)/5 – HDL\text{-}C\ (mg/dl).$$

3.3.8. Estimation of Lipid Peroxidation

The level of lipid peroxidation was determined using the thiobarbituric acid test as reported by Chatterjee and Agarwal, 1988 [13].

3.3.8.1. Reagents

1. Phosphate buffer
2. 20% trichloroacetic acid solution
3. Thiobarbituric acid (TBA)

3.3.8.2. Tissue Preparation

0.5 g of liver tissue was homogenized in 5 ml of phosphate buffer (0.1M; pH 7.5) and centrifugation at 7000 x g for 20 mins. The experiment was performed with the clear supernatant.

3.3.8.3. Procedure

2 ml of homogenate, 1 ml TCA and 2 ml TBA were taken in a Stoppard tube mixed thoroughly and heated for 10 mins in boiling water bath. Then quickly cooled and centrifuged for 10 minutes at 5000 x g). The OD was measured at 530 nm against a blank.

3.3.8.4. Calculation

The lipid peroxidation level was estimated by the malondialdehyde (MDA) level produced by the breakdown of hydroperoxide. The extinction coefficient for MDA is 1.56 x 105 / M/ cm [14]. The findings were represented as nanomole MDA per mg of protein.

3.3.9. Estimation of Antioxidant Enzymes

3.3.9.1. Estimation of Catalase (CAT)

Estimation of Catalase (CAT) Catalase was estimated using the enzyme-catalyzed breakdown of H_2O_2, as described by Cohen et al., 1970 [15].

3.3.9.1.1. Reagents

1. Phosphate buffer (0.01 M; pH 7.0)
2. H_2O_2 (6 mM)
3. H_2SO_4 (6 N)
4. $KMnO_4$ (0.01 N)

3.3.9.2. Tissue Preparation

Homogenizing 0.5 g of liver tissue in 5 ml of phosphate buffer (0.01M; pH 7.5), the sample was centrifuged for 20 mins at 7000 x g. The experiment was performed with the clear supernatant.

3.3.9.3. Procedure

For this experiment 3 glass tubes (dry, sterile) were taken and leveled as Sample, Blank, and Standard. Cold sample, buffer, and distilled water were all obtained in separate tubes at 0.5 ml each. The enzymatic reaction was initiated by thoroughly mixing in 5 ml of cold H_2O_2 for 3 minutes. 1ml of H_2SO_4 (6 N) was then added to stop the reaction. Optical density was measured at 480 nm as soon as 7 ml of $KMnO_4$ (0.01 N) was added.

3.3.9.4. Calculation

The catalase activity was expressed by the rate of decomposition of H_2O_2 using the formula:

$$K=\log (S_0/S_3) \text{ X } 2.3/T$$

$$K = (\text{Log } S_0/S_3) \text{ x } 2.3/ T$$

K = first-order reaction rate constant

T = time interval for which reaction was carried out (3mins)

S_0 = substrate concentration at zero time

S_3 = substrate concentration after 3mins

Catalase activity is expressed in nmol H_2O_2 decomposed / min/mg protein

3.3.9.5. Estimation of Superoxide Dismutase (SOD)

Beauchamp and Fridovich (1971) described, the enzymatic reduction of nitro tetrazolium to pharmazone by superoxides was used to estimate SOD activity [16].

3.3.9.5.1. Reagents

1. 1 mM Phosphate buffer (pH 7.8)
2. Methionine (650 mM)
3. Riboflavin (900 UM)
4. Nitro-blue tetrazolium (NBT) (7.5 mM)
5. EDTA (200mM)

3.3.9.5.2. Tissue Preparation

5 grams of liver tissue were mixed in 5 ml of phosphate buffer (0.01M; pH 7.8) and centrifuged for 20mins at 7000 x g. The clear supernatant was used for the test.

3.3.9.5.3. Procedure

300 µl NBT, 600 µl methionine, 150 µl EDTA, 1.7 ml phosphate buffer, and 50 µl tissue homogenate were collected in a glass tube. The "Control" tube was one that contained no tissue homogenate. Each tube received 200 µl of riboflavin, which was then maintained under tube light. optical density. was measured at different times intervals at 560 nm.

3.3.9.5.4. Calculations

$$Z = (X - Y)/ X *100$$

Z= SOD activity in nmol O2 decomposed / min / 100 mg protein

X= Optical Density at zero mins

Y= Optical Density at 2 mins

3.3.9.6. Estimation of Glutathione Peroxide (GPx)

GPx activity was determined by oxidizing selenol with H_2O_2 (Levander et al., 1983) [17].

3.3.9.6.1. Reagents

1. 50 mM Phosphate buffer (pH 7.0)
2. 50 mM EDTA
3. 200 mM Glutathione (GSH)

4. NADPH

The reaction mixture included 11.808 ml of phosphate buffer with EDTA, 20 µl glutathione reductase, 64.8 µl GSH, and 1.2 mg NADPH. The pH of the solution was then adjusted to 7.0 using 108 ml of phosphate buffer.

3.3.9.6.2. Tissue Preparation

Liver tissue (5 gm) was homogenized in 5 ml of phosphate buffer (50 mM; pH 7.8) and centrifuged at 7000 x g for 20 minutes. The experiment was performed with the clear supernatant.

3.3.9.6.3. Procedure

In a glass tube, 3 ml of reaction mixture were mixed with 50 µl of homogenate, and the optical density (OD) was measured at 340 nanometers. 10 µl of H2O2 was then added. After adding H2O2 for 0, 30, and 60 seconds, the OD was measured. In another tube, combine 3 ml of reaction mixture, 50 µl of phosphate buffer, and 10 µl of H2O2. Measure O.D. at 340 nm and use as 'Blank.

3.3.9.6.4. Calculation

Units/ml enzyme = sample optical density x 2 x total volume of assay x dilution factor (6.22) x volume of sample used.

GPx activity = Units/ml enzyme amount of protein value were expressed as n mole/ min/ mg protein

3.3.9.7. Calculation of Superoxide and Peroxide Handling Capacities (SPHC)

Superoxide and peroxide handling capacities (SPHC) were calculated as described by Nayak et al., 2014 [18].

GSH-independent SPHC= CAT/SOD

GSH-dependent SPHC = GPx/SOD

3.3.10. Total Protein Estimation from Tissues

The total protein content of several organs such as the liver, kidney, and ovary were determined using the Lowry technique [19].

3.3.10.1. Reagents

1. Phosphate buffer (pH 7.0)
2. Na2CO3 (2%) in 0.1 N NaOH
3. NaK Tartrate (1%) in H2O
4. CuSO4. 5 H2O (0.5%) in distilled H2O
5. Reagent I: A -48 ml + B – 1ml + C-1ml
6. Reagent II- Folin-Phenol [2 N]: water [1:1]
7. BSA Standard - 1 mg/ ml

3.3.10.2. Tissue Preparation

0.5 gm tissue (liver, kidney, ovary) was homogenized in phosphate buffer (5 ml; 50mM; pH 7.0) and centrifuged at 7000 x g for 20 minutes. The clear supernatant was used for the test.

3.3.10.3. Procedure

A standard graph was created using known concentrations of BSA. The quantity of protein in the test sample was determined using the standard graph provided. The amount of protein in samples was measured from the standard graph according to the respective OD values.

3.3.11. Protein Oxidation Assay

3.3.11.1. Reagents

1. Phosphate buffer (pH 7.4)
2. Trichloro acetic Acid
3. DNPH (10mM)
4. HCl (2N)
5. Ethanol
6. Guanidine hydrochloride (6 M)

3.3.11.2. Procedure

Protein oxidation was evaluated using the Levine et al. (1994) [20] approach, which involved measuring the carbonyl concentration of serum. The serum was centrifuged for 10 minutes at 12,000 rpm after being diluted with phosphate buffered saline at a 1:40 ratio. Centrifugation for 5 minutes was used to precipitate the serum protein after adding cold trichloroacetic acid (TCA, 20% final concentration). Solution 10 mM DNPH in 2 N HCl was combined with precipitated protein and stored in the dark for 1 hour with frequent vortexing at 15-minute intervals. The protein solution was then centrifuged for 5 minutes at 12000 rpm after being mixed with cold TCA (10% final concentration). The supernatants were discarded and protein

pellets were washed with 10% TCA by centrifugation. To remove any remaining free DNPH, 1 milliliter of ethanol/ethyl acetate (in a 1:1, v/v ratio) was added. After being suspended in 6 M guanidine hydrochloride, the samples were well mixed and incubated for 15 minutes at 37 °C. OD was calculated against 366 nm.

3.3.12. Estimation of Haemoglobin

The Sahli's Haemoglobinometer was used to estimate the percentage of hemoglobin [21].

3.3.12.1. Reagents and Accessories

1. Haemoglobinometer
2. HCl (0.1N

3.3.12.2. Procedure

Two tubes, 20 mm3 of hemolyzed with the same volume of 0.1N HCl and saturated CO gas, consist of the hemoglobinometer. This tube's color was considered the standard. In a separate tube, a blood sample was combined with an equal volume of HCl (0.1N), and color development was noted. The colour intensity of the sample adjusted with that of standard by adding HCl (0.1N).

3.3.13. DNA Extraction from Blood

The DNA was extracted from a blood sample, and its content was assessed using a slightly modified approach of the National Institute of Health procedure, 2004, as reported by Banerjee et al., 2012 [22].

3.3.13.1. Reagents

1. RBC lysis buffer
2. Nuclei lysis buffer
3. SDS (Sodium Dodecyl Sulphate) (10%)
4. Proteinase K
5. NaCl solution (6 M)
6. Ethanol
7. Tris-EDTA (T.E.) buffer (pH 8)

3.3.13.2. Procedure

DNA was extracted using the salting-out technique. RBC lysis buffer (200µl) was mixed with 200µl EDTA blood and incubated for 20 minutes at room temperature. The tube was centrifuged at 1000 x g for 10 minutes at 4 °C. The pellet was separated by removing the supernatant. Nuclei lysis buffer, proteinase K, and SDS were added to the pellet, which was well mixed and incubated at 65°C for 2-3 hours. Ice-chilled ethanol (100%) was added, and the contents were mixed by inverting the tube 10-20 times. The tube was centrifuged at 10,000 rpm for 10 minutes to precipitate DNA. Using a wide-mouthed spoon, the DNA was transferred to a microcentrifuge tube filled with 70% ethanol. At 4°C, the tube was centrifuged for 10 minutes at 13K. The precipitated DNA was dissolved in T.E. buffer (pH-8), and the supernatant was discarded. The Elico spectrophotometer was used to evaluate the DNA content and purity at A230, A260, and A280.

3.3.13.3. Calculation

Concentration of DNA (µg/ml) = (OD at A260 – OD at A320) X dilution factor X 50 µg/ml.

Total DNA isolated (µg) = Concentration of DNA X Final sample volume (ml).

Purity of DNA = A260/A280

3.3.14. DNA Extraction from Tissue Cells

The DNA was isolated from the tissue cell, and the content was assessed using a slightly modified technique reported by Gupta (1984) [23].

3.3.14.1. Reagents

1. Homogenizing buffer (1% SDS, 50 mM EDTA)
2. Tris HCl (1 mM; pH 7.4)
3. Proteinase K (100 µg/µL)
4. Phenol (Distilled)
5. Sevag (chloroform: isoamyl alcohol; 24:1)
6. Ethanol
7. NaCl (5 M)
8. Tris-HCl (50 mM; pH 4)
9. RNAse (100 µg/ml)
10. Tris-EDTA

3.3.14.2. Procedure

50 mg of liver tissue and homogenizing buffer were taken in a tube and homogenized with tissue rafter (REMI-RQ127A), proteinase K (100 µg/µL) was then added and incubated at 45ºC for 45 mins). 0.5 ml of 1 mM Tris HCl (pH 7.4) was added after incubation, and the mixture was centrifuged at 4000 x g for 10 minutes at 4ºC. The supernatant was discarded progressively with 1

volume of phenol, 1:1 combination of phenol and Sevag, and Sevag was mixed for 5 minutes, 3 minutes, and 3 minutes, respectively. The separation step involved centrifugation at 14000 x g for 25 minutes at 4ºC. NaCl (5 M) 0.1 volume and 1 ml ethanol (100%) was added and stored at -20ºC for one hour. Finally, the DNA was precipitated by centrifugation at14000 x g; for 15 mins. The precipitated DNA was rinsed twice with 70% ethanol and dissolved in T.E. buffer (0.5 ml). To remove RNA, RNAse (100 µg/ml) was added to Tris-HCl and incubated for 30 minutes at 38ºC. DNA was dissolved in 0.2 ml of T.E. buffer. The concentration, quantity, and purity were determined spectrophotometrically at A230, A260, and A280 (Elico Spectrophotometer).

3.3.14.3. Calculation

Concentration of DNA (µg/ml) = (OD atA260 – OD at A320) X dilution factor X 50 µg/ml.

Total DNA isolated (µg) = Concentration of DNA X Final sample volume (ml).

Purity of DNA = A260/A280

3.3.15. Assessment of DNA Damage by Comet Assay

The DNA damage was calculated using the comet test, as described by Singh et al., 1988 and Kido et al., 2006, with minor changes [24, 25].

3.3.15.1. Principle

Ostling and Johanson initially described the Comet Assay, which measures DNA damage in eukaryotic cells, in 1984. The assay uses the single cell gel electrophoresis (SCGE) technique to assess the type of breaking in single and/or double-stranded DNA. This approach immobilizes the cells on a double-layered gel slide made of low and high melting agarose. The immobilized

cells are then lysed in alkaline solution and electrophoresed to determine the type of comet structure. Because of its overall negative charge, the damaged and fragmented DNA loses its orderly and compact structure and moves towards the anode. Undamaged DNA, on the other hand, will remain strongly bound to its nuclear association. This movement of damaged and broken DNA will generate a distinctive pattern like that of a comet following ethidium bromide staining.

3.3.15.2. Reagents

1. High melting agarose (Melting point 40 – 42°C) (2%;)
2. Low melting agarose (0.5%)
3. Lysis buffer (Tris- EDTA) pH 10
4. Alkaline Electrophoresis Buffer (300 mM NaOH in 1 mM EDTA) pH 13
5. Neutralizing buffer (0.4 M Tris-HCl) pH 7.5
6. Homogenizing Buffer (0.075M NaCl; 0.024M EDTA)
7. Ethidium bromide (20 µg/ml)

3.3.15.3. Procedure

Kido et al., 2006 procedure was slightly modified for the comet assay [25]. On a frosted transparency slide, 200 µL of high melting agarose (Genei, India) was spread out and covered with a cover slip, and left to cast. 20 µL of whole blood was combined with 80 µL of melted low melting agarose (Genei, India) in a microcentrifuge tube. The low melting agarose blood mixture was carefully deposited in the groove created by removing the cover slip from the previously prepared slide. After covering it with a cover slip, it was left to cast for fifteen minutes. After carefully removing the cover slip, the freshly made cold lysis solution was incubated for two hours at 40C. The slides were immersed in alkaline electrophoresis solution at 40°C, put on a horizontal gel

electrophoresis device, and left in the dark for 15 minutes. Electrophoresis was initiated (1 v/cm; 250 mA) in the dark for 15 minutes. Following electrophoresis, the slides were washed with neutralizing buffer. Following Ethidium bromide staining, the slides were examined and photographed at 40 x magnification using the Leica fluorescence microscope Model 300 FX.

3.3.15.4. Measurement of comet parameter

The comet head diameter, tail length, and percentage of DNA damage were measured using the technique described by Uhl et al., 2000 [26]. A fluorescent microscope Model 300 FX was used to screen and evaluate 50 cells per animal at a magnification of 40 x. Using Image J software, the quantification of DNA damage in each cell was quantified as follows:

Total amount of DNA in the comet = (Total comet Area) × (Mean DNA intensity)

Total DNA in the comet head = (Total Head area) × (Mean DNA intensity)

$$\% \text{ Of DNA damage} = \frac{(\text{Total DNA in comet}) - (\text{Total DNA in comet head}) \times 100}{(\text{Total DNA in Comet})}$$

§ Tail length (lT): Distance between the end of the comet head and the end of the DNA migration

§ Head Area (A.H.): The area covered by comet head.

§ Comet Area (A.C.): The area covered by the total comet.

§ Mean Head intensity (I.H.): The mean intensity of the pixels located in the head area.

§ Head DNA (DNAH): The sum of the intensities located in the head area

DNAH = AH x IH

§ Tail Area (AT): The area covered by the comet tail.

$$AT = AC - AH$$

§ Tail DNA, DNAT: The difference between total comet DNA and head DNA.

$$DNAT = DNAC - DNAH$$

§ Percent Tail DNA (% DNAT): The percentage DNA in the tail.

$$\% DNAT = 100\ DNAT / (DNAT + DNAH)$$

§ Tail Moment (M.T.): The product of tail length and fraction of DNA in the tail.

$$MT = lT \times \% DNAT$$

3.3.16. Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA uses solid-phase enzyme immunoassay (EIA) to quantify the lowest range of ligand/antigen (ng/mL to pg/mL) in a liquid sample. It is a valuable diagnostic technique in medicine for quality control and detecting particular proteins in solutions [27]. A ligand-specific antibody measures the ligand. A solid surface (polystyrene microtiter plate) is coated with antigen (Ag) either non-specifically or specifically (also known as "sandwich" ELISA). Together with the antigen, the targeted antibody (Ab) immobilizes and forms an antigen-antibody complex. Any secondary antibody that has been bio-conjugated by an enzyme can detect the antibody. The aggregation of both antibodies (primary and secondary) is responsible for altering the color of the product, indicating the presence of an Ag: Ab complex. It is a diagnostic procedure for qualitative and quantitative detection of an antigen or antibody. The commercially available ELISA kit Genetix Puregene (India) was used in my work to quantify insulin, cytokines (IL-4, IL-6, TNF-α,

and IFN-γ), apoptotic markers (BAX and Bcl-2), and hormones (progesterone and estradiol). The Sandwich ELISA technique was utilized to quantify the above-mentioned parameters. Specific monoclonal antibody pre-coated microplates were utilized. In the microtitration plate wells designated for each individual parameter, the corresponding standard, control, and sample were collected. After washing off the unbound components, the wells were filled with an enzyme-linked polyclonal antibody that was specific to the parameter. Unbound antibodies, if any, were washed away and a particular substrate solution was added. The reaction was stopped by adding a stop solution. Different intensity of colour depending upon products was measured. Quantification of studied parameters was done by calculating the ODs of samples and standard and control from the standard curves.

3.3.17. Histo-Pathological Study of The Different Tissues

Following appropriate fixation and staining, histological analyses of the liver and kidney of treated and untreated rats were conducted.

3.3.17.1. Material / Accessory Required

1. Compound microscope
2. Glass Slides
3. Cover Slip
4. Absolute alcohol
5. Haematoxylin
6. Eosin
7. Bouin’s fluid (for fixation)

i) Fixation: Soft tissues from important organs such as the liver and kidney were sliced into pieces and immersed in a significant volume of Bouin's solution for 24 hours. Tissues were cleaned with 70% and 80% alcohol many times to remove the fixative solution, then stored in 70% alcohol.

ii)Embedding: Tissue specimens were gradually dehydrated with increasing alcohol concentrations (50%, 70%, 80%, and 95%). After that, the tissues were immersed in a combination of absolute ethanol and chloroform for 4 hours, followed by another 4 hours in chloroform. The specimens were then moved to a saturated paraffin and chloroform solution. The solution's temperature was maintained between 560C -580C. in a paraffin bath. The specimens were maintained in molten paraffin for three hours. The specimen was then put into an embedding frame consisting of two glass plates and two L-shaped metal bodies with a spatula. The paraffin was soon cooled by immersing the frame in cool water. The glass plate and metal frames were separated from the solid paraffin.

iii) Cutting and Handling of Sections: The tissues were sectioned with a rotary microtome. The specimen block was heated and put on a specific metal disk. By moving the block up and down against the knife, many pieces of tissue were formed and remained adhered to one another, making the ribbon. The ribbons were put on glass slides in a sequential order. An egg white and glycerine combination was utilized as an adhesive. Before staining, the paraffin was removed by soaking in xylol for 5 minutes.

iv) Staining (Haematoxylin and Eosin staining): Two distinct stains were used: haematoxylin, a basic dye that stained the nucleus, and eosin, an acidic dye that stained the cytoplasm. First, the slides with fixed sections were passed through different grades (95%, 80%, 70%, and 50%) of alcohols then to Water. After adequate hydration, slides were maintained in haematoxylin solution

for 5 minutes or until the sections became deep blue when seen under a microscope. The slides were placed in water for 15 to 30 minutes to remove any remaining stain. The portions were then subjected to several alcohol concentrations (50%, 70%, 80%, and 95%) for one minute each, and then to 95% alcohol for five minutes. After that, the portions were immersed in an alcoholic eosin solution for one to five minutes. Excess eosin was cleaned with 95% alcohol until no stains remained. After that, the stains were immersed in a solution of equal parts absolute alcohol and xylol for 5 minutes.

v) Clearing and Mounting: Clearing agents make sections visible and noticeable allowing histological characteristics to be viewed clearly. Xylol is an effective clearing agent for haematoxylin and eosin staining. Stained slides were immersed in fresh xylol for five minutes. Once the cleaning agent had been drained, a drop of Canada balsam / DPX was immediately applied to the tissue sections, followed by a gentle placement of a cover glass and drying.

3.4. Statistical Analysis

The experiments were done twice and all the data of different sets of experiments were entered in excel sheets. were averaged over N = 12 animals, and given mean + S.D. Significance levels were determined by using ANOVA, where * implied significant ($p < .01$) and ** implied highly significant ($p < .001$) of the data when compared with the data of nicotine treatment. Similarly, # implied significant ($p<.01$) and ## implied highly significant ($p<.001$) of the data when compared with the data of nicotine + curcumin treatments.

3.5. Cell Lines and Cell Culture

Two type cancer cell line used for this study were Human cervical cancer cell line (SiHa) and Lung cancer cell (A549) and Non- cancerous cell line is human embryonic kidney cell line, HEK293 which is served as a "Control". SiHa is a cell line isolated from fragments of a primary uterine tissue sample from a 55-year-old, female, Japanese patient with squamous cell carcinoma. It has been discovered that SiHa cells express the p53+ and pRB+ genes, which are implicated in DNA repair, tumor suppression, and cell cycle control. The karyotype of SiHa cells is hypertriploid, with an average of 69–72 chromosomes. One to two copies of the viral genome are integrated into each SiHa cell, indicating that the cells are HPV-16 positive. On the other hand, A549 cells were isolated from the lung tissue of a White, 58-year-old male with lung cancer. The A549 cell line is a hypotriploid human cell line that makes up 24% of cells. Its modal chromosome number is 66. Modal numbers between 64 and 67 are typical with higher ploidies occurring at an infrequent rate (0.4%).

These cell lines were procured from National Centre for Cell Science (NCCS), Pune, India. Moreover, these cell lines also require appropriate growth medium and a temperature-controlled environment to grow optimally. Dulbecco's Modified Eagle Medium (DMEM), which contains nearly double amount of amino acids used as the base culture medium for the cultivation of cells. To provide supplements of growth factors, and attachment factors, 5 – 10% of Foetal Bovine Serum (FBS) was added with the medium. To make it a 'complete' medium, 1 % of penicillin-streptomycin were also added. These cells were maintained in T-25 sterile flask within a 37ºC humidified temperature and 5% CO_2 environment, which is essential to maintain the required pH for the cells to survive. Cells were checked for the presence of any contaminants like *Mycoplasma* before passage and commencement of any experiments. DNA extracted from PBS washed cell

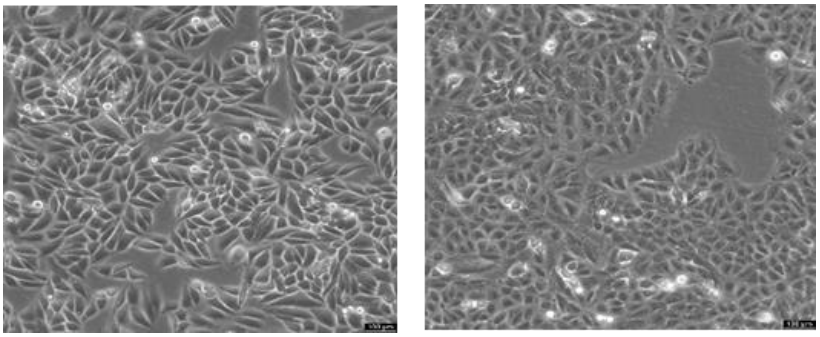


Figure 3.1: Morphology of Siha and A549 cell lines as observed under phase contrast microscope [28, 29].

lines is subjected to 16S rRNA detection. The absence of 16S rRNA molecules ruled out the presence of Mycoplasma in cell line culture. The sequences of the forward and reverse primers adopted in this study are:

U1 (forward) - 5′GTTTGATCCTGGCTCAGGAYDAAC3′;

U8 (reverse) - 5′ GAAAGGAGGTRWTCCAYCCSCAC3′,

qPCR tests are performed to detect 16S rRNA by using the universal U1 and U8 set of degenerate primer pairs targeting 1.5 kb fragment. RT-PCR and microscopic DNA negative cultures are considered for downstream studies only. The medium was changed every alternative day and after attaining 80 – 90% confluence, the cells were detached from the flask surface by 2 ml of Trypsin-EDTA solution, containing 0.25% trypsin and 0.02% EDTA in phosphate buffered saline, which constitutes of 137 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4. Then the cells were centrifuged at 3000 rpm for 3 minutes and the pellet was resuspended in the culture media and seeded in new sterile flasks for further experiments and maintenance. For storage of cells, the pellet was suspended in cell freezing media, containing FBS and DMSO in 9:1 ratio, within a cryo-vial. The cells were then kept at -20ºC for two hours followed by -80ºC for overnight and then

stored at liquid nitrogen. To have the frozen cells back to culture, they are defrosted and washed in cell culture medium and seeded in a T-25 sterile flask with 5 ml of culture medium.

3.5.1. Drugs Used in the Study

The drug used for this experiment is Curcumin and nanocurcumin. Curcumin was purchased from Sigma Chemicals Company, St. Louis. Nanocurcumin is prepared according to the protocols reported 27.

3.5.2. Cell Viability Assay

To assess the direct cytotoxic effect of curcumin and nanocurcumin on Siha and A549 cells which could lead to cell death, the MTT tetrazolium reduction assay was performed. This assay is based on the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), a water-soluble yellowish tetrazolium dye, by the activity of mitochondrial dehydrogenases of metabolically active cells to form purple coloured formazan crystal [30]. Comparing the intensity of the colour produced by dissolution of the formazan crystals in Dimethyl sulfoxide (DMSO) between untreated control cells and treated cells can, therefore, effectively provide the amount of change in percentage of metabolically active cells or viable cells. Siha and A549 cells were plated in 96-well plates at a density of 2×10^4 cells/well using phenol red free cell culture medium and treated with different concentrations of curcumin and nanocurcumin, ranging from 10 -60 µM), for 24 hours and 48 hrs. A 5 mg/ml stock solution of MTT (G-Biosciences) was prepared in PBS, which was applied onto the cells at a volume of 20 µl/well and incubated at 37ºC in dark for 3 – 4 hours following completion of the treatment. Following incubation, the medium containing MTT

solution was removed from the wells and 100 µl (0.2 mL of DMSO was added in each well to solubilize the formazan crystals formed by the activities of the viable cells. The plates were then gently shaken for 10 minutes in dark condition, ensuring proper solubilization of formazan and the absorbance of the final-coloured product was measured using Bio-Rad iMark^{TM} Microplate Reader (Bio-Rad Laboratories) at 590 nm. The relative cell survival rate is calculated based on the ratio of the absorbance of the sample treatment relative to the absorbance of the control treatment.

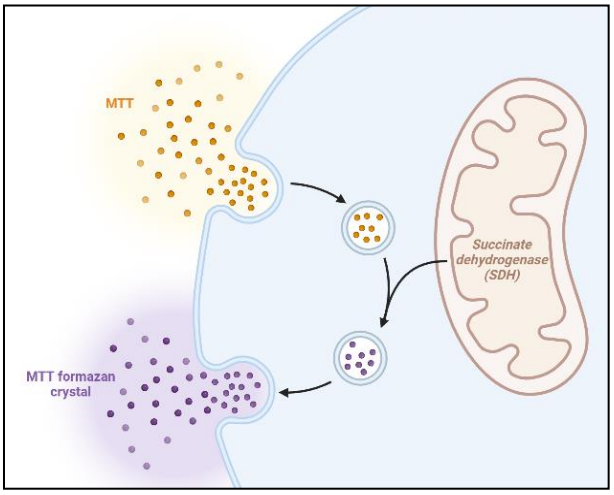


Figure 3.2: Graphical representation of formation of formazan crystals from MTT reagent.

SiHa and A549 cells were plated in 6-well plates at a density of 2×10^5 cells/well plate and cultured overnight for adhesion of cells. After removing the culture medium, cells are treated with inhibitory concentrations of curcumin and nanocurcumin for 48 hrs. SiHa and A549 (2×105) cells are seeded in a 6 well plate and cultured overnight for adhesion of cells. After removing the culture medium, cells are treated with inhibitory concentrations of curcumin and nanocurcumin for 48 h, observed under a microscope (Olympus CKX53, Japan) and images are captured with Magvision software.

3.5.3. Cell Morphology Assay

The evaluation of the cell morphological study was carried out using ELISA assay kit, procured from Cayman Chemicals (USA), following manufacturer's protocol. SiHa and A549 cells were plated in 6-well plates at a density of 2×10^5 cells/well plate and cultured overnight for adhesion of cells. After removing the culture medium, cells are treated with inhibitory concentrations of curcumin and nanocurcumin for 48 h. Morphological features of the cells were done using by inverted microscopy (Olympus CKX53, Japan) and images are captured with Magvision software.

3.5.4. Detection of ROS by H_2DCFDA Staining

Intracellular ROS generation was directly measured by H_2DCFDA staining method. In this study use oxidation-sensitive fluorescent dye, 2', 7',-dichlorofluorescein diacetate. H_2DCFDA is a cell permeable dye which remains non-fluorescent till it reacts with ROS SiHa and A549 cells are grown in 24 well plates and treated for 24 hrs with curcumin and nanocurcumin at IC_{50}. After removing the medium from the plate, the cells are rinsed with PBS and treated with 1mM H2DCFDA dye for 20 mins at 37 ^{0}C in dark. Then the cells are washed twice with PBS and examined under a microscope (Zeiss) for fluorescence emitting cells to determine ROS generation.

3.5.5. DAPI Staining to Determine Apoptotic Nuclei

DAPI (4',6-diamidino-2-phenylindole) is a fluorescent stain that binds strongly to adenine-thymine (A-T) rich regions in DNA. It is commonly used in fluorescence microscopy and flow cytometry to visualize and quantify cellular nuclei. DAPI emits blue fluorescence when bound to DNA, allowing for the detection and analysis of DNA content and distribution within cells. To

analyze the morphological changes in the apoptotic cell nuclei, cells are stained with DAPI and observed under microscope. 2×105 cells of SiHa and A549 per well are seeded in 6-well plates and cultured overnight in DMEM medium supplemented with 10% FBS. The medium is then replaced with DMEM medium supplemented with 0.2% FBS and treated with curcumin and nanocurcumin for 48 h. Thereafter, the medium is completely removed and the cells are washed with ice cold PBS twice, fixed in 100% ethanol for 20 min at room temperature, and washed twice again with cold PBS. Then the cells are incubated with 100 nm of DAPI in PBST for 5 min in room temperature and observed under a fluorescence microscope (Zeiss).

3.5.6. Quantification of DNA Fragmentation

DNA fragmentation is one of the hallmarks of late apoptosis in majority of cell types. DNA fragmentation is qualitatively analyzed by agarose gel electrophoresis by observing the DNA ladder formation. Total DNA is isolated from cells according to the manufacturer's protocol (ROCHE Apoptotic DNA-Ladder Kit, Germany). Samples are mixed with loading dye (0.25% bromophenol blue, 30% glycerol; v/v) and dispensed in wells of a 1% agarose gel. The gel is run at 5 V/cm for about 2 hrs, and the DNA is detected by ethidium bromide under UV light (GelDoc; Bio-Rad XRS+)

REFERENCES

1. Hawk PB, Oser BL, Summerson WH, Oser BL Ltd.Practical Physiological Chemistry, Mc Graw Hill Book Comp, New York, London. 1954; 13th Ed.

2. Bessey DA, Lowery OH, Brocks MJ. In: H.U. Bergmeyer, (Ed), J Biol Chem. Methods of Enzymatic Analysis. Acad Press. NY.1946; 321-329.

3. Kind PRN, King EJ. Estimation of Plasma Phosphatase by Determination of Hydrolysed Phenol with Amino-Antipyrine. J Clin Path. 1954; 7(4):322-326.

4. Reitman S, Frankel S. A Colorimetric Method for the Determination of Serum Glutamic Oxalacetic and Glutamic Pyruvic Transaminases. Amer J Clin Pathol. 1957; 28(1):56-63.

5. Friedman RB, Young DS. Effects of Disease on Clinical Laboratory Tests. AACC.2000; 5th Ed.

6. Moss GA, Bondar RL, Buzzelli DM. Kinetic Enzymatic Method for Determining Serum Creatinine. Clin Chem. 1975; 21:1422-1426.

7. Walker HK, Hall WD, Hurst JW. BUN and Creatinine In: Clinical Methods: The History, Physical, and Laboratory Examinations. 1990; 3rd Ed.

8. Allain CC, Poon LS, Chan CS, Richmond W, Fu PC. Enzymatic determination of total serum cholesterol. Clin Chem. 1974 Apr; 20(4):470-5.

9. Grundy MS. National Cholesterol Education Program. Summary of the Second Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel II). JAMA. 1993; 269(23):3015-3023.

10. Werner M, Gabrielson DG, Eastman G. Ultra-Micro Determination of Serum Triglyceride by Bioluminescent Assay. Clin Chem. 1981; 27(2):268-271.

11. Suchanda S. Calculation of VLDL-Cholesterol from Triglycerides and Total Cholesterol Levels. Biomedicine. 2008; 28:219-221.

12. Oliveira AJM, Deventer VEH, Bachmann ML, et al. Evaluation of Four Different Equations for Calculating LDL-C with Eight Different Direct HDL-C Assays. Clin Chim Acta. 2013;23 (423):135-140.

13. Chatterjee SN, Agarwal S. Liposomes as Membrane Model for Study of Lipid Peroxidation. Free Radic Biol Med. 1988; 4(1):51-72.

14. Sinnhuber RO, Yu TC, Yu TC. Characterization of the Red Pigment Formed inthe 2-Thiobarbituric Acid Determination of Oxidative Rancidity. Food Res. 1958; 23:626-634.

15. Cohen G, Dembiec D, Marcus J. Measurement of Catalase Activity in Tissue Extract. Anal Biochem. 1970; 34(1):30-38.

16. Beauchamp C, Fridovich I. Superoxide Dismutase: Improved Assays and an Assay Applicable to Acrylamide Gels. Anal Biochem. 1971; 44(1):276-287.

17. Levander OA, DeLoach DP, Morris VC, Moser PB. Platelet glutathione peroxidase activity as an index of selenium status in rats. J Nutr. 1983; 113(1):55-63.

18. Narake SS, Gupta PC. Nasal use of snuff. Indian J Cancer. 2014; 51(1):S88.

19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin¬ phenol reagent. J Biol Chem. 1951; 193(1): 265-275.

20. Levine RL, Williams JA, Stadtman ER, Shacter E. Carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol. 1994; 233: 346 - 357.

21. Chatterjee CC, In: Human Physiology. 1994; 11th Reprint Ed.

22. Banerjee S, Chattopadhyay K, Chhabra JK, Chattopadhyay B. Protein dependent fate of hepatic cells under nicotine induced stress and curcumin ameliorated condition. Eur J Pharmacol. 2012; 684(1-3):132-45.

23. Gupta RC. Nonrandom Binding of the Carcinogen N-Hydroxy-2 Acetylaminofluorene to Repetitive Sequence of Rat Liver DNA in Vivo. Biochemistry. 1984; 81:6943-6947.

24. Singh NP, Mccoy MT, Tice RR, et al. A Simple Technique for Quantification of Low Levels of DNA Damage in Individual Cells. Exp Cell Res. 1988; 175:184-191.

25. Kido R, Sato I, Tsuda S. Detection of in Vivo DNA Damage Induced by Ethanol in Multiple Organs of Pregnant Mice Using the Alkaline Single Cell Gel Electrophoresis (Comet) Assay. J Vet Med Sci. 2006; 68(1):41-47.

26. Uhl M, Helma C, Knasmuller S. Evaluation of the Single Cell Gel Electrophoresis Assay with Human Hepatoma (Hep G2) Cells. Mutat Res. 2000; 468(2):213-225.

27. Savige JA, Paspaliaris B, Silvestrini R, Davies D, Nikoloutsopoulos T, Sturgess A, Neil J, Pollock W, Dunster K, Hendle M. A review of immunofluorescent patterns associated with antineutrophil cytoplasmic antibodies (ANCA) and their differentiation from other antibodies. J Clin Pathol. 1998; 51(8):568-75.

28. Aarbiou J, Ertmann M, van Wetering S, van Noort P, Rook D, Rabe KF, Litvinov SV, Van KJHJM, de Boer WI and Hiemstra PS. Human neutrophil defensins induce lung epithelial cell proliferation in vitro: J. Leukoc. Biol. 2002; 72: 167-174.

29. Wang C,Gu W,Zhang Y,Ji Y,wen Y, Xu X.Nicotine promotes cervical carcinoma cell line HeLa migration and invasion by activating PI3k/Akt/NF-κB pathway in vitro. Exp Toxicol Pathol.2017; 0940-2993

30. Berridge MV, Herst PM, Tan AS. Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. Biotechnol Annu Rev. 2005; 11: 127-52.

CHAPTER IV

INSTRUMENTS & APPARATUS

4. Instruments and Apparatus Used in the Research

4.1. Centrifuge

Antonin Prandtl invented centrifuged apparatus in 1864 to remove fat from milk. In my research, the Plastocraft ROTA 3R centrifuge was used for experimentation. This machine is used to separate fluids from particles of various sizes and densities. It is mostly utilized in my research to separate protein, DNA, plasma, and serum from animal peripheral blood. This centrifuge machine maintains the sedimentation principle and keeps the proper temperature throughout the process.

4.1.1. Working Principle of Centrifuge

Under the action of gravity, the particles eventually sink to the bottom of the container. Increasing the gravitational force acting on the particles can significantly speed up the settling process. A tube filled with a suspension of particles, such tissue homogenate, is inserted into a centrifuge's rotor and rapidly circulated. The force exerted on suspended particles is significantly increased by the ensuing acceleration, which speeds up their deposition to the tube's bottom along pathways perpendicular to the axis of rotation.

The force acting on a particle at the time of centrifugation is explained by the equation

$F = m\omega 2x$

Where m = mass of the particle.

ω = angular velocity of the spinning rotor in radians per second (one unit of circumference contains 2π radians).

X = Distance of the particle from the axis of rotation

During centrifugation, the value which has given for the force applied to the particles is a relative one. It is contrasted with the force that the gravity of earth would have on the same particles. It is called relative centrifugal force or RCF and is given by the following equation.

$$\mathbf{RCF} = \frac{F_{centrifugation}}{F_{gravity}} = \frac{m\omega^2 \mathbf{X}}{mg} = \frac{\omega^2 x}{g}$$

Where "g" is the acceleration due to gravity and equals 980cm/sec/sec.

In my research, the Plastocraft ROTA 3R centrifuge was employed for experimentation. This machine is used to separate fluids from particles of varying sizes and density. It is mostly utilized in my research to extract protein, DNA, plasma, and serum from animals' peripheral blood. This centrifuge machine follows the sedimentation principle and keeps the needed temperature constant throughout the process.

4.2. Spectrophotometer

This device was firstly discovered by Arnold. O. Beckman in 1940. The spectrophotometer is an instrument used to determine the quantity of transmittance and reflectance in a solution. The UV-3101PC (Shimadzu, Japan) UV-VIS single-beam spectrophotometer was employed in this investigation. This UV-Visible spectrophotometer was used to perform a number of quantitative estimates of DNA and biochemical tests as well as numerous interaction studies. The spectrophotometer is commonly used in chemistry, biochemistry, and biophysical chemistry to quantitatively analyze solutes in a solution by measuring the amount of light absorbed in a cuvette. It is the most useful technique rapidly used in various biochemical experiments which involve DNA, RNA, enzyme kinetics Protein, etc.

4.2.1. Working Principle

Spectrophotometers function on the basis of Beer-Lambert's law. This equation is expressed as

$$A = \log_{10} (I_0/I_T)$$

$$= \log_{10} (1/T)$$

$$= -\log_{10} T = \epsilon cl = OD$$

Where, A = Absorbance.

I_0 = intensity of the incident radiation.

I_T = intensity of the transmitted radiation.

ϵ = Molar Absorptivity or molar extinction coefficient.

C = Concentration of the absorbing substance.

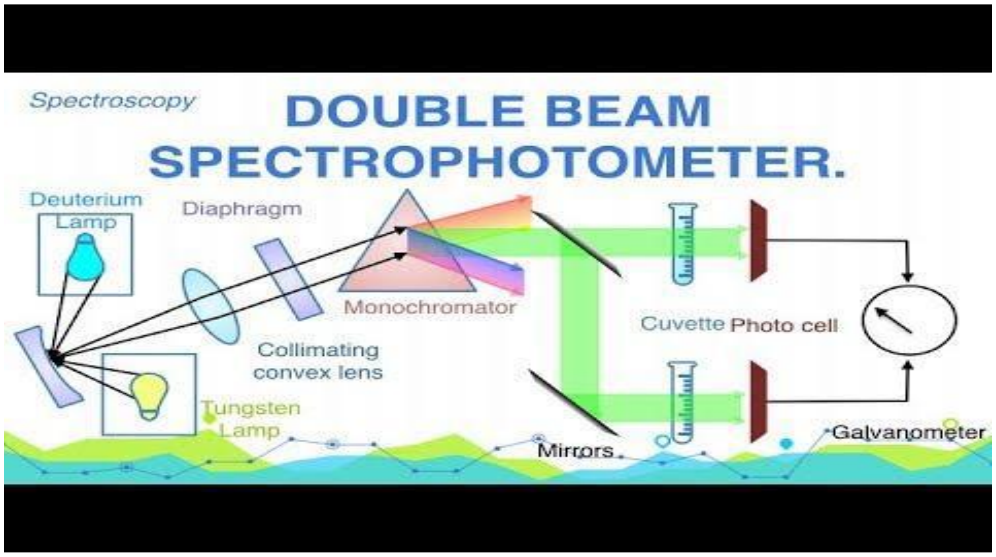
L = Optical path length.

A spectrophotometer can determine a compound's wavelength based on its color. It measures the quantity of light absorbed by chemical components by analyzing the intensity of light flowing through a sample. This measurement is helpful in determining a sample's quantification

Depending on the light source's wavelength range, there are two main types of spectrophotometers.

- UV –Visible spectrophotometer.
- IR Spectrophotometer.

There are two main types of spectrophotometers: single beam and double beam. The most crucial tool is a double beam spectrophotometer, which divides beams into two distinct routes and compares the light intensities of the two directions. One light path contains the reference sample, while another includes the test sample. In contrast, single-beam spectrophotometers employ a reference standard to standardize the instrument before measuring samples.



4.3. Fluorescence Spectroscopy

Fluorescence spectroscopy (also known as fluorimetry or spectrofluorometry) is a form of electromagnetic spectroscopy that analyses the fluorescence of a material. Fluorescence spectroscopy is a technique for analyzing the fluorescence characteristics of a sample by stimulating it with a specific wavelength of light and measuring the released photons. It employs a light beam typically ultraviolet light to stimulate the electrons in specific compounds' molecules, causing them to release light.

LS 50B, Fluorescence spectroscopy, was employed in my investigation to analyze fluorescence from a sample.

4.4. Fourier Transform Infrared Spectroscopy (FTIR)

In 1880s, Albert A. Michelson pioneered Fourier-transform infrared (FT-IR) spectroscopy. FTIR stands for The Fourier transform infrared spectrometer, the preferred method of infrared spectroscopy. It is the most advanced type and is preferred over other dispersive spectrometers. It

is due to its excellent precision, accuracy, speed, increased sensitivity, ease of use, and sample non-destructiveness. The fundamentals of infrared spectroscopy technology are based on atomic vibrations in a molecule, which only absorbs specified frequencies and energy of infrared light. FTIR can identify and classify molecules since various compounds have distinct infrared spectrums. The FTIR spectrometer simply employs an interferometer to detect the energy transferred to the sample. The infrared radiation released by the black body reaches the interferometer, where signals are spectrally encoded. The resulting interferogram signal passes through or bounces off the sample surface, absorbing specific energy wavelengths. The beam ultimately passes through the detector and is then sent on to the processing computer for Fourier transformation of energy signals.

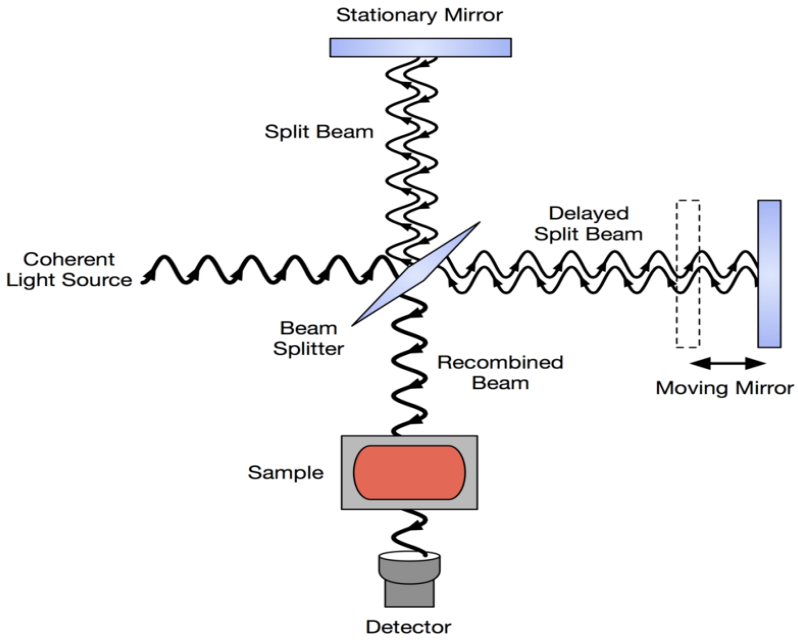


Figure 4.1: Schematic diagram of a Michelson interferometer, configured for FTIR

Fourier transform infrared spectroscopy (Perkin Elmer -FTIR-1600, USA) was employed in my research to determine the functional group present in nanocurcumin.

4.5. Ultrasonic Probe Sonicator

Ultrasonic probe sonicators are used in scientific and industrial applications to homogenize samples, destroy cells, and reduce particle size. It disturbs particles or cells in a liquid by producing high-frequency sound waves, also known as ultrasonics, using a probe. This approach is excellent for sample preparation and processing in fields including biology, chemistry, and nanotechnology. In nanotechnology probe sonicator mainly used to break the particle size and made it nano form. In my study for preparing nanocurcumin Hielscher Ultrasonic Processor- UP100H (Germany) was used.

4.6. Lyophilizer

A lyophilizer, also known as a freeze dryer, is a device that eliminates water from a substance via a process known as freeze-drying or lyophilization. Earl W. Flosdorf and Ronald produced the first commercial lyophilizer. In 1906, Jacques-Arsène d'Arsonval and Frédéric Bordas collaborated to construct the first modern lyophilizer. This procedure is used to preserve

perishable products, such as food, pharmaceuticals, and biological samples. It extends their shelf life and makes them simpler to carry.

Lyophilizer works through 3 stages:

(i) **Freeze**: The sample is frozen to a very low temperature.

(ii) **Reduce pressure**: The pressure around the sample is reduced.

(iii) **Sublimate**: The frozen water in the sample turns directly from a solid to a gas without going through a liquid state.

Lyophilizers are utilized in numerous areas, including medicines, biotechnology, food preservation, and research facilities. It is mostly used to improve the shelf life of products by

eliminating water from frozen materials. The orange powder of nanocurcumin was obtained using an EYELA-FDU-2000 (Japan) lyophilizer.

4.7. Field Emission Scanning Electron Microscope (FESEM)

Scanning electron microscopy is a flexible and non-destructive tool for analyzing the structure and composition of natural and manmade materials. A scanning electron microscope uses a narrow electron beam from an electron cannon to rapidly scan a specimen's surface. The signals generated when the electrons interact with the atoms that make up the sample offer information on its surface topography, composition, and electrical conductivity. The FESEM can detect several signals, including current transmitted electrons, cathodoluminescence, X-rays, secondary electrons, and back-state electrons (BSE). While secondary electron detectors are a standard component of FESEMs, it is uncommon for a single machine to have detectors for all potential signals. Signals are formed when atoms near the sample's surface interact with an electron beam. Secondary electron imaging (SEI) is the most often used or standard method of detection. It produces high-resolution pictures of a sample surface, revealing details smaller than 1 nm in size. SEM micrographs, with their narrow electron beam, provide a detailed three-dimensional view of a sample's surface structure.

The particle size of the nanocurcumin was determined using a FE-SEM, the FEI INSPECT F50 (FEI Europe BY, the Netherlands). FESEM is dried, gold-coated, and stored in desiccators before examination using an INSPECT F50. Elastic scattering reflects backscattered electrons (BSE) from the material. Since the intensity of the BSE signal is closely correlated with the specimen’s atomic number (Z), BSE is frequently utilized in analytical FESEM along with spectra generated from characteristic X-ray’s. BSE pictures can provide information about the distribution

of different components in the sample. The distinctive X-rays are produced when an electron from the sample's inner shell is removed by the electron beam, causing a higher-energy electron to fill the shell and release energy. Because of these characteristics, X-rays are used to quantify the elemental abundance of the sample and ascertain its composition.

4.8. Dynamic Light Scattering

Dynamic light scattering (DLS) can measure the size of particles based on their Brownian motion. When particles are scattered in a liquid, they travel randomly in every direction. The notion of Brownian motion states that particles are continually interacting with solvent molecules. These collisions convey a certain amount of energy, inducing particle movement. Energy transmission is more or less constant, hence smaller particles are affected more. As a result, smaller particles are traveling faster than bigger ones. If you know all of the other factors that impact particle movement, you can calculate the hydrodynamic diameter by measuring the particles' speeds.

Dynamic light scattering is a well-established, standardized technique for measuring particle size in the nanoscale range and particle size distribution. In current study DLS is most commonly used to analyze nanoparticles.

4.9. X-ray diffraction (XRD)

X-ray diffraction is a commonly used method to analyze atomic spacing and crystal structure. It operates by employing constructive interference between a crystalline sample and monochronic X-rays. A cathode ray tube generates these X-rays, which are then converted into monochromatic radiation, collimated, and directed towards the sample. When Bragg's Law ray (2d $Sin\Theta = n\lambda$, where n is an integer, λ is the wavelength in angstroms, d is the interatomic spacing in angstroms, and Θ is the diffraction angle in degrees) is satisfied, the interaction between the incident rays and the sample results in both constructive interference and diffracted rays. This law establishes how a crystalline sample's diffraction angle and lattice spacing affect the wavelength

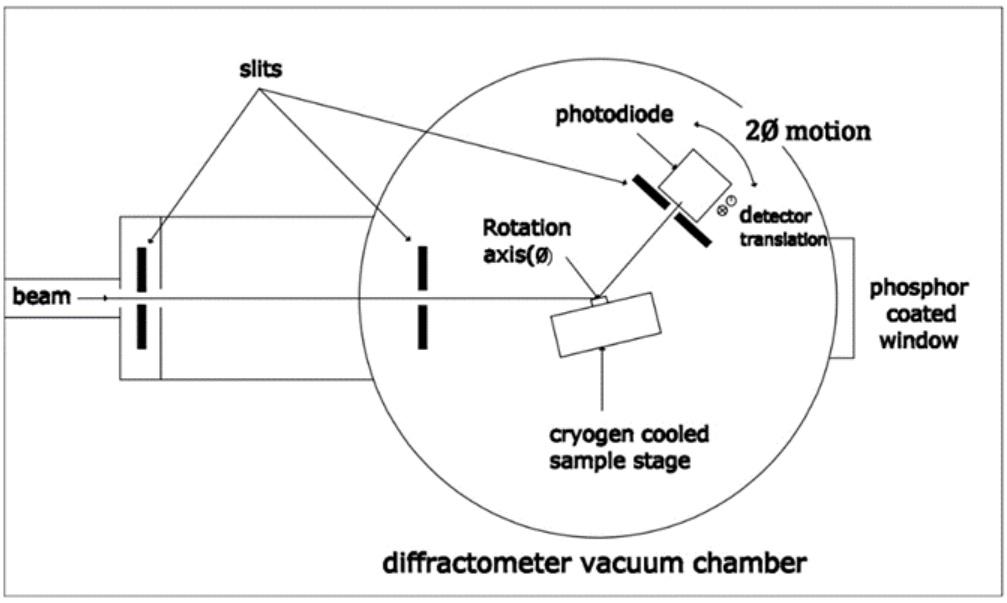


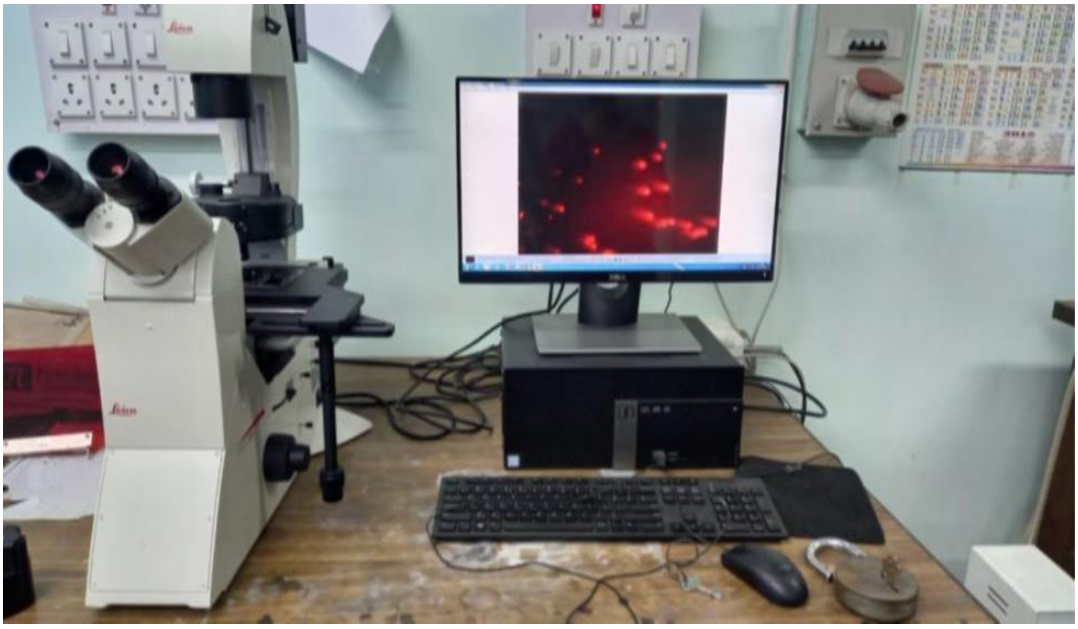
Figure 4.2: Graphical presentation of XRD test.

of electromagnetic radiation. The diffracted X-rays are next analyzed and tallied. The random orientation of powdered materials requires scanning the sample across a range of 2Ɵ angles to identify all potential diffraction directions of the lattice. XRD is used to determine the crystallographic state of a material. The crystallinity of nanocurcumin was determined using a Burker AXS, Inc. model D8, WI.

4.10. Fluorescent Microscope

The fluorescent microscope is the most powerful optical instrument utilized in cell imaging. Fredrick W. Herschel created the microscope in 1845. Fluorescent microscopy measures the absorption of light by a particular fluorescent probe. Fluorescent probes can be a single synthetic molecule that fluoresces or a mix of fluorescent and indicator molecules. Usually, they are either toxins or antibodies. The process of fluorescence starts with light absorption and concludes with light emission. The phenomena rely on the quantum behavior of light, rather than its wave-like nature. Fluorescence occurs when a material or probe is triggered by another light

source. A fluorescent microscope uses an exciter filter between the light source and condenser lens to transmit only excitation light. The condenser lens focuses the beam on the specimen, forcing fluorescent chemicals to pass through the objective lens. A barrier filter eliminates the excitation wavelengths. This allows the emission wave lengths to form the final picture. The resulting image seems brilliant on a dark background. Fluorescent dyes, also known as fluorophores or fluorochromes, are used to stain cellular components. These dyes are stimulated by a certain wavelength of light and emit light at a longer wavelength. The application of fluorescence microscopy in biology is expanding quickly. It is a very helpful method for identifying the cell's dynamic activity. The comet test method was employed in this investigation to identify DNA damage within the cells using a fluorescence microscope. In the current investigation, the fluorescent microscope was employed in the comet assay to calculate the proportion of DNA damage in whole blood cells. This experiment was conducted using a Leica Model 300 FX fluorescent microscope.



4.11. ELISA Reader

The ELISA reader, also known as a micro-plate reader, is widely used in fields such as biological science, chemistry, biotechnology, and pharmaceutical science. It is commonly used in research to quickly identify biological events and bioassays. Reactions typically take place in 96-well microtitre plates, with volumes ranging from 100-200µL per well. The 1-1536 well format is typically used for research and diagnostic purposes. Microplates with a greater density are commonly utilized for screening applications. Microplate assays offer several detection techniques, including absorbance, luminescence, fluorescence, time-resolved fluorescence, fluorescence polarization, light scattering, and nephelometry. Absorbance detection is commonly employed in ELISA experiments to quantify proteins, nucleic acids, cell viability, ROS generation, and enzymes.

CHAPTER V

RESULTS

5. Results

5.1. Nanocurcumin Characterization

Nanocurcumin was prepared by ultra-sonication method from curcumin. The prepared nanocurcumin looks as a yellow powder. The morphology of the as prepared nanocurcumin is shown in Figure 5.1a, where the average particle size of the nanocurcumin was 60 ± 10 nm approximately. DLS analysis was utilized to determine size distribution profile of nanocurcumin and the average hydrodynamic diameter of prepared nanocurcumin was 65 nm which is shown in Figure 5.1b. The zeta potential of the as prepared nanocurcumin was less than - 25 mV as seen from Figure 5.1c.

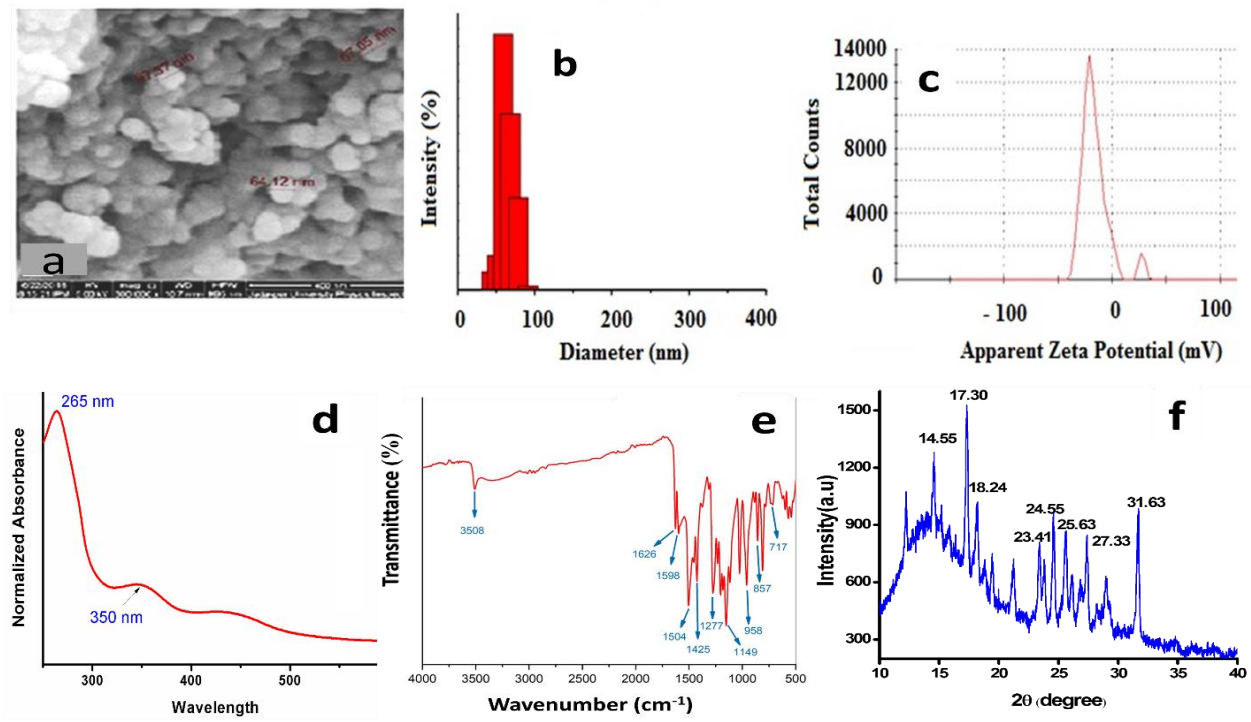


Figure 5.1: (a). Physical appearance and average particle size (60 ± 10 nm) as measured by FESEM. (b) DLS study of nanocurcumin. (c) Zeta potential of nanocurcumin. (d) Absorption peak showed at 350 nm by UV-visible spectrophotometer. (e) FTIR spectrum of synthesized nanocurcumin. (f) Crystalline properties of prepared nanocurcumin as evidenced by XRD.

The powder is soluble in water, and gives a reddish yellow solution, with an absorption peak at 350 nm as determined by a UV-visible spectrophotometer (Figure 5.1d). The as prepared nanocurcumin showed a weak peak at 717 cm^{-1} assigned for the C−H vibrational stretching of the aromatic ring. Another peak of nanocurcumin at 857 cm^{-1} correspond to the C-H bending and 958 cm^{-1} corresponds to benzoate trans-C−H vibration. Peak of nanocurcumin at 1149 cm^{-1} is due to the C−H stretching. Other peak at 1277 cm^{-1} represents the C−O vibrational stretching, peak at 1425 cm^{-1} assigned to phenolic C−O stretching and peak at 1504 cm^{-1} correspond to C=O. The strong absorption peak of nanocurcumin at 1598 cm^{-1} can be designated for the symmetric stretching vibrations of aromatic C=C group. Additional absorption peak observed at 1626 cm^{-1} which may be assigned for aliphatic (C=C) stretching. The peak at 3508 cm−1 expresses that there is presence of OH stretching in nanocurcumin. X-ray diffraction confirmed the crystalline nature of prepared nanocurcumin (Figure 5.1f).

5.2. Nanocurcumin Ameliorates Nicotine-Induced Toxicity in Female Rats Under Protein Restricted Condition

Nicotine is recognized to be hazardous to human health. Long-term nicotine use has harmful consequences at the biochemical, cellular, histopathological, immunological, and molecular levels. Curcumin demonstrates the capacity to counteract the harmful effects of nicotine. However, due to its poor pharmacokinetic qualities, it has limited medicinal use. The current study evaluated the potential of nanocurcumin against nicotine toxicity and compared it to that of curcumin.

5.2.1. Effects of Nanocurcumin on Nicotine-Induced Alterations in Hepatic Enzymes Under Protein Restricted Condition

The activity of all four hepatic enzymes, ACP, ALP, ALT, and AST, were considerably raised

(42.6%, 106.5%, 78.6%, and 90.77%, respectively) in the nicotine-treated rats as compared to the control group. Both curcumin and nanocurcumin demonstrated strong protective benefits against nicotine-induced liver damage due to which both reduce the activity level of those enzymes which are due to nicotine treatments. However, nanocurcumin showed to be more helpful in this regard than curcumin. Table 5.1 shows the results of hepatic enzymes levels.

Table 5.1: Levels of Four Hepatic Enzymes in Different Study and Control Groups of Female Rats Under Protein Restricted Condition

Enzymes	Control	Nicotine	Nicotine + Curcumin	Nicotine + Nanocurcumin
ACP (mmol/ h/100 ml	1.36 ±0.10	1.94±0.11 (42.6 ↑)	1.52± 0.12** (11.8 ↑)	1.42± 0.10**## (4.4 ↑)
ALP (mmol/ h/100 ml	9.30± 0.60	19.20± 0.30 (106.5 ↑)	12.95± 0.30* (39.2 ↑)	10.42± 0.23**## (12.0 ↑)
AST (IU/L)	9.80± 1.60	17.50± 4.00 (78.6 ↑)	11.32± 0.70** (15.5 ↑)	10.35± 0.90**## (5.61 ↑)
ALT (IU/L)	37.40± 2.60	71.35± 1.61 (90.77 ↑)	42.91± 3.49** (14.7 ↑)	39.99± 2.86**## (6.9 ↑)

The experimental setup was repeated twice and all data were averaged over n = 12 animals and given mean ± S.D. Significance levels were determined by using ANOVA, where * implied significant ($p < 0.01$) and ** implied more significant ($p < 0.001$) of the data when compared with the control. Similarly, # implied significant ($p < 0.01$) and ## implied more significant ($p < 0.001$) of the data when compared with nicotine treatment.

5.2.2. Effects of Nanocurcumin on Nicotine Induced Changes on Renal Function Parameters

Nicotine-exposed animals had significantly higher blood urea and creatinine levels 48.3% and 37.0

%, respectively than normal mice (Table 5.2). The administration of curcumin and nano-curcumin along with nicotine showed beneficial effects by declining the levels of urea and creatinine. In nicotine-treated rats, nanocurcumin reduced blood urea and creatinine levels more effectively 2.4 % and 3.7 %, respectively than natural curcumin 22.7 % and 15.6 % respectively. Table 5.2 shows the findings of serum urea and creatinine levels.

Table 5.2: Levels of serum urea and creatinine in different study and control groups of female rats under protein restricted condition

Parameter	Control	Nicotine	Nicotine + Curcumin	Nicotine + Nanocurcumin
Urea (mg/100 ml))	38.30 ± 2.15	56.80 ± 2.60 (48.3 ↑)	47.00±2.78* (22.7 ↑)	39.23 ± 2.06**## (2.4 ↓)
Creatinine (mg/100 ml)	1.35 ± 0.12	1.85 ± 0.21 (37.0 ↑)	1.56 ± 0.50* (15.6 ↑)	1.30 ± 0.10**## (3.7 ↓)

The experimental setup was repeated twice and all data were averaged over n = 12 animals and given mean ± S.D. Significance levels were determined by using ANOVA, where * implied significant ($p < 0.01$) and ** implied more significant ($p < 0.001$) of the data when compared with the control. Similarly, # implied significant ($p < 0.01$) and ## implied more significant ($p < 0.001$) of the data when compared with nicotine treatment.

5.2.3. Effects of Nanocurcumin on Nicotine Induced Changes in Lipid Profile

A significant increase in triglyceride, cholesterol, VLDL-C and LDL-C (75.9%, 29.3%, 74.4% and 75.9 % respectively) and decreased level of HDLC concentration (24.6%) in the serum of the nicotine treated rats were recorded in respect to control arm (Table-5.3). However, curcumin and nanocurcumin demonstrated potential ameliorative benefits by lowering the amounts of triglycerides, cholesterol, VLDL-C, and LDL-C in the rats' serum. When compared to the nicotine-

treated arm, both curcumin and nanocurcumin arms showed higher levels of HDL-C. Nanocurcumin performed better than unformulated curcumin in preserving the lipid profile owing to nicotine toxicity.

Table 5.3: Levels of Lipid Profile in Different Study and Control Groups of Female Rats Under Protein Restricted Condition

Parameter	Control	Nicotine	Nicotine + Curcumin	Nicotine + Nanocurcumin
Triglyceride(mg/dl)	105.6 ± 8.4	185.8 ± 9.1 (75.9 ↑)	109.5 ± 8.6** (3.7 ↑)	106.4 ±2.5**## (0.8 ↑)
Cholesterol (mg/dl)	107.3 ± 7.9	138.7 ± 9.0 (29.3 ↑)	115.0 ± 5.6** (7.2 ↑)	109.3 ±2.4**## (1.9 ↑)
HDL (mg/dl)	39.5 ± 7.0	29.8 ± 4.0 (24.6 ↓)	31.7 ± 1.0* (19.7 ↓)	33.5 ± 1.5*# (15.2 ↓)
VLDL (mg/dl)	21.1 ± 1.6	36.8 ± 0.7 (74.4 ↑)	30.7 ± 1.8* (45.5 ↑)	21.5 ± 0.4**## (1.03 ↑)
LDL (mg/dl)	46.1 ± 2.1	81.8 ± 4.1 (75.9 ↑)	64.79 ± 1.2* (40.5 ↑)	43.5 ± 1.5**## (5.6 ↓)

The experimental setup was repeated twice and all data were averaged over n = 12 animals, and given mean ± S.D. Significance levels were determined using ANOVA, where, * implies $P < 0.01$ and ** implies $P < 0.001$ compared to control. The data presented in the parenthesis showed average (%) increase (↑) or decrease (↓) in respect to the control.

5.2.4. Effects of Nanocurcumin on Nicotine Induced Changes on MDA

MDA levels are used to measure the rate of lipid peroxidation by various free radicals in plasma and the liver. Nicotine-induced lipid peroxidation by free radicals resulted in elevated

MDA levels in plasma and liver cells 42.2% and 53.0%, respectively. Both curcumin and nanocurcumin reduced MDA levels in nicotine-treated rats. Nanocurcumin was more efficient than curcumin at decreasing MDA levels in nicotine-treated rats (Table 5.4).

Table 5.4: Levels of MDA in Different Study and Control Groups of Female Rats Under Protein Restricted Condition

MDA level	Control	Nicotine	Nicotine + Curcumin	Nicotine + Nanocurcumin
Serum (nmol/ml)	5.61 ± 0.6	7.98 ± 1.3 (42.2 ↑)	6.30 ± 0.5** (12.3 ↑)	6.32 ± 1.1** (12.7 ↑)
Liver (nmol/mg protein)	14.98 ± 0.4	22.92 ± 0.5 (53.0 ↑)	16.01 ± 2.96** (6.9 ↑)	15.50 ± 0.9**# (3.5 ↑)

The experimental setup was repeated twice and all data were averaged over n = 12 animals, and given mean ± S.D. Significance levels were determined using ANOVA, where, * implies $P < 0.01$ and ** implies $P < 0.001$ compared to control. The data presented in the parenthesis showed average (%) increase (↑) or decrease (↓) in respect to the control.

5.2.5. Effects of Nanocurcumin on Nicotine Induced Changes on Antioxidant Enzymes

Antioxidants are enzymes that neutralize free radicals, reducing oxidative stress. In my current investigation, it was noticed that the levels of SOD, CAT, GSH, and GPx were considerably lower in the nicotine treated group compared to the control group by 54.7%, 27.3%, 12.02%, and 10.2%, respectively. Curcumin and nanocurcumin increased the levels of antioxidants in nicotine-treated rats. It was discovered that the nanocurcumin group had a greater capacity than the curcumin group (Table 5.5).

Table 5.5: Levels of Antioxidant Enzymes in Different Study and Control Groups of Female Rats Under Protein Restricted Condition

Enzymes	Control	Nicotine	Nicotine + Curcumin	Nicotine + Nanocurcumin
SOD (nmol/O2 decomposed/ min/100 mg protein)	9.02 ± 0.2	4.09 ± 0.1 (54.7 ↓)	6.72 ± 0.14* (25.5 ↓)	7.15 ± 0.5**# (20.7 ↓)
CAT (nmol/H2O2 decomposed/ min/mg protein)	40.21 ± 1.0	29.22 ± 1.0 (27.3 ↓)	32.9 ± 0.5* (18.2 ↓)	35.50 ± 1.0**# (11.7 ↓)
GSH (µg/mg protein)	18.75 ± 1.76	12.02 ± 1.13	14.00 ± 1.41*	16.00 ± 1.41**#
GPx (nmol/ min/mg protein)	150.52 ± 2.5	135.11 ±2.9 (10.2 ↓)	144.34 ± 2.2* (4.1 ↓)	149.50 ± 2.5**# (0.6 ↓)

The experimental setup was repeated twice and all data were averaged over n = 12 animals, and given mean ± S.D. Significance levels were determined using ANOVA, where, * implies $P < 0.01$ and ** implies $P < 0.001$ compared to control. The data presented in the parenthesis showed average (%) increase (↑) or decrease (↓) in respect to the control.

5.2.6. Effects of Nanocurcumin on Nicotine Induced Changes on Haemoglobin Under Protein Restricted Condition

The concentration of hemoglobin dropped to 25.52% in the nicotine treated group, but both natural curcumin and nanocurcumin increased haemoglobin concentration in the nicotine treated group (Table 5.6).

Table 5.6: Levels of Haemoglobin Content in Different Study and Control Groups of Female Rats Under Protein Restricted Condition

Parameter	Control	Nicotine	Nicotine + Curcumin	Nicotine + Nanocurcumin	Nanocurcumin
Haemoglobin (g/dL^{-1}) (n=12)	12.3±0.34	9.16±0.29** (25.52 ↓)	11.66±0.18*## (5.48 ↓)	12.3±0.18*## (0.0)	13.4±0.5 (8.94 ↑)

The experimental setup was repeated twice and all data were averaged over n = 12 animals, and given mean ± S.D. Significance levels were determined using ANOVA, where, * implies P < 0.01 and ** implies P < 0.001 compared to control. The data presented in the parenthesis showed average (%) increase (↑) or decrease (↓) in respect to the control.

5.2.7. Effects of Nanocurcumin on Nicotine Induced Changes in Total Protein in Different Tissues

The total protein content of several organs such as the liver, kidney, and ovary were dramatically reduced in nicotine-treated animals (7.12%, 7.5% and 12.02% respectively) (Table 5.7). Nanocurcumin treatment considerably raised the protein level of those tissues ($p < 0.001$) compared to curcumin ($p < 0.01$).

Table 5.7: Contents of Total Protein in Different Tissues of Different Study and Control Groups of Female Rats Under Protein Malnourished Condition

Parameter	Control	Nicotine	Nicotine + Curcumin	Nicotine + Nanocurcumin
Liver (mg/g)	15.00 ± 0.70	7.12 ± 1.59	12. 85 ± 0.91*	13.77± 0.70$^{**\#}$
Kidney (mg/g)	15.5 ± 2.12	7.5 ± 1.41	11.50 ± 2.53*	14.00 ± 1.4$^{**\#\#}$
Ovary (mg/g)	18.75 ± 1.76	12.02 ± 1.13	14.00 ± 1.41*	16.00 ±1.41$^{**\#}$

The experimental setup was repeated twice and all data were averaged over n = 12 animals, and given mean ± S.D. Significance levels were determined by using ANOVA, where * implied significant (P < 0.01) and ** implied more significant (P < 0.001) of the data when compared with the data of nicotine treatment. Similarly, # implied significant (P < 0.01) and ## implied more significant (P < 0.001) of the data when compared with the data of nicotine + curcumin treatments. The data within the parenthesis represent the average percentage of increase (↑) or decrease (↓) relative to the control.

5.2.8. Effects of Nanocurcumin on Nicotine Induced Changes in Total DNA Content in Different Tissues of Animals and Percentage of DNA Damage

The whole blood DNA contents was drastically reduced (29.81 %) by nicotine as observed in nicotine treated rats (Table-5.8). Nicotine hampered DNA synthesis in the tissues of the blood liver, kidney, and ovary of the rats under protein restricted diet as seen in Table 5.8. DNA contents in those tissues were reduced to more than 20%, which was significant ($P < 0.001$). Nicotine treatment was responsible for a massive DNA damage (48 %) of the whole blood cells in female rats in protein restricted condition where as it was only 13.3 % in control group (Table 5.9). The ameliorative action of nanocurcumin against nicotine in DNA synthesis was found to be significant ($P < 0.001$) than that of curcumin.

Administration of curcumin or nanocurcumin alone did not produce any negative effect on DNA content of blood cells rather they showed healthy condition of blood cells (Figure 5.2).

Table 5.8: Total DNA Content in Blood Cells and Different Tissues in Different Study and Control Groups of Female Rats Under Protein Restricted Condition

parameter	Control	Nicotine	Nicotine + Curcumin	Nicotine + Nanocurcumin	Nanocurcumin
Whole blood (µg/300µL blood)	110.67± 5.31	77.67± 6.75 (29.81 ↓)*	87.36±9.39 (21.06 ↓)*#	104.82±4.08 (5.28 ↓)*#	113.94±3.86 (2.95 ↑)
Liver (µg/mg)	17.00 ± 2.13	13.29 ± 1.60 (21.82 ↓)*	14.89 ± 0.51 (12.41 ↓)*#	16.16± 3.91 (4.94 ↓)*#	17.06±1.41 (0.35 ↑)
Kidney (µg/mg)	17.66 ± 0.42	13.49 ± 1.54 (23.61 ↓)*	15.63± 0.23 (11.49 ↓)*#	16.41 ± 2.36 (7.07 ↓)*#	17.74±0.75 (0.45 ↑)
Ovary (µg/mg)	19.72 ± 0.67	13.84 ± 0.36 (29.81 ↓)*	15.29 ± 0.24 (22.46 ↓)*#	18.44 ±0.05 (6.49 ↓)*#	19.39±0.04 (1.67 ↑)

Data are Mean ± SD. * and # indicate significant differences with Control and Nicotine groups, respectively. Values in parenthesis indicate the percentage of increase (↑) or decrease (↓) relative to the control.

Table 5.9: Percentage of DNA Damage and Tail Moment in Different Study and Control Groups of Female Rats Under Protein Restricted Condition

Parameter	Control	Nicotine	Nicotine + Curcumin	Nicotine + Nanocurcumin	Nanocurcumin
% of DNA damaged	13.36±0.5	48.00±4.2*	26.89±2.17*	20.06±0.5*#	3.12±0.14
Tail moment arbitrary unit	80.93±2.50	636.95±5.04*	210.8±6.42*	184.27±8.44*#	19.44±8.35

Data are Mean ± SD. * and # indicate significant differences with Control and Nicotine groups, respectively. Values in parenthesis indicate the percentage of increase (↑) or decrease (↓) relative to the control.

5.2.9. Effects of Nanocurcumin on Nicotine Induced Changes in Cytokine Molecules

Cytokines have a significant function in influencing the pathophysiological manifestation of several clinical characteristics controlled by the host immune system. The concentration levels of all the observed cytokine molecules (IL-4, IL-6, TNF-α, and IFN-γ) were increased due to the action of nicotine in female rats maintained under protein restriction conditions. Nicotine therapy resulted in substantial increases in blood levels of IL-4, IL-6, TNF-α, and IFN-γ to 1.4, 1.8, 2.1 and 1.3 folds respectively compared to the control group (Figure 5.3). All four cytokine levels were significantly reduced in nicotine-induced rats treated with curcumin or nanocurcumin. Supplementation of nano-curcumin to nicotine- exposed and protein-restricted animals reduced those cytokine levels very significantly ($p < 0.001$), due to which the concentration levels of IL-4, IL-6, TNF-α, and IFN-γ b became 1.0, 1.3, 1.2 and 1.1 folds respectively.

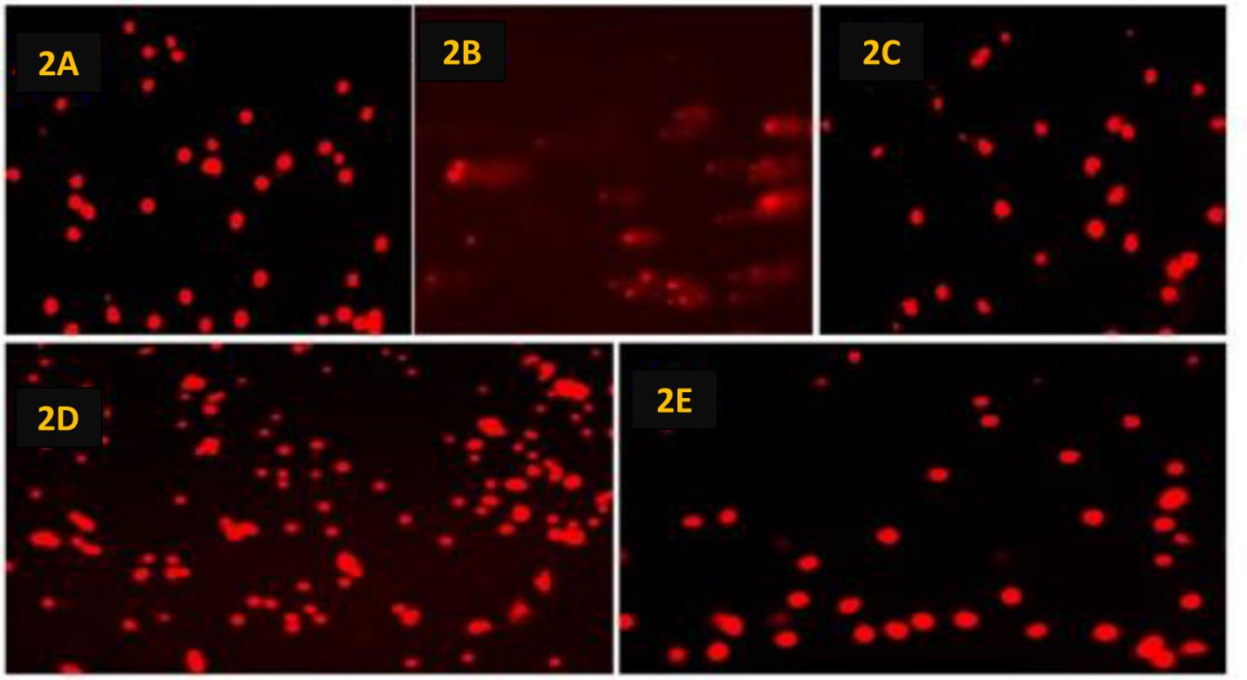


Figure 5.2: Photomicrographs of the comet assay of blood cell DNA of animals under protein- restricted conditions. (2A) represents the comet picture of blood cell DNA in protein-restricted diet-fed rats. (2B) represents the comet picture of blood cell DNA in the nicotine-exposed group. (2C) represents the comet picture of blood cell DNA in the nicotine + curcumin-exposed group. (2D) represents the comet picture of blood cell DNA in the nicotine + nano-curcumin-exposed group. (2E) represents the comet picture of blood cell DNA in the nanocurcumin supplemented group.

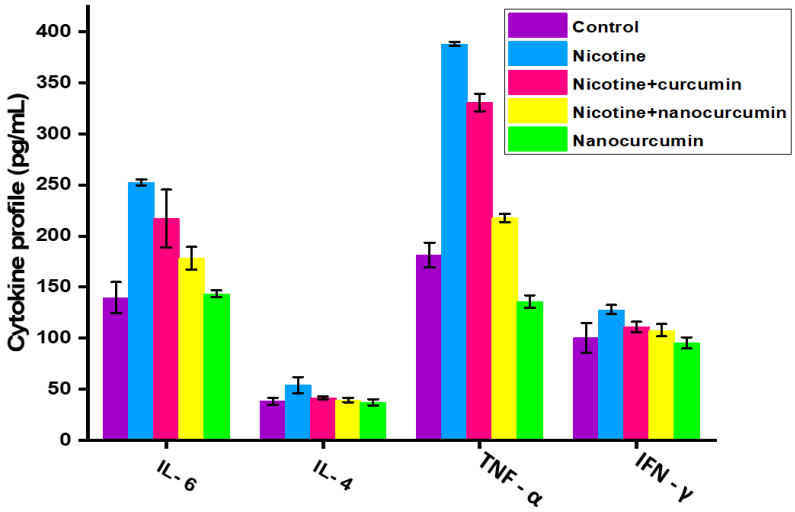


Figure 5.3: Cytokine profile determined by ELISA. Columns are Mean ± SD. * and # indicate significant differences with Control and Nicotine groups, respectively.

5.2.10. Effects of Nanocurcumin on Nicotine Induced Changes in Apoptosis Regulator Proteins

A significantly declined level (78.3 %) of apoptosis regulator protein, BCL-2 was recorded among nicotine treated animals than the animals of control arm whereas the opposite event was noticed with increased level of BAX (62.4%). Nicotine induced lower level of BCL-2 was increased effectively ($p < 0.01$) by curcumin and more significantly ($p < 0.001$) by nanocurcumin.

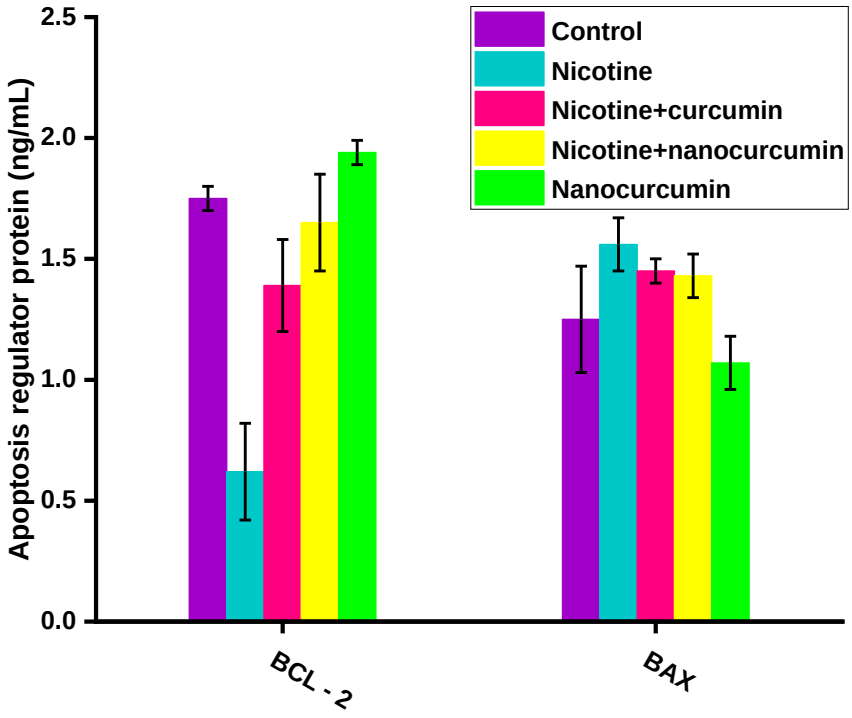


Figure 5.4: Apoptosis regulator protein determination by ELISA.

Columns are Mean ± SD. * and #indicate significant differences with Control and Nicotine groups, respectively.

5.2.11. Effects of Nanocurcumin on Nicotine Induced Changes in Steroidogenic Female Hormones

Nicotine substantially lowered steroidogenic female hormone levels, including estradiol (26.9%) and progesterone (29.6%) in female rats maintain under a protein restricted diet compare to their restricted control (Table 5.10). Curcumin efficiently restored nicotine-induced reduced levels of these hormones, and nanocurcumin almost nullified the action of nicotine and restored the normal concentration levels of estradiol and progesterone more significantly ($p < 0.001$) than curcumin in the nicotine-exposed and protein restricted diet fed female rats.

Table 5.10: Levels of Steroidogenic Hormones in Different Study and Control Groups of Female Rats Under Protein Restricted Condition.

Parameter	Control	Nicotine	Nicotine + Curcumin	Nicotine + Nanocurcumin	Nanocurcumin
Estradiol(pg/mL)	79.62±5.25	63.69±13.57 (26.9↓)**	71.11±10.1 (16.4↓)*	78.88±5.09 (6.2↓)*##	84.06±5.01 (4.3 ↑)
Progesterone(ng/mL)	11.59±0.23	8.95±0.04 (29.6↓)**	9.49±0.121 (20.4↓)	10.7±0.19 (10.5↓)*##	13.28±0.20 (11.7 ↑)

The experimental setup was repeated twice and all data were averaged over n = 12 animals, and given mean ± S.D. Significance levels were determined using ANOVA, where, * implies $P < 0.01$ and ** implies $P < 0.001$ compared to control. The data presented in the parenthesis showed average (%) increase (↑) or decrease (↓) in respect to the control.

5.2.12. Histological Study

Figure 5.5A depicts the histological analysis of liver tissue from control group mice. Figure-5.5B depicts the altered cell arrangement and central vein (indicated by an arrow head) of

the nicotine exposed liver. Curcumin and nanocurcumin supplemented liver pictures are shown in Figures 5.5C and 5.5D, respectively. The histological studies of the kidney tissues of control rats, nicotine exposure distorted kidney tissue, curcumin and nanocurcumin supplemented kidney tissues are presented in Figures. 5.6A – 5.6 D respectively. The histological studies of the ovary tissues of control rats, nicotine exposure distorted ovary tissue, curcumin and nanocurcumin supplemented ovary tissues are presented in Figures. 5.7A – 5.7D respectively.

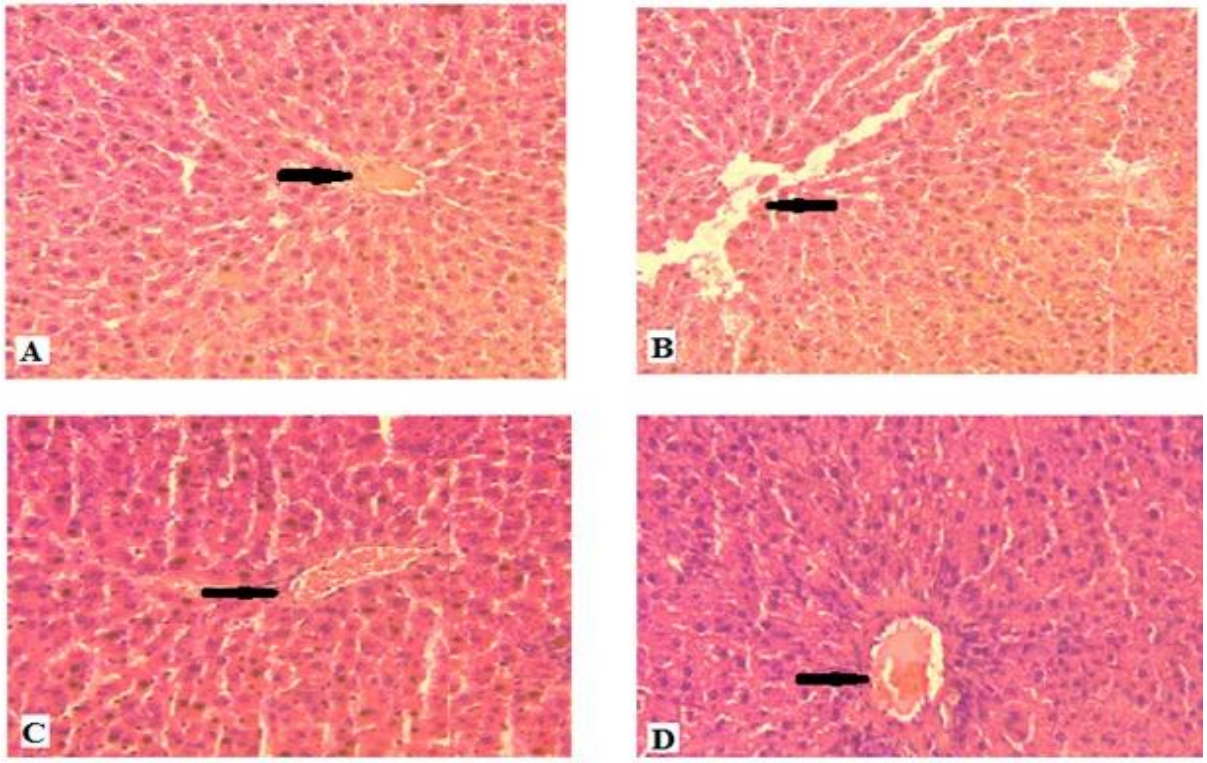


Figure 5.5: Photographs showing the histopathological changes of liver: (A). Control group, (B). Nicotine treated group, (C). Nicotine plus curcumin treated group and (D). Nicotine plus nanocurcumin treated group.

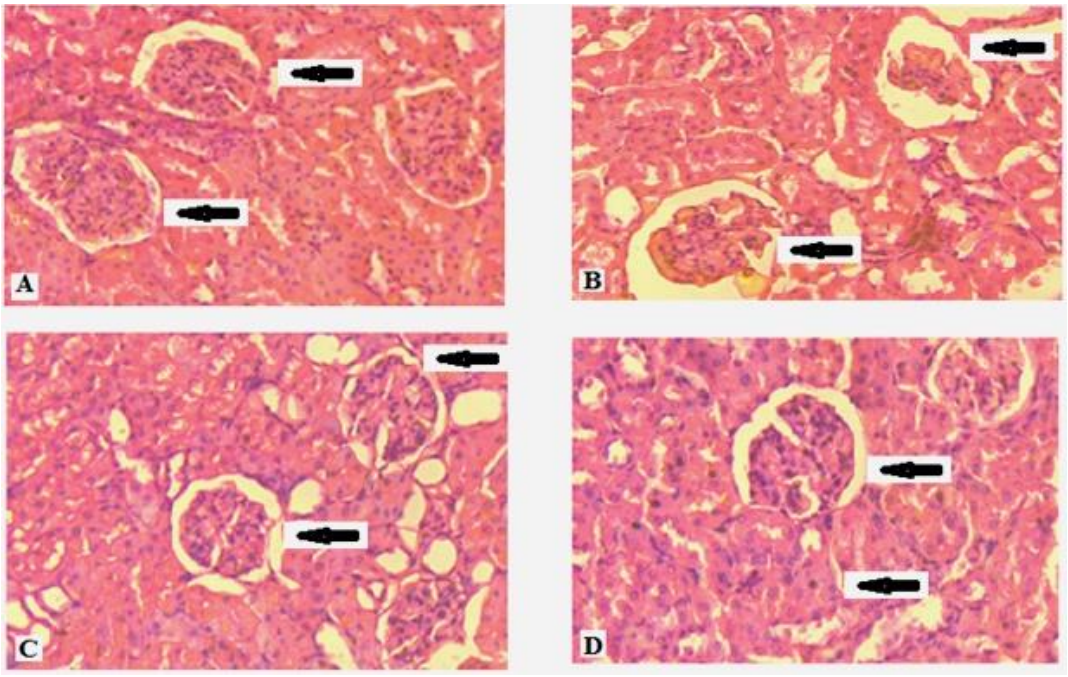


Figure 5.6: Photographs showing the histopathological changes of kidney: (A). Control group, (B). Nicotine treated group, (C). Nicotine plus curcumin treated group and (D). Nicotine plus nanocurcumin treated group.

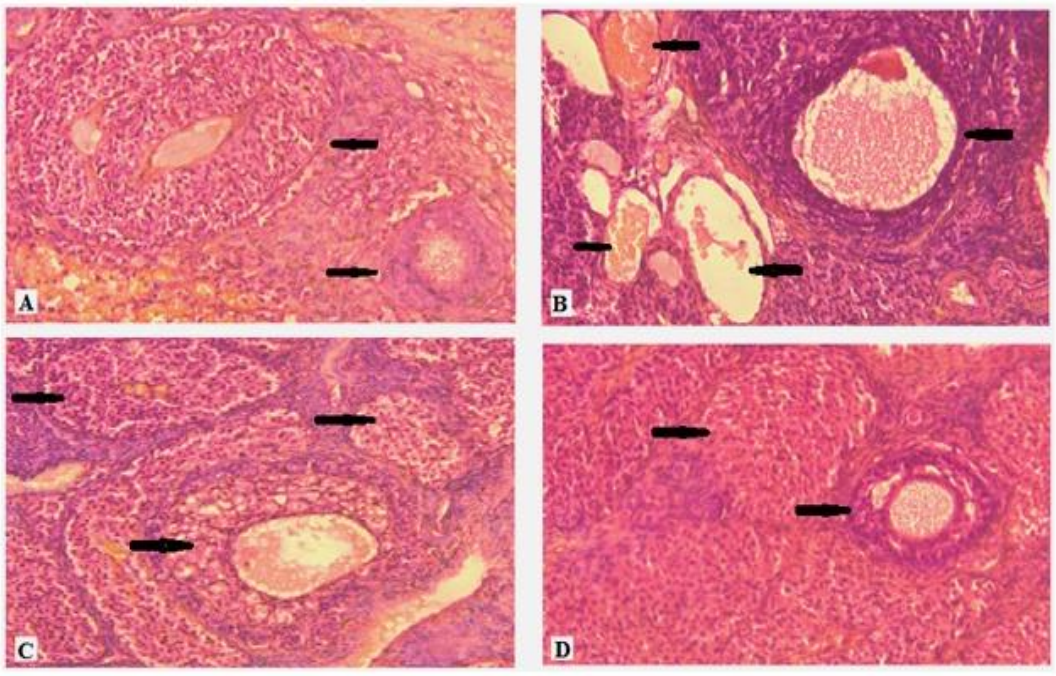


Figure 5.7: Photographs showing the histopathological changes of ovary: A). Control group, B). Nicotine treated group, C). Nicotine plus curcumin treated group and D). Nicotine plus nanocurcumin treated group

5.2.13. Cytotoxic Effects Induced by Curcumin and Nanocurcumin in Cancer Cell Lines

The effect of different doses of curcumin and nanocurcumin on cervical cancer cell line SiHa and lung cancer cell line A549, as well as on normal epithelial cell line HEK293 are shown in Figure 5.8A and 5.8B. The IC50 value of Nanocurcumin for SiHa and A549 cells within 48 h is about 10.66 ± 1.04 and7.65 ± 1.35 µM (Table 5.11). Curcumin treatment at 10µM, the cell viability of SiHa and A549 declines to 60.22% and 54.21% respectively compared to normal cell line, HEK293 (Figure 5.8A). Cancerous cell line SiHa and A549, the increasing concentrations of nano curcumin (10-60 µM) in the media decreases the percentage of viable cells significantly after 48h (Figure 5.8B).

Table 5.11: Inhibitory Effects (IC_{50} In µM) Of Curcumin and Nanocurcumin on Cancer Cells (Siha and A549) and Normal Epithelial Cell Line (HEK293) After 24 H and 48 H Incubation.

Cell Line	Curcumin IC_{50} values(µM) 24h	Nano-curcumin IC_{50} values (µM) 24h	Curcumin IC_{50} values(µM) 48h	Nano-curcumin IC_{50} value (µM) 48h
HEK293	57.36 ±2.08	51.31 ± 0.59	46.79 ± 1.14	36.10 ± 1.09
SiHa	25.64 ± 0.87	19.27 ± 1.34	14.57 ± 1.17	10.66 ± 1.04
A549	25.33 ± 0.74	17.80 ± 1.13	12.17 ± 0.44	7.65 ±1.35

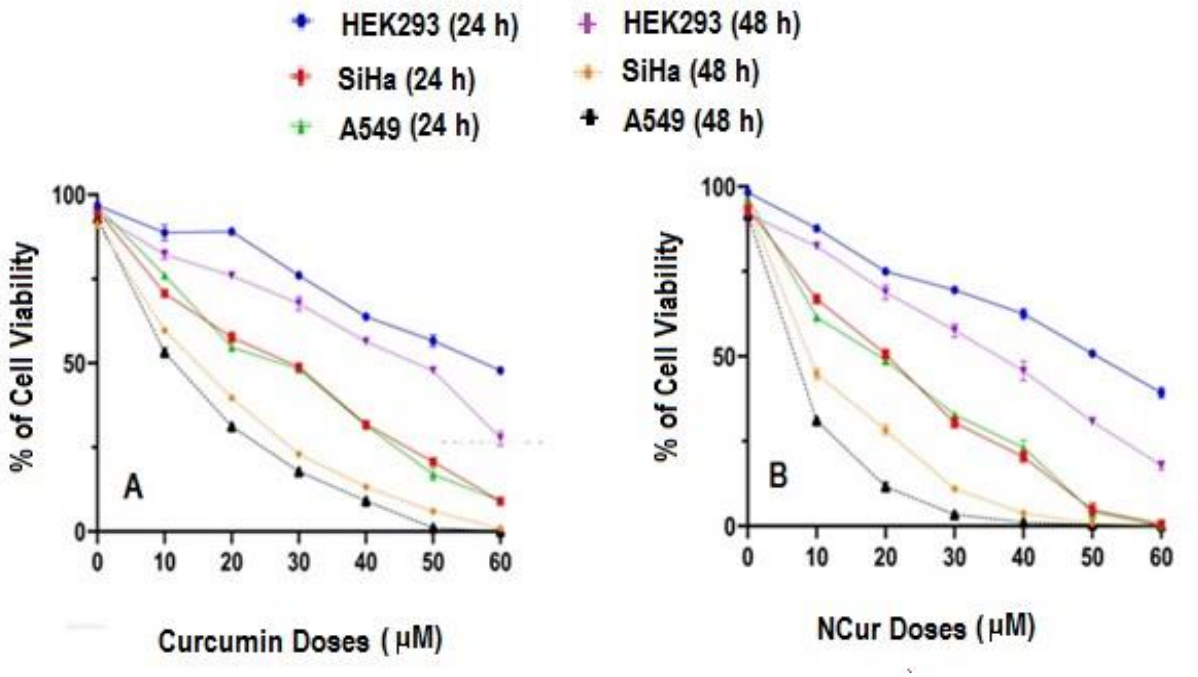


Figure 5.8: Cell viability changes of SiHa, A549 and HEK293 cells, where (A) Viability of the cells after 48hrs of 10-60 µM of curcumin treatment. (B) Viability of the cells after 48hrs of 10-60 µM of Nanocurcumin treatment.

5.2.14. Changes in Cellular Morphologies Induced by Curcumin and Nanocurcumin in Cancer Cell Line

The morphological changes (indicated by arrows) of SiHa and A549 cells by curcumin and nanocurcumin are depicted in Figure 5.9A and 5.9B. Potent morphological changes in cancer cells (SiHa and A549) including cell shrinkage and star-shape with membrane blabbing which represents a typical apoptosis. During treatment, at inhibitory concentration of curcumin and nanocurcumin both SiHa and A549 cells became round in shape. Morphological changes in cell nuclei have also been analyzed by fluorescence microscope. The treatment group showed shrunken, emarginated nuclei and fragmented apoptotic bodies compared to the control. The large

nucleus in the untreated cells is demonstrating the apoptotic efficacy of curcumin and nanocurcumin. Apoptosis causes many morphological changes such as nuclear fragmentation, chromatin condensation and emargination of nucleus (marked by arrows in Figure 5.9B) for both cancer cells.

5.2.15. Effect of Curcumin and Nanocurcumin on the Level of Intracellular ROS Generation and In Promoting Apoptosis and DNA Damage

Curcumin induced apoptosis in SiHa and A549 lung cancer cells when cells are exposed to 14 µM and 12 µM curcumin respectively while nanocurcumin induced apoptosis when cells are exposed to 10 µM and 7 µM respectively. The fluorescence microscopic images show an increased proportion of cells with higher fluorescence intensity when cells are treated with nanocurcumin for 48h (Figure 5.10A and 5.10B). This is an indication of increased level of ROS in SiHa and A549 cancer cells by nanocurcumin. Curcumin can induce similar magnitude of ROS generation only at very higher doses in comparison to nanocurcumin. DNA laddering indicating nanocurcumin ability to induce apoptosis in both SiHa and A549 cells (L2 and L4 respectively in Figure 5.11). Lesser magnitude of DNA laddering is observed in the treatment of curcumin (L1 and L3 respectively) on SiHa and A549 cancer cell lines Figure 5.11). Lesser magnitude of DNA laddering is observed in the treatment of curcumin (L1 and L3 respectively) on SiHa and A549 cancer cell line.

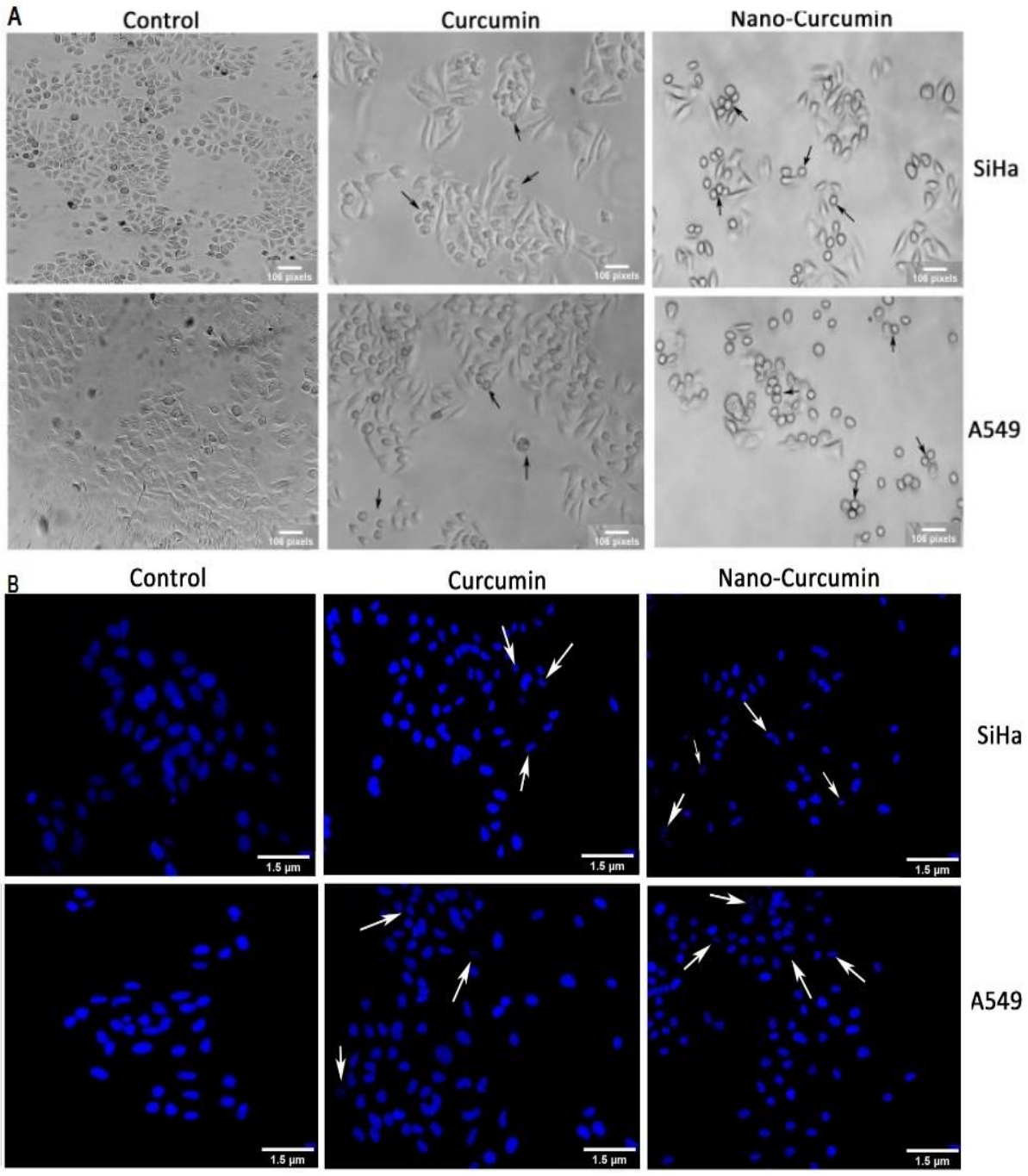


Figure 5.9: (A) Morphological changes (shown by arrows) of SiHa and A549 cells by curcumin and nanocurcumin. (B) Fluorescence microscopic images of SiHa and A549 cells treated with respective IC50 concentrations of curcumin (14µM for SiHa and 12 µM for A549) and nanocurcumin (10 µM for SiHa and 7 µM for A549) and stained with 100 nM DAPI for 5 min. The curcumin and nanocurcumin induced DNA damage/ chromatin condensation are shown by arrows. Data represent mean ± SD of two experiments. $^{*}P < 0.05$.

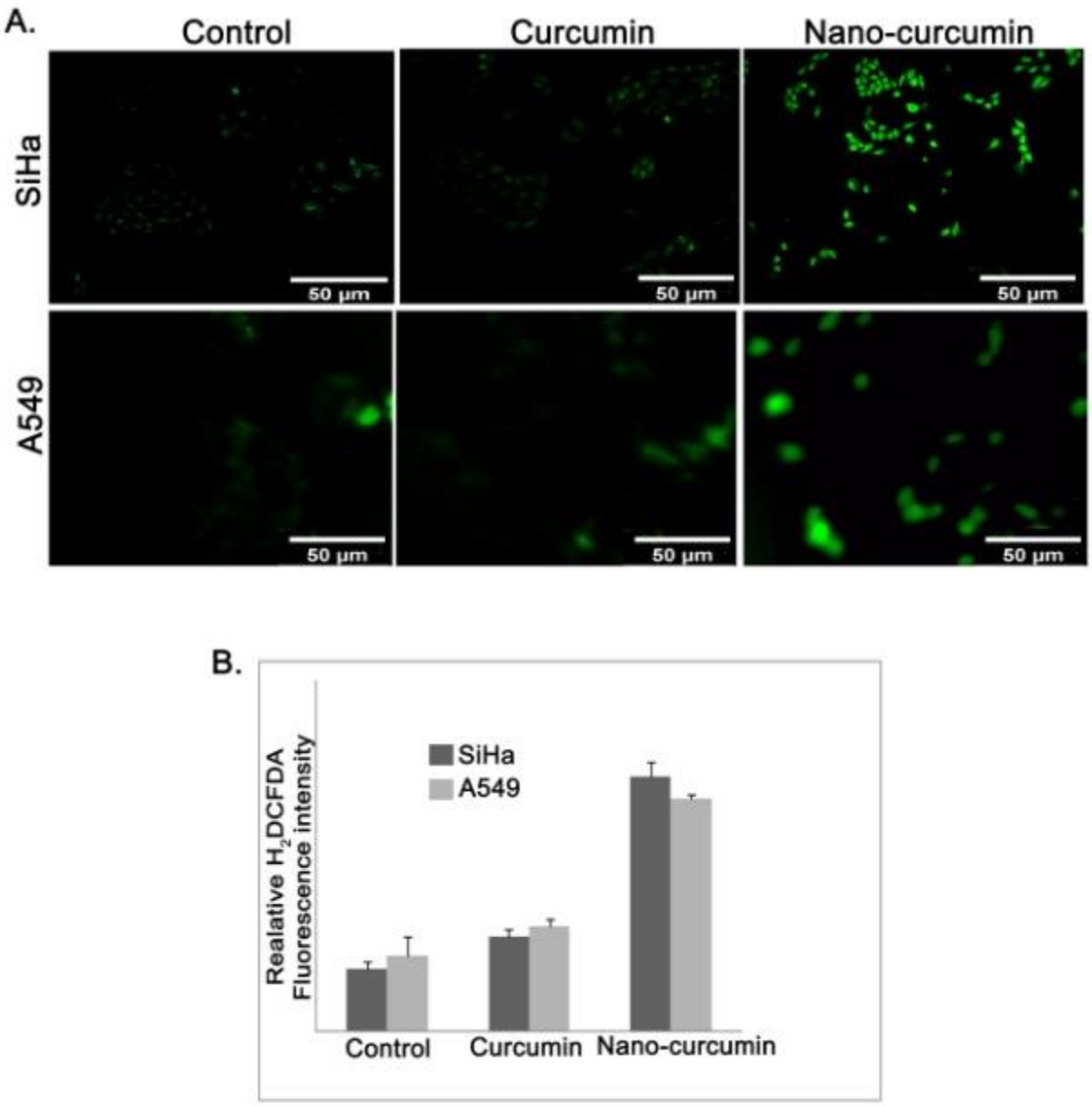


Figure 5.10: (A) Determination of ROS generation and apoptotic cell death in SiHa and A549 cell lines. Where, SiHa and A549 cells treated with IC_{50} doses of curcumin and nanocurcumin and stained with H_2DCFDA. ROS generation is observed under fluorescence microscope. (B) Determination of ROS generation and apoptotic cell death in SiHa and A549 cell lines. Where, representative fluorescence intensities of H_2DCFDA which are plotted in the bar diagram after treatment with curcumin and nanocurcumin.

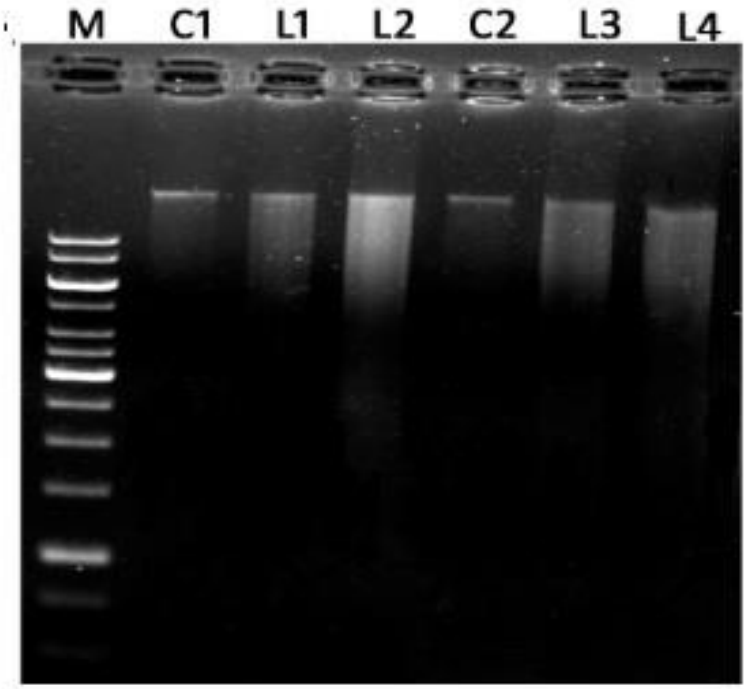


Figure 5.11: DNA fragmentation study, were M -represents molecular weight marker (1Kb DNA ladder), C1 - SiHa cell control, (L1) - exposed with curcumin for 48h, (L2) - SiHa cell incubated for 48hrs with nanocurcumin, (C2) - A549 cell control, (L3) - incubated with curcumin for 48h, (L4) - A549 cells exposed to nanocurcumin for 48h.

CHAPTER VI

DISSCUSSION

Numerous studies in animal models maintained at normal dietary condition have shown that curcumin effectively mitigates nicotine induced toxicities. The clinical effectiveness of curcumin in human subjects is limited by its undesirable pharmacokinetic properties, which include poor absorption, poor bioavailability, chemical instability, rapid metabolism, and rapid systemic elimination of curcumin [1]. Nanocurcumin is water soluble and more bioavailable than curcumin [2]. For these advantages Scientists are attempting to employ nanocurcumin against a variety of disorders to generate therapeutic applications [3,4]. The benefits of curcumin nanoparticles included greater drug-loaded capacity in tissues and increased bioavailability in bodily fluids and tissues with a long half-life [5,6]. The current study provides a helpful use of nanocurcumin against the exacerbated toxic effects generated by nicotine in rats under protein deficient conditions.

Nanocurcumin, in contrast to curcumin, dissolves readily in water without any surfactants [7]. The solubility testing of synthesized nanocurcumin in water showed a dense reddish coloured solution. The UV-visible spectrum investigation of prepared nanocurcumin revealed the distinctive peak of nanocurcumin at 350 nm, as demonstrated by Alam et al., 2012 and Ghosh et al., 2011 [8,9]. FTIR spectrum of nanocurcumin showed a weak peak at 717 cm^{-1} assigned for the C−H vibrational stretching of the aromatic ring. Another peak at 857 cm^{-1} correspond to the C-H bending and 958 cm^{-1} corresponds to benzoate trans -C−H vibration. Peak at 1149 cm^{-1} is due to the C−H stretching. Other peak at 1277 cm^{-1} represents the C−O vibrational stretching, peak at 1425 cm^{-1} assigned to phenolic C−O stretching and peak at 1504 cm^{-1} correspond to C=O. The strong absorption peak at 1598 cm^{-1} can be designated for the symmetric stretching vibrations of aromatic C=C group. Additional absorption peak observed at 1626 cm^{-1} which may be assigned for aliphatic (C=C) stretching. The peak at 3508 cm^{-1} expresses that there is presence of OH

stretching in nanocurcumin. Similar findings were reported by others [10-14]. The XRD pattern verified the nanocurcumin's crystalline state, which has previously been reported by numerous studies [15,16]. FE-SEM analysis revealed that the produced nanocurcumin particles were 60±10 nm in size. DLS analysis showed the size distribution profile of nanocurcumin, and averaged hydrodynamic diameter (65 nm) of the as prepared nanoparticles. The zeta potential of the as prepared nanocurcumin was less than - 25 mV. Therefore, curcumin's chemical characteristics in its nano form stayed unchanged. It is well known that nicotine is a hazardous substance that has a number of negative consequences on people. Although curcumin has demonstrated beneficial benefits against nicotine toxicity [17-19], nanocurcumin's effects have not been previously investigated.

Autopsy samples from smokers revealed that the liver, kidney, spleen, and lungs had the highest affinity for nicotine [20]. Nicotine biotransformation, which mostly occurs in the liver, has disastrous consequences. Nicotine is easily absorbed via the lungs and processed in the liver during smoking. Nicotine damages the hepatic cell membrane, increasing the release of hepatocytic cytosomal enzymes and raising their concentration in serum [21]. Nicotine causes oxidative stress, which raises the activity of ACP and ALP in serum. Increased oxidative stress promotes tissue damage, which may be linked to the ROS generation. [22]. The ACP and ALP levels were dramatically raised in protein-deficit conditions, indicating a more severe impact of nicotine on the tissues (Table 5.1). Nanocurcumin administration significantly improved the antioxidant state of protein starved female rats ($P < 0.001$), resulting in restored ACP and ALP activity. Nicotine also ruptures the cell membranes of liver tissues, resulting in liver injury. As a result of the loss of functional integrity in liver cells, AST and ALT activities rise [21]. Curcumin has already been shown to correct liver enzyme levels to some extent in normal protein conditions [23,24]. The

condition became more severe in protein malnourished situation because of exacerbated liver damage. The current investigation found that the activities of nanocurcumin at a dose of 4 mg/kg body weight are superior to that of native curcumin (Table 5.1).

Nicotine triggers the pathophysiology of kidney disease. Nicotine-mediated albumin discharge and proteinuria are exacerbated by nicotine and contribute to renal dysfunction [25]. Nicotine-induced oxidative stress impairs the kidney's ability to filter. Nicotine causes damage to the glomerulas and raises the levels of creatinine and urea in the serum [26]. The levels of urea and creatinine were considerably higher in the serum of the nicotine-exposed protein starved group than in the control group (Table 5.2), perhaps due to increasing kidney failure, as described by Addo et al. [24]. It is evident that progressive kidney failure is clearly associated with a slow fall in nicotine excretion, both renal and non-renal, which may enhance the incidence of nephrotoxicity [24]. Heavy metals included in tobacco, such as cadmium, mercury, and lead, may also contribute to kidney injury [27]. Several studies have shown that curcumin functions as an anti-inflammatory agent, lowering the risk of kidney injury [28-30]. In my research the mean difference of urea and creatinine are significantly elevated ($P < 0.001$) in nicotine exposed group in compared to controls under protein malnourished condition. According to my research, nanocurcumin supplementation is a superior restorative phenomenon than curcumin for the repair of kidney injury.

Cholesterol is an essential biomolecule that helps to maintain membrane structure and synthesize steroid hormones and bile acids. According to Balakrishnan and Menon (2007) [22], nicotine increases the production of catecholamines, which in turn promotes the lipolysis of adipose tissues and raises serum cholesterol and triglyceride levels. According to my current study, increase in triglyceride (75.9%), VLDL (74.4%), LDL (75.9%), and cholesterol (29.3%) and decrease in HDL (24.6%) levels in the serum of rats treated with nicotine that nicotine had a

significant negative impact on the lipid profile in protein malnourished condition (Table 5.3). Similar observations were also reported by various researchers [22,31-33]. Curcumin reduces total cholesterol levels in the blood via increasing CYP7A1 gene expression (a rate-limiting enzyme in the production of bile acid from cholesterol) [34]. Curcumin may reduce the triglyceride level by interacting with multiple targets like peroxisome proliferator-activated receptor PPAR-γ, alpha (PPAR-α), lipoprotein lipase, and cholesteryl ester transfer protein (CETP) [35]. Curcumin has an effect on lipoprotein metabolism [36]. In my research it was shown that curcumin raises blood levels of HDL-C and decreases levels of triglycerides, cholesterol, LDL-C, and VLDL-C in serum under protein malnourished condition. These findings are consistent with previous research [23,37]. The decreased cholesterol levels in the curcumin-treated group might be attributed to increased expression of the CYP7A1 gene [38]. The significant amelioration of lipid peroxidation by nanocurcumin was observed in this study. Because of nanocurcumin superior pharmacokinetic qualities, it showed better hypocholessterolaemia effect than curcumin in protein malnourished condition. It might be attributed to an increase in total cholesterol absorption [39] or breakdown and rapid removal of total cholesterol [40].

Several in vitro and in vivo investigations demonstrated that nicotine can cause oxidative stress and disrupt the peroxidant/antioxidant balance in blood cells, plasma, serum, and tissues [31,37]. Nicotine disrupts the mitochondrial respiratory chain, increasing the formation of different free radical ions (e.g., hydrogen peroxide ions, superoxide ions etc.) and enhancing lipid peroxidation on human circulating lymphocytes. [41]. It was also reported that nicotine could reduce the level of antioxidant enzymes (SOD, Catalase, GSH, GPX [41-43]. Oxidative stress generates free radicals, which target lipid membranes and cause the generation of malonialdehyde (MDA), resulting in peroxidative tissue damage [44,45]. Previous research found that curcumin

reduced MDA levels by inhibiting the generation of free radicals [46,47]. Nanocurcumin's substantial ameliorative impact demonstrated its ability to scavenge free radicals while maintaining cell membrane interiority and functionality by suppressing membrane lipid peroxidation. The higher protein content in diverse tissues shows that nanocurcumin can better protect tissues from nicotine-induced injury. Nicotine reduces muscle mass by decreasing protein synthesis, promoting proteolysis and also reduces the protein content in kidney, liver and ovarian tissues owing to its harmful action. Nicotine impacts the expression of genes involved in muscle protein degradation. Nicotine also causes organ failure in the liver, kidney, and ovary [48]. The current data demonstrated that nicotine reduces 20 to 25% tissue protein either by lowering protein production or by increasing the rate of protein catabolism (Table 5.7). Reduction of muscle protein causes structural and functional abnormalities in the cells. Curcumin modulates the action of a variety of targets, including apoptotic proteins, cell cycle regulators, growth factors, receptors, protein kinases, and transcription factors [49], which safeguard our bodies. Curcumin and nanocurcumin effectively inhibited nicotine induced stress in several tissues in protein restricted condition.

Previous research found that nicotine dramatically lowered the content of haemoglobin in blood under protein restricted diet conditions. Thomas and Lumb (2012), showed that carbon monoxide from tobacco smoking impacted the binding of oxygen to haemoglobin [50]. Carbon monoxide has a higher binding affinity (>300 times) for hemoglobin than oxygen. Banerjee et al. (2010) found a nicotine induced drop in RBC count, which might be attributed to peroxidative damage to the RBC membrane, resulting in a decrease in total haemoglobin content of the blood [51]. In the current investigation, the Hb% level was considerably lower in the nicotine-treated

group. Table 5.6 clearly reveals that nanocurcumin has a better effect on nicotine-induced Hb content than unformulated curcumin.

In protein restricted condition, 300 µL of blood contains 110.67µg of DNA. Banerjee et al. (2010) reported comparable results [51]. The DNA content reduced considerably in the nicotine-treated group. The drop in total DNA content might be attributed to nicotine-induced oxidative damage. The decreased level of total DNA content of the blood cells was more effectively ($p < 0.001$) corrected by nanocurcumin. This was proven by a similar quantity of total DNA in the nanocurcumin supplemented nicotine-induced group compared to the control group of mice. Compared to native curcumin, the ameliorative impact of nanocurcumin was observed to be more pronounced (Table 5.8). It was shown that the percentage of DNA damaged in protein restricted control group is 13.36%. However, compared to the control group, the average DNA damage in the nicotine-treated group was considerably larger (48%; $p<0.001$). Due to various physiological stress amount of DNA damage (<10%) always occurs in normal condition. In my study it was observed that protein restricted causes additional stress which may cause a slightly higher percentage of DNA damage. The DNA damage might be caused by nicotine induced chromosomal aberration, sister chromatid exchange, or single-strand DNA breakage [52]. Banerjee et al. (2010) found that nicotine-induced oxidative stress enhanced DNA damage in the blood and liver tissues of female albino rats. They reported that curcumin efficiently interacted with both nicotine and DNA, reducing nicotine induced oxidative stress and thereby DNA damage. The current study shown that nanocurcumin reduces the percentage of DNA damage more efficiently than curcumin against nicotine induced toxicity.

IL-6 is vital to the cytokine network. During the first stage of inflammation, it is released from the local lesion and transported to the liver via the bloodstream. It promotes the release of

serum amyloid A (SAA), α1-antichymotrypsin, haptoglobin, fibrinogen, CRP, and fibrinogen. Long term preservation of serum amyloid A (SAA) at greater concentrations causes various chronic inflammatory disorders [53]. A high dose of IL-6 decreases serum iron and zinc levels [54]. According to Wang et al. (2012), increased IL-6 expression is linked to higher tiredness ratings [55]. TNF-α is a pro-inflammatory cytokine. Abnormal TNF-α signaling can lead to autoimmune and inflammatory disorders [56]. TNF-α is also found to be associated with fatigue condition [57]. The primary cytokine that causes allergic inflammation is IL-4 [58]. Another pro-inflammatory cytokine that contributes to a number of inflammatory conditions, including exacerbate chronic lung disease, is interferon gamma (IFNγ) [59]. According to earlier research, patients with schizophrenia and bipolar disorder, particularly those who experienced childhood trauma, have greater levels of pro-inflammatory cytokines (IL-6, TNF-α) [60].

In my current investigation, it was shown that nicotine treated group had higher levels of pro-inflammatory cytokines under protein restricted condition. A similar finding was made earlier by Maiti et al., 2015, where nicotine toxicity increased pro-inflammatory cytokine release and caused an overabundance of reactive oxygen species [61]. In a variety of cell types and pathological situations, curcumin, a natural medicinal drug, was shown in many studies to exhibit anti-inflammatory properties by downregulating numerous pro-inflammatory cytokines and chemokines [62,63]. In my research, I found that nanocurcumin was more effective than curcumin at lowering the levels of pro-inflammatory cytokines.

BCL-2 is important because it influences cellular survival and inhibits the function of pro-apoptotic proteins. Any damage to the BCL-2 gene has been linked to several types of malignancies, as well as schizophrenia and autoimmune illnesses [64]. Hardwick et al. (2013) discovered that the defective BCL-2 gene inhibited the effectiveness of anticancer medicines [65].

They also discovered that a hetero-dimmer of the apoptosis regulator protein BAX with BCL-2 acts as an apoptotic activator. In the current study, nicotine stressed rats exhibited a drop in anti-apoptotic protein BCL-2 and an increase in pro-apoptotic protein BAX. Thus, nanocurcumin had a greater immunomodulatory impact against apoptosis than normal curcumin under protein restricted dietary condition.

The study found that nicotine significantly reduced 17 β-estradiol and progesterone levels in female rats on a protein restricted dietary condition. Nicotine may have an anti-estrogenic impact, leading to a decrease in 17 β-estradiol levels in nicotine treated group under protein restricted dietary condition. Nicotine reduces progesterone levels by upregulating PGF2α and VEGF-mRNA expression. Curcumin's strong affinity for estradiol allows it to prevent nicotine's effect on estrogen metabolism. Nanocurcumin was shown to be more effective in boosting 17β-estradiol and progesterone levels among nicotine induced rats, perhaps due to its increased bioavailability and binding affinity with nicotine.

The histology of liver tissue was investigated after eosin haematoxylin staining using a compound microscope (40X). The control group animals' liver tissue showed a regular arrangement of hepatic cells, normal morphology of hepatocytes, and a central vein. Under protein restricted dietary conditions, nicotine-exposed rats had altered hepatic cell arrangements and dilated central venous and sinusoidal spaces (Figure 5.5B). Curcumin administration helps restore normal hepatic cell shape and central vein diameter (Figure 5.5C). Nanocurcumin demonstrated more efficacy than native curcumin in restoring the natural arrangement of hepatocytes and the central vein (Figure 5.5D).

Histological examination of the kidney tissues revealed the usual configuration of the glomerulus, bowman space, and proximal/distal tubules of kidney tissue of control rats under protein restricted condition. Nicotine administration considerably altered this pattern, as shown in Figure 5.6B. Curcumin treatment partially restored the natural organization of the glomerulus, Bowman space, and proximal/distal tubules in kidney tissue (Figure 5.6C). Nanocurcumin supplementation efficiently restored the natural organization of the glomerulus, Bowman space, and proximal/distal tubules in kidney tissue (Figure 5.6D). In the ovary of female rats on a protein-deficient diet, nicotine therapy caused the regression of graafian follicles with damaged cellular structures generating vacuoles (Figure 5.7B). This was most likely owing to the combined effect of protein malnutrition stress and nicotine toxicity on ovarian hormone forming in such conditions, as documented by Sinha et al. 2012 [66]. Both curcumin (Figure 5.7C) and nanocurcumin (Figure 5.5D) restored graafian follicular structures, although nanocurcumin had a greater effect on restoring normal ovarian structure than curcumin.

cytotoxicity is an essential part of the immune system, a highly complex and controlled process that is executed by various cytotoxic cells of our immune system. The cytotoxic granzyme B/perforin pathway produces cytokines like IFN-γ and TNF-α by which cytotoxic CD8+T cells, NK, and NK-like T cells generate immune responses against cancer. IFN-γ suppresses angiogenesis and cellular proliferation which encourages apoptosis of the cancer cells. The reduction of IFN-γ is likely the reason for the suppression of pro-tumor microenvironment and the survival of cancer cells. Furthermore, it stimulates the adaptive immune system, which helps in efficient production and processing of antigen [67]. According to some research, curcumin reduces inflammation by downregulating TNF-α, IL-1β, and IL-6 and altering antioxidant enzymes. Curcumin inhibits the paclitaxel-induced NFκB pathway in cancer cells and has anti-apoptotic

effects [68]. The current experiment's findings support curcumin's anti-inflammatory properties and concurred with earlier research showing curcumin decreased the production of pro-inflammatory cytokines. The study also verifies the importance of nanocurcumin in cancer treatment by utilizing nano-sized herbal powder to overcome the limits of curcumin's low solubility and absorption for increased anti-inflammatory properties.

In my present study it was observed that nanocurcumin treatments possessed a higher impact toward the cervical cancer cell like SiHa and lung cancer cell line like A549 cells compared to curcumin. Interestingly, following an increase of curcumin and nanocurcumin concentration in the medium, the growth of normal cell line HEK293 was not affected significantly ($P<0.05$), but at doses greater than 35µM concentration, the growth inhibition of HEK was also significantly affected ($P<0.05$). Moreover, at the 50% inhibitory concentrations of nanocurcumin towards the cancer cell lines, no significant effect was observed in the normal HEK293 cells. Effective morphological changes in cancer cells (SiHa and A549) including cell shrinkage and star-shape with membrane blabbing which represent a typical apoptosis. When cells are treated with inhibitory concentration of curcumin and nanocurcumin cells become round in shape (Figure 5.9A).

Assessing apoptosis is essential to distinguishing it from necrosis. One of the hallmarks of the apoptotic process is nuclear fragmentation which leads to cell death. Using fluorescent microscope, morphological changes in cell nuclei are identified. Identifying the condensed nuclei of apoptotic cells using blue fluorescent Hoechst 33342 cell permeable nucleic acid dye, chromatin condensation and disintegration can be determined. Fluorescence microscopy is used to assess the nuclear morphology of cells after DAPI staining. The distinctive nuclear alterations that distinguish apoptosis from necrosis. DAPI is a nuclear stain that exhibits blue fluorescence when

stimulated under a fluorescence microscope. DAPI staining revealed the changes associated with apoptosis in SiHa and A549 cells when both were treated with curcumin and nanocurcumin. The treatment group showed fragmented apoptotic bodies, shrunken and emarginated nuclei, as opposed to the control and large nucleus in the untreated cells, demonstrating the apoptotic potential of curcumin and nanocurcumin. Both cancer cells exhibit morphological alterations consistent with apoptosis, such as chromatin condensation, nuclear fragmentation, and nucleus emargination, upon treatment (Figure 5.9B).

ROS generation usually associated with cell apoptosis. Fluorescent microscopy images revealed that the proportion of cells with higher fluorescence intensity is increased in cells treated with nanocurcumin for 48h (Figure. 5.10A and 5.10B), indicating that nanocurcumin significantly increased the level of ROS in SiHa and A549 cancer cells. Curcumin could only induce ROS generation at very higher doses as compared to nanocurcumin. It was clearly observed that the elevation of intracellular ROS is perhaps the attributing factor for nanocurcumin dependent cell death which might be due to initiation of apoptosis in SiHa and A549 cells. Apoptotic induction can also be facilitated by ROS intermediates [69]. It has been observed that ROS may have a role in downregulating BCL-2 [70] in mitochondria, while releasing the cytochrome C into the cytoplasm, enabling Fas-associated proteins leads to caspase 3 activation and apoptosis [71]. Increased levels of ROS in the treatment group indicate the apoptotic mode of cell death induced by nanocurcumin through the generation of ROS. These observations clearly suggest that that nanocurcumin has better efficacy to increase the intracellular ROS supporting apoptosis of both SiHa (cervical cancer) and A549 (lung cancer) cells. Also, the nanocurcumin doses are found to be harmless and causes no-side effect to normal human body cells. On the other hand, curcumin

needs higher doses to cause apoptosis of the same cancer cells and more time to initiate therapeutic activities.

DNA laddering is one of the defining indicators that helps differentiate apoptosis from necrosis [72]. In the treatment group, the existence of the laddering (smear) pattern of DNA fragments and the lack of the necrotic stripe further support apoptosis, as shown in Figure 5.11. DNA fragmentation assay, which is an indicative of apoptosis, is used to define the mechanism of cell death. DNA is extracted from cell lines after the administration of various doses of curcumin and nanocurcumin and subjected to agarose gel electrophoresis for evaluation. DNA laddering indicating nanocurcumin ability to cause apoptosis in both SiHa and A549 cells (L2 and L4 respectively). Laddering is also observed in treatment of curcumin which was comparatively lesser (L1 and L3 respectively) on these two cancer cell lines.

Formulation of nano particulate curcumin may bring about reduction in dose and improvement its efficacy than native curcumin against nicotine induced toxicities and higher efficacy of nano sized curcumin in inhibiting the proliferation of both human SiHa (cervical cancer) and A549 (lung cancer) cells in comparison to normal sized curcumin. Natural curcumin exhibits slow and low therapeutic activity. Thus, the study re-establishes the scope of nanotechnology in developing alternate nature based herbal drugs in the treatment of cancer.

REFERENCES

1. Lopresti AL. The Problem of Curcumin and Its Bioavailability: Could Its Gastrointestinal Influence Contribute to Its Overall Health-Enhancing Effects? Adv Nutr. 2018; 9(1):41-50.

2. Sreekala S, Indira M. Effects of exogenous selenium on nicotine-induced oxidative stress in rats. Biol Trace Element Res. 2009; 130: 62-71.

3. Danafar H. Study of the Composition of Polycaprolactone/Poly (Ethylene Glycol)/Polycaprolactone Copolymer and Drug-to-Polymer ratio on drug loading efficiency of curcumin to nanoparticles. Jundishapur J Nat Pharm Prod. 2017; 12: 34179.

4. Nosrati H, Sefidi N, Sharafi A, Danafar H, Manjili HK. Bovine Serum Albumin (BSA) coated iron oxide magnetic nanoparticles as biocompatible carriers for curcumin-anticancer drug. Bioorg Chem. 2018; 76: 501-509.

5. Shaikh J, Ankola DD, Beniwal V, Singh D, Kumar MN. Nanoparticle encapsulation improves oral bioavailability of curcumin by at least 9- fold when compared to curcumin administered with piperine as absorption enhancer. Eur J Pharm Sci. 2009;37(3-4):223-30.2019;10:97

6. Yadav A, Lomash V, Samim M, Flora SJ. Curcumin encapsulated in chitosan nanoparticles: a novel strategy for the treatment of arsenic toxicity. Chem Biol Interact. 2012 Jul 30; 199(1):49-61.

7. Arunraj T, Rejinold NS, Mangalathillam S, Saroj S, Biswas R, Jayakumar R. Synthesis, characterization and biological activities of curcumin nanospheres. J. Biomed. Nanotechnol. 2014;10: 238-250

8. Alam S, Panda J, Chauhan V. Novel dipeptide nanoparticles for effective curcumin delivery. Int. J. Nanomed. 2012; 7:4207-4222

9. Ghosh M, Singh AT, Xu W, Sulchek T, Gordon LI, Ryan RO. Curcumin nanodisks: formulation and characterization. Nanomedicine. 2011; 7(2):162-7.

10. Singh PK, Wani K, Kaul-Ghanekar R, Prabhune A, Ogale S. From micron to nano-curcumin by sophorolipid co-processing: Highly enhanced bioavailability, fluorescence, and anti-cancer efficacy. RSC Adv. 2014; 4: 60334–60341.

11. Sayyar Z, Malmiri HJ. Preparation, Characterization and Evaluation of Curcumin Nanodispersions Using Three Different Methods–Novel Subcritical Water Conditions, Spontaneous Emulsification and Solvent Displacement. Z. Phys. Chem. 2019; 233: 1485–1502.

12. Raghavendra GM. Jayaramudu T, Varaprasad K, Ramesh S, Raju KM. Microbial resistant nanocurcumin-gelatin-cellulose fibers for advanced medical applications. RSC Adv. 2014; 4: 3494–3501.

13. Pandit RS, Gaikwad SC, Agarkar GA, Gade AK, Rai M. Curcumin nanoparticles: Physico-chemical fabrication and its in vitro efficacy against human pathogens. 3 Biotech. 2015; 5: 991–997.

14. Bhawana BR, Buttar HS, Jain V, Jain N. Curcumin nanoparticles: Preparation, characterization, and antimicrobial study. J. Agric. Food Chem. 2011; 59: 2056–206.

15. Sav A, Khetrapal N, Amin P. Preparation of curcumin solid dispersions using hydrophilic polymers by different techniques and in vitro characterization. Asian J. Pharm. Sci. 2012; 7(4): 271-279.
16. Abd El-Rahman SN, Al-Jameel SS. Protection of Curcumin and Curcumin Nanoparticles against Cisplatin Induced Nephrotoxicity in Male Rats. Sch Acad J Biosci. 2014; 2(3): 214-223.
17. Bandyopadhyaya G, Sinha S, Chattopadhyay BD, Chakraborty A. Protective role of curcumin against nicotine-induced genotoxicity on rat liver under restricted dietary protein. Eur J Pharmacol. 2008; 588(2-3):151-7.

18. Banerjee S, Chattopadhyay K, Chhabra JK, Chattopadhyay B. Protein dependent fate of hepatic cells under nicotine induced stress and curcumin ameliorated condition. Eur J Pharmacol. 2012;684(1-3):132- 45

19. Chattopadhyay BD, Tamang A, Kundu D. Protective role of raw-turmeric rhizomes against nicotine-induced complications of Beedi (Indian cigarette) workers. Pharmaceut. Anal. Act. 2015; 6:386.

20. Benowitz NL. Pharmacology of nicotine: addiction, smoking-induced disease, and therapeutics. Annu Rev Pharmacol Toxicol. 2009; 49:57-71.

21. Salahshoor M, Mohamadian S, Kakabaraei S, Roshankhah S, Jalili C. Curcumin improves liver damage in male mice exposed to nicotine. J Tradit Comp Med. 2015; 6: 176-183.

22. Balakrishnan A, Menon VP. Antioxidant properties of hesperidin in nicotine-induced lung toxicity. Fund Clin Pharmacol. 2007; 21: 535-544.

23. Chattopadhyay K, Samanta A, Mukhopadhyay S, Chattopadhyay BD. Potential amelioration of nicotine-induced toxicity by nanocurcumin. Drug Dev Res. 2018; 79: 119-128.

24. Addo MA, Gbadago JK, Affum HA, Adom, T, Ahmed K, Okley GM. Mineral profi le of ghanaian dried tobacco leaves and local snuff: A comparative study. J Rad Nucl Chem. 2008; 277: 517-524.

25. Salahshoor MR, Roshankhah S, Motavalian V, Jalili C. Effect of Harmine on Nicotine-Induced Kidney Dysfunction in Male Mice. Int J Prev Med. 2019; 10:97.

26. Chaturvedi A, Natarajan A, Sharma V, Yaparthy N, Shetty JK, Virupaksha D, et al. Association of age related severity in oxidative stress and blood urea nitrogen levels in patients with dementia: A coastal Karnataka study. Asian J Biomed Pharm Sci. 2015; 5:06.

27. Usunobun U, Adegbeg J, Ademuyiwa O, Okugbo TF, Evuen U, Osibemhe M, et al. N-Nitrosodimethylamine (NDMA), liver function enzymes, renal function parameters and oxidative stress parameters: a Review. British J Pharm Toxicol. 2012; 3: 165-176.

28. Ghosh SS, Gehr TW, Ghosh S. Curcumin and chronic kidney disease (CKD): major mode of action through stimulating endogenous intestinal alkaline phosphatase. Molecules. 2014; 19(12):20139-56.

29. Ali BH, Al-Salam S, Al Suleimani Y, Al Kalbani J, Al Bahlani S, Ashique M, Manoj P, Al Dhahli B, Al Abri N, Naser HT, Yasin J, Nemmar A, Al Za'abi M, Hartmann C, Schupp N. Curcumin Ameliorates Kidney Function and Oxidative Stress in Experimental Chronic Kidney Disease. Basic Clin Pharmacol Toxicol. 2018; 122(1):65-73.

30. Ghelani H, Razmovski-Naumovski V, Chang D, Nammi S. Chronic treatment of curcumin improves hepatic lipid metabolism and alleviates the renal damage in adenine-induced chronic kidney disease in Sprague-Dawley rats. BMC Nephrol. 2019;20(1):431

31. Chattopadhyay K, Chattopadhyay BD. Effect of nicotine on lipid profi le, per oxidation and antioxidant enzymes in female rats with restricted dietary protein. Ind. J. Med. Res. 2008; 127: 571-576.

32. Rao CS, Subash YE. The effect of chronic tobacco smoking and chewing on the lipid profile. J Clin Diagn Res. 2013; 7(1):31-4.

33. Singh DP, Gulati D, Singh P. Smoking and its association with serum lipid levels. Int J Med Res Rev. 2016; 4(11):2064-2070.

34. Miró O, Alonso JR, Jarreta D, Casademont J, Urbano-Má rquez A, Cardellach F. Smoking disturbs mitochondrial respiratory chain function and enhances lipid peroxidation on human circulating lymphocytes. Carcinogenesis. 1999; 20: 1331-1336.

35. Kang Q, Chen A. Curcumin suppresses expression of low-density lipoprotein (LDL) receptor, leading to the inhibition of LDL-induced activation of hepatic stellate cells. Br J Pharmacol. 2009; 157:1354-1367.

36. Asai A, Miyazawa T. Dietary curcuminoids prevent high-fat diet-induced lipid accumulation in rat liver and epididymal adipose tissue. J Nutr. 2001; 131(11):2932-5.

37. Chattopadhyay K, Mondal S, Chattopadhyay BD. Ameliorative effect of sesame lignans on nicotine toxicity in rats. Food Chem Toxicol. 2010; 48: 3215-3220.

38. Kim M, Kim Y. Hypocholesterolemic effects of curcumin via up-regulation of cholesterol 7a-hydroxylase in rats fed a high fat diet. Nutr Res Pract. 2010; 4(3):191-5.

39. Hasimun P, Sukandar EY, Adnyana IK, Tjahjono DH. Synergistic effect of curcuminoid and s-methyl cysteine in regulation of cholesterol homeostasis. Int J Pharmacol. 2011; 7:268-272.

40. Arafa HM. Curcumin attenuates diet-induced hypercholesterolemia in rats. Med Sci Monit. 2005; 11(7):BR228-234.

41. Aspera-Werz RH, Ehnert S, Heid D, Zhu S, Chen T, Braun B, Sreekumar V, Arnscheidt C, Nussler AK. Nicotine and Cotinine Inhibit Catalase and Glutathione Reductase Activity Contributing to the Impaired Osteogenesis of SCP-1 Cells Exposed to Cigarette Smoke. Oxid Med Cell Longev. 2018; 2018:3172480.

42. Agarwal P, Bagewadi A, Keluskar V, Vinuth DP. Superoxide dismutase, glutathione peroxidase, and catalase antioxidant enzymes in chronic tobacco smokers and chewers: A case-control study. Indian J Dent Res. 2019; 30(2):219-225.

43. Raddam QN, Moafaq MZ, Mostafa AA, Nahla KA. Smoking Effects on Blood Antioxidants Level: Lactate Dehydrogenase, Catalase, Superoxide Dismutase and Glutathione Peroxidase in University Students. J Clin Exp Pathol. 2017; 7: 331.

44. Mohammadghasemi F, Khanaki K, Moravati H, Faghani M. The amelioration of nicotine-induced reproductive impairment in male mouse by Sambucusebulus L. fruit extract. Anat Cell Biol. 2021; 54(2):232-240.

45. Khademi F, Totonchi H, Mohammadi N, Zare R, Zal F. Nicotine-Induced Oxidative Stress in Human Primary Endometrial Cells. The Neuroscientist. 2019; 38(3):432-439.

46. Alizadeh M, Kheirouri S. Curcumin reduces malondialdehyde and improves antioxidants in humans with diseased conditions: a comprehensive meta-analysis of randomized controlled trials. Biomed (Taipei). 2019; 9(4):23.

47. Lin X, Bai D, Wei Z, Zhang Y, Huang Y, Deng H, Huang X. Curcumin attenuates oxidative stress in RAW264.7 cells by increasing the activity of antioxidant enzymes and activating the Nrf2-Keap1 pathway. PLoS One. 2019; 14(5): 0216711.

48. Petersen AM, Magkos F, Atherton P, Selby A, Smith K, Rennie MJ, Pedersen BK, Mittendorfer B. Smoking impairs muscle protein synthesis and increases the expression of myostatin and MAFbx in muscle. Am J Physiol Endocrinol Metab. 2007; 293(3):E843-8.

49. Zhou H, Beevers CS, Huang S. The targets of curcumin. Curr Drug Targets. 2011;12(3):332-47

50. Thomas C, Lumb A. Physiology of haemoglobin, Continuing Education in Anaesthesia and Critical Care Pain. 2012; 12(5):251-256.

51. Banerjee S, Bandyopadhyaya G, Chattopadhyhay K, Chattopadhyhay BD. Amelioration of nicotine-induced damage of blood cells in protein malnourished female rats by curcumin. Int J Pharmacol. 2010; 6(4):609- 620.

52. Sanner T, Grimsru TK. Nicotine: Carcinogenicity and Effects on Response to Cancer Treatment – A Review. Front Oncol 2015; 5:196.

53. Castell JV, Gómez-Lechón MJ, David M, Andus T, Geiger T, Trullenque R, Fabra R, Heinrich PC. Interleukin-6 is the major regulator of acute phase protein synthesis in adult human hepatocytes. Febs Lett. 1989; 242(2):237-9.

54. Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. Cold Spring Harb Perspect Biol. 2014; 6(10):016295.

55. Wang XS, Williams LA, Krishnan S, Liao Z, Liu P, Mao L, Shi Q, Mobley GM, Woodruff JF, Cleeland CS. Serum sTNF-R1, IL-6, and the development of fatigue in patients with gastrointestinal cancer undergoing chemoradiation therapy. Brain Behav Immun. 2012; 26(5):699-705.

56. Jang DI, Lee AH, Shin HY, Song HR, Park JH, Kang TB, Lee SR, Yang SH. The Role of Tumor Necrosis Factor Alpha (TNF-α) in Autoimmune Disease and Current TNF-α Inhibitors in Therapeutics. Int J Mol Sci. 2021; 22(5):2719.

57. Tian T, Wang M, Ma D. TNF-α, a good or bad factor in hematological diseases? Stem Cell Investig. 2014; 1:12.

58. Steinke JW, Borish L. Th2 cytokines and asthma. Interleukin-4: its role in the pathogenesis of asthma, and targeting it for asthma treatment with interleukin-4 receptor antagonists. Respir Res. 2001; 2(2):66-70.

59. Zhu Y, Song D, Song Y, Wang X. Interferon gamma induces inflammatory responses through the interaction of CEACAM1 and PI3K in airway epithelial cells. J Transl Med. 2019; 17(1):147.

60. Quidé Y, Bortolasci CC, Spolding B, Kidnapillai S, Watkeys OJ, Cohen-Woods S, Berk M, Carr VJ, Walder K, Green MJ. Association between childhood trauma exposure and pro-inflammatory cytokines in schizophrenia and bipolar-I disorder. Psychol Med. 2019; 49(16):2736-2744.

61. Maiti M, Chattopadhyay K, Verma M, Chattopadhyay B. Curcumin protects against nicotine-induced stress during protein malnutrition in female rat through immunomodulation with cellular amelioration. Mol Biol Rep. 2015; 42(12):1623-37.

62. Yadav R, Jee B, Awasthi SK. Curcumin Suppresses the Production of Proinflammatory Cytokine Interleukin-18 in Lipopolysaccharide Stimulated Murine Macrophage-Like Cells. Indian J Clin Biochem. 2015; 30(1):109- 12.

63. Gorabi AM, Razi B, Aslani S, Abbasifard M, Imani D, Sathyapalan T, Sahebkar A. Effect of curcumin on proinflammatory cytokines: A meta-analysis of randomized controlled trials. Cytokine. 2021; 143:155541.

64. Jalili C, Salahshoor MR, Moradi MT, Ahookhash M, Taghadosi M, Sohrabi M. Expression Changes of Apoptotic Genes in Tissues from Mice Exposed to Nicotine. Asian Pac J Cancer Prev. 2017; 18(1):239-244.

65. Hardwick JM, Soane L. Multiple functions of BCL-2 family proteins. Cold Spring Harb Perspect Biol. 2013; 5(2): 008722.

66. Sinha S, Maiti M, Chattopadhyay K, Chattopadhyay BD. Potential amelioration of curcumin against nicotine-induced toxicity of protein malnourished female rats. J Pharmacol Toxicol. 2012; 7: 166-180.

67. Hodge G, Barnawi J, Jurisevic C, Moffat D, Holmes M, Reynolds PN, Jersmann H, Hodge S. Lung cancer is associated with decreased expression of perforin, granzyme B and interferon (IFN)-γ by infiltrating lung tissue T cells, natural killer (NK) T-like and NK cells. Clin. Exp. Immunol. 2014; 178(1):79–85.

68. Singh AK, Vinayak M. Curcumin attenuates CFA induced thermal hyperalgesia by modulation of antioxidant enzymes and down regulation of TNF-α, IL-1β and IL-6. Neurochem. Res. 2015; 40(3):463–472.

69. Meshkini A, Yazdanparast R. Involvement of oxidative stress in taxol-induced apoptosis in chronic myelogenous leukemia K562 cells. Exp Toxicol Pathol.2012; 64(4):357-65.

70. Li D, Ueta E, Kimura T, Yamamoto T, Osaki T. Reactive oxygen species (ROS) control the expression of Bcl-2 family proteins by regulating their phosphorylation and ubiquitination. Cancer Sci. 2004; 95(8): 644–650.

71. Chen KC, Kao PH, Lin SR, Chang LS. Upregulation of Fas and FasL in Taiwan cobra phospholipase A2-treated human neuroblastoma SK-N-SH cells through ROS- and Ca2+-mediated p38 MAPK activation. J. Cell. Biochem.2009; 106(1): 93–102.

72. Pajaniradje S. Mohankumar K, Subramanian RS, Rajagopalan R. Antiproliferative and apoptotic effects of Sesbania grandiflora leaves in human cancer cells.Bio. Med. Res. Int. 2014; 2014:474953.

CHAPTER VII

SUMMARY

The ultrasonication process was used to synthesis nanocurcumin, which was then physically characterized. The plasmon resonance (SPR) band pattern, as measured by a UV-Vis spectrophotometer, verifies the distinctive absorbance peak of 350 nm, which is identical to that of nanocurcumin. The morphological analysis of nanocurcumin using Field Emission Scanning Electron Microscopy revealed an average particle size of 60±10 nm, falling within the range of nanoparticles (below 100nm). DLS analysis showed the size distribution profile of nanocurcumin, and averaged hydrodynamic diameter (65 nm) of the as prepared nanoparticles. The zeta potential of the synthesis nanocurcumin was less than -25 mV. Fourier transform infrared spectroscopy (FTIR) demonstrated that the functional groups of lab-synthesized nanocurcumin were similar to curcumin. The crystalline nature of the nanocurcumin powder was evaluated using an X-ray diffractometer. Reddish yellow aqueous solution demonstrated its water solubility.

A variety of animal investigations demonstrated its protective benefits against nicotine toxicity at the biochemical, cellular, and molecular levels.

As demonstrated by changed hepatic enzymes, lipid profiles, SODs, free radicals, apoptotic proteins, LFT, RFT, levels of various cytokines, and disastrous alterations in molecular levels, nicotine-mediated toxicities were clearly established in the animals in the control group.

Nanocurcumin outperformed native curcumin at substantially lower doses in terms of ameliorating nicotine-mediated toxicity. Nanocurcumin was shown to be more efficient than curcumin in preventing adverse histopathological alterations at the cellular level in important organs such as the liver and kidney caused by nicotine use.

Nanocurcumin's superior protective performance over native curcumin may be attributed to its higher effective surface area, water solubility, rapid intestinal absorption, cellular dispersion, and long half-life.

There were no adverse side effects documented in the nanocurcumin treated group, showing that it is safe to use.

The anti-carcinogenic and antiproliferative properties of curcumin and nanocurcumin on two different cancer cell was examined. Anticancer efficacy of nanocurcumin and curcumin in terms of cell viability (MTT assay), apoptosis (DAPI staining) and proliferation (morphological studies) to human cervical cancer cells (SiHa) and lung cancer cells (A549).

The MTT assay establishes the viability and cytotoxic effects of curcumin and nanocurcumin on cancerous cell lines in vitro.

The inhibitory mechanism of nanocurcumin on the proliferation of SiHa and A549 cancer cells through ROS induced apoptosis. Nanocurcumin is more potent than curcumin in inhibiting the proliferation of cancer cell through apoptosis and reveals the importance of curcumin size in mounting potent anticancer activity.

We demonstrate the use of safe herbal drugs (curcumin and nanocurcumin) in effective treatment of cancer using in vitro experiments. Nano sized curcumin displays better anticancer property at a relatively smaller dose in comparison to standard curcumin powder. Therefore, we hypothesize that nano-sized curcumin may be considered for treating cervical and lung cancer.

To conduct a human experiment on nanocurcumin's effectiveness against nicotine toxicity in smokers, adequate guidelines, clearance from the Drug Control Authority of India, and approval from an authorized Human Ethics committee are required.

PUBLICATIONS

RESEARCH ARTICLE

Efficacy of nano-curcumin on nicotine-induced genotoxicity and immunomodulatory disruption in protein-malnourished female rats

Somashree Biswas, Krishna Chattopadhyay, Brajadulal Chattopadhyay *

ABSTRACT

Background: Aggravated nicotine-induced DNA damage and immunomodulatory disruption are more prominent in female populations under proteinrestricted conditions. Females seem to be more susceptible to nicotine-induced complications due to their low innate immunity. Though the anti-inflammatory and anti-genotoxic properties of curcumin are able to overcome effectively nicotine-induced complications, still its therapeutic use is hindered due to its poor aqueous solubility and low bioavailability. Nano-curcumin appears to be a better therapeutic agent because of its increased aqueous solubility and higher bioavailability than curcumin. *Methods:* This study investigated the effects of nicotine (2.5 mg/kg body weight injected subcutaneously for three consecutive weeks) on genotoxicity and immunomodulatory disruption in female rats maintained under a protein-restricted diet (5% casein). It also measured the ameliorative efficacy of nanocurcumin (4 mg/kg body weight supplemented orally after one hour of nicotine exposure) against nicotine. *Results:* It is observed that nicotine decreases hemoglobin and DNA contents and causes severe DNA damage in blood cells under protein-restricted conditions. It disrupts the immune system and affects the endocrine functions of the female rats maintained under protein- a restricted diet. Nano-curcumin ameliorates the nicotine-mediated genotoxic effects significantly, maintains the female sex hormonal level effectively, and restores the normalcy of immune responses in protein-restricted diet rats more efficiently than curcumin. Conclusion: Nano-curcumin looks like a potential blocker of nicotine due to its enhanced bioavailability and acts as a prospective therapeutic herbal agent to protect the health of the protein-malnourished female population.

Keywords: Genotoxicity; Nano-curcumin; Nicotine; Protein malnutrition.

Indian Journal of Physiology and Allied Sciences (2024); DOI: 10.55184/ijpas.v76i01.211 **ISSN:** 0367-8350 (Print)

Introduction

The tobacco epidemic is one of the biggest public health threats the world has ever faced. It kills more than 8 million people a year, including around 1.2 million deaths from exposure to second-hand smoke[1] N′-Nitrosonornicotine, a tobacco-specific nitrosamine, is one of the strong carcinogens and is accused of the addictive potential of smoking. It is mediated by neuronal nicotinic acetylcholine receptors in the central nervous system.[2] Nicotine promotes endothelial cell migration, proliferation, and nitric oxide (NO) production *in-vitro*, mimicking the effect of other angiogenic growth factors.[3, 4] It also stimulates growth factors, fibroblast proliferation, collagen release, and expression of myofibroblast markers.[5] Cigarette smoke causes airway epithelial cell damage[6] and global epigenetic changes, including DNA methylation and chromatin remodeling, which negatively impact genes involved in physiologic lung repair and regeneration.[7, 8] Smoking yields chemicals with carcinogenic potential, and the metabolism of nicotine produces reactive intermediates capable of binding to proteins and DNA, which increases the risk of hepatocellular carcinoma.[9] The genotoxic effect of nicotine and its reactive metabolites induce chemical modifications of DNA (DNA adducts) and play a key role in chemically induced carcinogenesis. DNA adducts can lead to mutations during cell division and may ultimately disrupt the regular functioning of the systems regulating normal cell growth.

Department of Physics, Jadavpur University, Kolkata, India

***Corresponding author:** Brajadulal Chattopadhyay, Department of Physics, Jadavpur University, Kolkata, India, Email: bdc_physics@yahoo.co.in

How to cite this article: Biswas S, Chattopadhyay K, Chattopadhyay B. Efficacy of nano-curcumin on nicotine-induced genotoxicity and immunomodulatory disruption in protein-malnourished female rats. *Indian J Physiol Allied Sci* 2024;76(1):25-31.

Conflict of interest: None

Submitted: 01/01/2024 **Accepted:** 23/02/2024 **Published:** 31/03/2024

Recently, nicotine has been shown to increase the frequency of micronuclei in human gingival fibroblasts,[10] and DNA strand breaks in human spermatozoa.[11]

At the cellular level, the induction of autoimmunity is a manifestation of an imbalance between pathogenic effectors versus protective regulatory responses.[12] Nicotine-induced production of pro-inflammatory cytokines (IL-1β; IFN-γ from Th1 cells and IL-4 and IL-5 from Th2 cells) trigger the signaling pathway, which plays an essential role in the cellular survival, death, and various pathological states.[13-16] IFN-γ has antitumor, antiviral, and immunomodulatory activities. It coordinates both innate and adaptive immune responses.[17] IL-4, a cytokine produced by activated Th2 lymphocytes, mast cells, and basophiles, is involved in many immunologic processes, such as Th2 differentiation, class switching, and B cell proliferation.[18] Cigarette smoke exerts

Cytotoxic and both pro-inflammatory and anti-inflammatory effects on nasal epithelial cells, leading to increased reactive oxygen species (ROS) production, Toll-like receptor (TLR) 4 expression, and lipopolysaccharide (LPS).[19,20] Changes in the redox status within the cell initiate the activation of redox-sensitive transcription factors, such as nuclear factor-κB (NF-κβ) and activator protein-1 (AP-1). NF-κβ plays a significant role in gene expression of pro-inflammatory mediators (IL-6 and TNF-α), linked to cigarette smoke exposure and altered cytokine production.[21,22] The imbalance between oxidants and antioxidants resulting from exposure to tobacco smoke leads to oxidative stress and increases mucosal inflammation and inflammatory cytokines (IL-6 and TNF-α) expression. The primary role of TNF-αlies in the regulation of immune cells. It is an 11-enigmatic cytokine controlling signaling pathways towards cell proliferation and death. Complex-I and complex-II are the two complexes formed by TNF-α binding with TNF-R.[23] Complex-II induces the pro-apoptotic protein BAX that releases Cytochrome-C and reactive oxygen species (ROS) from the mitochondria. Cytochrome-C causes apoptosis, where ROS induces necrosis of the cell.[24] BCL-2 is localized to the outer membrane of mitochondria, where it plays an important role in promoting cellular survival and inhibiting the actions of pro-apoptotic proteins.[25]

Nicotine also alters the endocrine function, perhaps at the level of the ovary, which in turn affects the release of female hormones. Smoking is an established modifiable risk factor for a number of serious complications in pregnancy and maternal-fetal health. These complications include preterm delivery, intrauterine growth restriction, placental abruption, and prenatal mortality, *etc*. The endocrine disruption likely contributes to the reported associations of smoking with adverse reproductive outcomes, including menstrual dysfunction, infertility, and earlier menopause.[26]

Protein Energy Malnutrition (PEM) continues to be a major public health problem in developing countries and affects mostly all age groups like infants, young children, pregnant and lactating mothers and poorer segments of the population. Low dietary protein imposes a constraint on biosynthetic activity, disposition, and toxicity.[27] Though it is already proven that in normal dietary conditions, nicotine causes various damage to our body, the toxic effects of nicotine, particularly in protein-restricted dietary situations, are still a cause for concern. The therapeutic use of curcumin, a natural yellow polyphenolic pigment isolated from the rhizomes of the plant *Curcuma longa L*. (turmeric), in diet can inhibit the antigen-mediated activation of mast cells, IgE production, and airway inflammation. It shows a wide spectrum of biological and pharmacological effects, such as anti-inflammatory, antioxidant, antimicrobial, anti-hepatotoxic, hypolipidemic, and anticancer properties. Curcumin also has immunomodulatory and anti-allergic activities. Poor aqueous solubility, low bioavailability, poor absorption, and rapid excretion from our body are the basic drawbacks of curcumin against its use in clinical practice. Curcumin, in the form of nanoparticles, is more active than curcumin because of its more aqueous solubility and increased bioavailability. Nano-particles of curcumin are therefore proposed as nano-drugs for oral supplementation to increase their bio-availability against nicotine-induced toxicities under protein-restricted conditions.

Materials And Methods

Nicotine hydrogen tartrate and curcumin were purchased from Sigma Aldrich Chemicals Company, St. Louis, USA. The preparation and characterization of nano-curcumin was done in our laboratory, which had already been discussed elaborately elsewhere.[28] Spectrochem Pvt. Ltd. India and Merck India supplied all others analytical grade chemicals. PUREGENE-made ELISA Kits were supplied by Genetix Biotech Asia Pvt. Ltd. and were used for the detection of cytokines, apoptotic proteins, and steroidogenic hormones.

Protein Restricted Diet and Animals

Female albino rats of Wistar strain (*Rattus norvegicus)* having 130-150 gm body weight were procured from the Animal housing facility of Jadavpur University and maintained one week by feeding with the standard pellet diet (Hindustan Liver Ltd., India) and water ad libitum. The animals were fed a protein-restricted diet during the nicotine exposure period, which was three consecutive weeks. The protein-restricted diet contains carbohydrates (83%), protein(5%), fat (7%), salt mixture (4%), and vitamin mixture (1%).[29] The rats were maintained with a natural lighting schedule, *i.e.*, 12 hours light and 12 hours dark cycles, by following strictly the guidelines of the Institutional Animal Ethics committee of the Jadavpur University, Kolkata, India (Ref. No.: AEC/PHARM/1502/14/2015, Dated: 30/07/2015). There were 30 rats were taken and divided equally into five groups of six rats each.

Group –C: Animals in this group received no nicotine exposures and served as a control group.

Group–NT: Animals in this group were exposed to nicotine. Nicotine tartrate solution was subcutaneously injected at an effective dose of 2.5 mg nicotine/ kg body weight.

Group–NTCS: Animals in this group received an effective dose of nicotine followed by a supplemented effective dose of curcumin (80 mg/kg body weight), which was given orally after one hour of nicotine injection.

Group –NTNCS: Animals in this group received an effective dose of nicotine followed by a supplemented effective dose of nano-curcumin (4 mg/kg body weight), which was given orally after one hour of nicotine injection.

Group –NCS: Animals in this group received an effective dose of nano-curcumin (4 mg/kg body weight) only, which was given orally after one hour of nicotine injection.

After the completion of exposure for three consecutive weeks, all animals were kept fasting overnight and sacrificed the next day by decapitation.

Sample Collection

After decapitation, blood samples were collected from the heart immediately, divided into two parts, and stored with or without

an anticoagulant (Heparin). The serum was separated from the blood, kept in anticoagulant containers by centrifugation, and stored at - 20 °C for further analysis. The liver, kidney, and ovary were dissected out and stored in vacuum desiccators at -20°C to prevent auto-oxidation for future studies.

Hemoglobin Estimation

Percentages of hemoglobin estimation were done using the method described elsewhere.[30]

DNA Content Estimation

DNA contents from blood and tissues (liver, kidney, and ovary) were determined by following the protocol as described by Bandyopadhyay *et al.*[31] After the extraction of DNA, it was dissolved in TE buffer (pH 8) and its concentration and purity were measured by using a spectrophotometer at the range of 230, 260 and 280 nm.

Comet Assay

The whole procedure of comet assay was followed by a slightly modified technique as described by Bandyopadhyay *et al.*[31] The photomicrograph of each slide was taken in Leica Fluorescent Microscope at the 40x magnification, Model 300 FX. Measurements of the total comet tail length and mean DNA density, *etc.* were done by using the Perceptive Comet Assay IV software version 4.3. The parentage of DNA damage and tail moment were done accordingly. A total of 50 cells were screened per animal, and the data were averaged. Quantification of DNA damage for each call was as follows:

Total DNA in comet = (Total comet area) × (mean DNA intensity)

Total DNA in comet head= (Total head area) × (mean DNA intensity)

% DNA damage = (Total DNA in comet) - (Total DNA in comet head) ×100

Tail moment = (Tail length) × (Mean DNA intensity in tail)

Cytokines and Apoptotic Molecules by ELISA

Various cytokines such as IL-6, IL4, TNF-α, and IFN-γ and apoptosis regulatory proteins (BCL-2 and BAX), which were induced by nicotine and nano-curcumin, were measured from the serum of protein-restricted rats. This process was conducted with the help of Quantikine Immunoassay Kits (Genetix Biotech Asia Pvt.Ltd.) as per the manufacturer's protocols. The quantitative sandwich enzyme immunoassay technique did this assay. The intensity of the color measure was proportional to the amount of rats IL-4 and IL-6 and bound in the initial step. After the completion of this assay, the plates were read for optical density at 450 nm and then compared with the standard curve.

Female Hormones by Radioimmunoassay

Un-conjugated forms of female hormones, viz., estradiol and progesterone hormones, were estimated with the help of an ELISA kit supplied by Genetix Biotech Asia Pvt. Ltd. as per the manufacturer's protocol. The concentration of the hormones in the serum of control and experimental rats was determined by interpolating the measured OD values at 450 nm from the standard curves.

Statistical Analysis

Each experiment was repeated twice, and data were averaged (N = 12) and tabulated as a mean ± standard deviation (SD). The statistical analysis of the data obtained from control, nicotine, nicotine+nano-curcumin, and only nano-curcumin supplemented animals was performed by one-way analysis of variance (ANOVA). The significant levels of the observed data were determined at $p < 0.05$.

RESULTS

Nicotine exposure caused a significant ($p < 0.001$) decrement in total hemoglobin content (25%) in the RBC of the animals under protein-restricted diet conditions. It was observed that both curcumin and nano-curcumin increased the hemoglobin concentration in RBC effectively (Table 1). Nicotine hampered DNA synthesis in the tissues of the rats' blood, liver, kidney, and ovary under a protein-restricted diet, as seen in Table 2. DNA contents in those tissues were reduced to more than 20%, which was significant ($p < 0.001$). The ameliorative action of nano-curcumin against nicotine in DNA synthesis was found to be significant ($p < 0.001$) than that of curcumin (Table 2).

Comet assay (Figure 1 and Table 3) showed that nicotine caused aggravated DNA damage (almost 50%) to the blood cells of female rats in protein-restriction conditions. Both curcumin and nano-curcumin resisted the deleterious action of nicotine against DNA damage. Table 3 shows that nano-curcumin possesses better ameliorative efficacy ($p < 0.001$) than curcumin in the case of repairing DNA damage.

The concentration levels of all the observed cytokine molecules (IL-4, IL-6, TNF-α, and IFN-γ) were increased due to the action of nicotine in female rats maintained under protein-restriction conditions (Figure 2). Results suggested that IL-4 increased 1.4 folds in nicotine-exposed and protein-restricted animals compared to control protein-restricted

Table 1: Hemoglobin levels of animals in different groups

	Groups				
Parameter	*(C)*	*(NT)*	*(NTCS)*	*(NTNCS)*	*(NCS)*
Haemoglobin (g/dL^{-1}) (n=12)	12.3 ± 0.34	9.16 ± 0.29** (25.52↓)	11.66 ± 0.18$^{*\#\#}$ (5.48↓)	12.3 ± 0.18$^{*\#\#}$ (0.0)	13.4 ± 0.5 (8.94↑)

Data are Mean ± SD. * and# indicate significant differences with C and NT groups, respectively.

Values in parenthesis indicate the percentage of increase (↑) or decrease (↓) relative to the control.

Table 2: Total DNA content in different tissues of animals in different groups

	Groups				
Parameter	*(C)*	*(NT)*	*(NTCS)*	*(NTNCS)*	*(NCS)*
Blood (µg/300 µL)	110.67 ± 5.31	77.67 ± 6.75 (29.81 ↓) *	87.36 ± 9.39 (21.06 ↓) *#	104.82 ± 4.08 (5.28 ↓) *#	113.94 ± 3.86 (2.95 ↑)
Liver (µg/mg)	17.0 ± 2.13	13.29 ± 1.60 (21.82 ↓) *	14.89 ± 0.51 (12.41 ↓) *#	16.16 ± 3.91 (4.94 ↓) *#	17.06 ± 1.41 (0.35 ↑)
Kidney (µg/mg)	17.66 ± 0.42	13.49 ± 1.54 (23.61 ↓) *	15.63 ± 0.23 (11.49 ↓) *#	16.41 ± 2.36 (7.07 ↓) *#	17.74 ± 0.75 (0.45 ↑)
Ovary (µg/mg)	19.72 ± 0.67	13.84 ± 0.36 (29.81 ↓) *	15.29 ± 0.24 (22.46 ↓) *#	18.44 ± 0.05 (6.49 ↓) *#	19.39 ± 0.04 (1.67 ↑)

Data are Mean ± SD. * and # indicate significant differences with C and NT groups, respectively.

Values in parenthesis indicate the percentage of increase (↑) or decrease (↓) relative to the control.

Table 3: DNA damage percentage and tail moment in whole blood of different groups

	Groups				
Parameter	*(C)*	*(NT)*	*(NTCS)*	*(NTNCS)*	*(NCS)*
% DNA damaged	13.36 ± 0.5	48.00 ± 4.2*	26.89 ± 2.17*	16.06 ± 0.5*#	13.12 ± 0.14
Tail moment arbitrary unit	80.93 ± 2.50	636.95 ± 5.04*	210.8 ± 6.42*	164.27 ± 8.44*#	19.44 ± 8.35

Data are Mean ± SD. * and # indicate significant differences with C and NT groups, respectively.

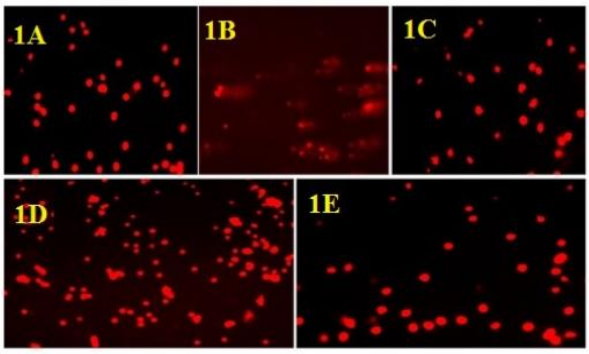


Figure 1: Photomicrographs of the comet assay of blood cell DNA of animals under protein-restricted conditions. (1A) represents the comet picture of blood cell DNA in protein-restricted diet-fed rats. (1B) represents the comet picture of blood cell DNA in the nicotine-exposed group. (1C) represents the comet picture of blood cell DNA in the nicotine + curcumin-exposed group. (1D) represents the comet picture of blood cell DNA in the nicotine+nano-curcumin-exposed group. (1E) represents the comet picture of blood cell DNA in the nano-curcumin-supplemented group.

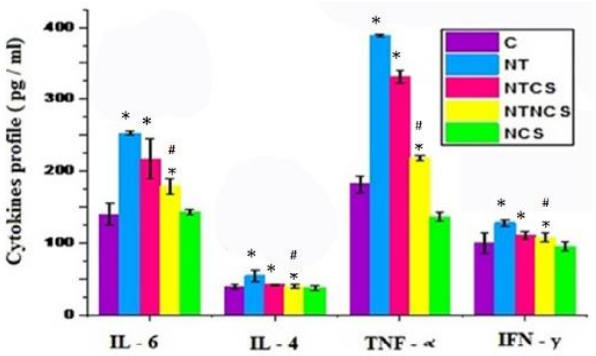


Figure 2: Cytokine profile determined by ELISA. Columns are Mean ± SD. * and # indicate significant differences with C and NT groups, respectively

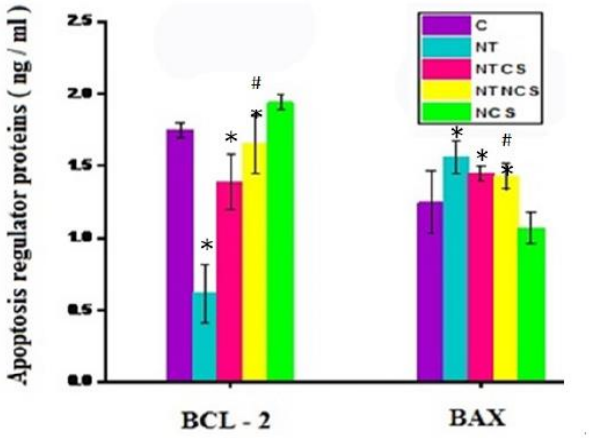


Figure 3: Apoptosis regulator protein determination by ELISA Columns are Mean ± SD* and # indicate significant differences between the C and NT groups, respectively

animals. Similarly, IL-6, TNF-α, and IFN-γ were increased by 1.8, 2.1, and 1.3 folds, respectively, in nicotine-exposed and protein-restricted animals than in control protein-restricted animals. Curcumin supplementation reduced the concentration levels of all these cytokines, and the concentration levels of IL-4, IL-6, TNF-α, and IFN-γ became 1.1, 1.6, 1.8, and 1.1 folds, respectively, in nicotine-exposed and protein-restricted animals. Supplementation of nano-curcumin to nicotine-exposed and protein-restricted animals reduced those cytokine levels very significantly ($p < 0.001$), due to which the concentration levels of IL-4, IL-6, TNF-α, and IFN-γ b became 1.0, 1.3, 1.2 and 1.1 folds, respectively.

Table 4: Levels of steroidogenic hormone released in serum of rats under protein restricted dietary condition determined by ELISA

Steroidogenic hormone	*control*	*Nicotine*	*Nicotine + curcumin*	*Nicotine + Nanocurcumin*	*Nanocurcumin*
Estradiol (pg/mL)	79.62 ± 5.25	63.69 ± 13.57	71.11 ± 10.1	78.88 ± 5.09	84.06 ± 5.01
Progesterone (ng/mL)	11.59 ± 0.23	8.95 ± 0.04	9.49 ± 0.121	10.7 ± 0.19	13.28 ± 0.20

The concentration of anti-apoptotic molecule (BCL-2) was drastically reduced (2.8-fold) by nicotine exposure in female rats under protein-restricted conditions (Figure 3). The concentration level of BCL-2 became 1.2 folds ($p < 0.01$) by curcumin and 1.0 folds ($p < 0.001$) by nano-curcumin supplementation in the nicotine-exposed and protein-restricted diet-fed female rats (Figure 3).

Nicotine also caused a decrement of female sex hormones (estradiol by 1.3 folds and progesterone by 1.3 folds) in the female rats maintained under a protein-restricted diet compared to their respective controls (Table 4). Nano-curcumin almost nullified the action of nicotine and restored the normal concentration levels of estradiol and progesterone more significantly ($p < 0.001$) than curcumin in the nicotine-exposed and protein-restricted diet-fed female rats.

Discussion

Different forms of tobacco consumption are widely spread over socio-economically handicapped populations in developing countries, particularly in India, and therefore, this study is worthy of investigation. The study shows that nicotine causes a significant reduction ($p < 0.001$) of total hemoglobin concentration in the blood cells of female rats when maintained under protein-restricted diet conditions. This is in agreement with our previous study,[30] where it is shown that nicotine induces a low RBC count and affects the binding efficacy of hemoglobin with oxygen. Hemoglobin concentration is therefore becoming low in nicotine exposure. Numerous studies have demonstrated that low hemoglobin (Hb) is a strong prognostic indicator of poor disease control. The observed increased concentration of hemoglobin by curcumin or nano-curcumin is due to their protective efficacy against nicotine, as explained by Banerjee *et al.*[30] The more bio-available nano-curcumin shows enhanced ameliorative efficacy than curcumin against the nicotine-induced effect.

Nicotine causes suppression of cellular proliferation and increases DNA breakdown, resulting in increased cell death (Mohammed *et al.*, 2004). The present finding supports the earlier studies[31] as nicotine causes a very significant ($p < 0.001$) reduction of DNA content in various tissues (blood, liver, kidney, and ovary) of the female rats under protein-restricted conditions (Table 2). Chattopadhyay and Chattopadhyay[32] have shown that nicotine enhances lipid peroxidation in female rats under a protein-restricted diet, which results in increased oxidative DNA damage and, therefore, the total DNA content in various tissues becomes less compared to their respective controls in such conditions (Table 2). Though curcumin and nano-curcumin both are able to minimize the toxic effect of nicotine against the DNA content of the tissues, nano-curcumin shows better efficacy against nicotine due to its more bioavailability, resulting in less decrement of total DNA content in various tissues of nicotine-exposed animals. It is reported that nano-curcumin possesses higher binding efficacy with DNA than nicotine.[33] Therefore, the nano-curcumin vs. DNA complex is more stable than the nicotine vs. DNA complex. Nano-curcumin thus protects DNA from nicotine, resulting in increased DNA concentration in various tissues.

The comet assay results, as shown in Figure 1, clearly indicate that nicotine can break DNA strands, which is why the comet-like appearance is seen in the picture. The comet assay is a well-established genotoxicity test for *in-vitro* testing of chemicals against biological macromolecules.[34] The retrenched head diameter, extended tail length, decreased head diameter *vs.* tail length ratio, and amplified percentage of DNA damage are seen to be significantly higher in nicotine-exposed protein-restricted animals. In the comet assay, it is observed that the percentage of DNA damage in the protein-restricted control group is 13.36.%. Some amount of DNA damage (< 10%) always occurs in normal conditions due to various physiological stresses. Protein restriction causes additional stress, due to which a slightly higher percentage of DNA damage is observed in our study. Figure 1 for comet assay clearly indicates that nicotine plays a significant role in genotoxicity to the blood cells of female rats in protein-restricted conditions. This may be due to the increased production of free radicals and reactive oxygen species (ROS) that attack all types of macromolecules, including DNA, as reported in an earlier study.[35] Curcumin significantly reduces DNA damage due to its anti-mutagenic, tumor-inhibiting, and anti-oxidant properties. Nano-curcumin supplementation ($p < 0.001$) significantly prevents nicotine-induced DNA damage in the protein-restricted group. It suggests that more bio-available nano-curcumin has better efficacy in combating nicotine and restoring DNA damage than in nicotine-stressed conditions.

ELISA test was performed to determine the concentration of cytokines (IL-6, IL-4, TNF-α, IFN-γ) in serums of rats exposed to nicotine, curcumin, and nano-curcumin under protein-restricted dietary conditions. The results of this study demonstrated that cytokine levels were influenced by nicotine. At the physiological level, IL-6 plays an important role in the cytokine network. However, excessive production of IL-6 in response to exposure to lipopolysaccharide has an inflammatory effect, resulting in injury.[36] IL-4 in extravascular tissues promotes alternative activation of macrophages into M2 cells and inhibits the classical activation of macrophages into M1 cells. It stimulates the activation of B-cells and the proliferation of T-cells, and it differentiates B-cells into

plasma cells.[37] TNF- α, along with IL-1beta, up-regulates the expression of VCAM-1 on hepatic sinusoidal endothelium *in-vivo*, which promotes cancer cell adhesion and liver metastasis. IFN-γ possesses immune regulatory property that alters the transcription of~30 genes producing a variety of physiological and cellular responses.[38] Activated T cells release macrophage-activating factor IFN-γ which activates macrophages to secrete many inflammatory proteins such as IFN-γ inducible protein (IP-10), IFN inducible T cell α – chemoattachment (I-TAC), and monokine induced by IFN-γ to orchestrate the inflammatory process. ELISA results for the cytokines of serum demonstrate that the cytokines are elevated rapidly after nicotine exposure in normal protein dietary conditions, and curcumin prevents that elevation, whereas nano-curcumin supplementation helps more effectively than curcumin to regain the normal level of cytokines as released under normal dietary conditions.

Apoptosis plays an important role in regulating a variety of diseases. In the case of schizophrenia, a neurodegenerative disease, an abnormal ratio of pro- and anti-apoptotic factors is observed.[39] BCL-2 is localized to the outer membrane of mitochondria, where it plays an important role in promoting cellular survival and inhibiting the actions of pro-apoptotic proteins. The pro-apoptotic proteins in the BCL-2 family, including BAX and BCL-2, normally act on the mitochondrial membrane to promote permeabilization and release of cytochrome C and ROS, which are important signals in the apoptosis cascade. These pro-apoptotic proteins are, in turn, activated by BH3-only proteins and are inhibited by the function of BCL-2 and its relative BCL-XI.[40] ELISA test shows that nicotine exposure decreases the concentration level of anti-apoptotic BCL-2 molecules and increases the concentration of pro-apoptotic BAX molecules. The more bio-available nano-curcumin supplementation regains the normal concentration levels of both apoptotic molecules more significantly than that of curcumin released under protein-restricted dietary conditions.

Cigarette smoking has major effects on the reproductive potential of humans. Being pro-androgenic, smoking is also anti-estrogenic, which means it has a negative effect on estrogen levels in women.[41] Nicotine significantly reduces 17 β-estradiol and progesterone levels in female rats maintained on a protein-restricted diet. Nicotine has an anti-estrogenic effect of which affects the hepatic estrogen metabolism,[42] thereby reducing 17 β-estradiol levels in the serum of those female rats. It also induces a decrease in progesterone levels by increasing PGF-2α, an important factor for autolysis, and also by increasing VEGF mRNA expression. Both curcumin and nano-curcumin combat the anti-estrogenic effect of nicotine, and increased 17β-estradiol level and progesterone levels are noted when supplemented in rats maintained under a protein-restricted diet. This increment occurs because of curcumin, which inhibits microvascular endothelial cell angiogenesis through the inhibition of COX-2 expression.[43] Nano-curcumin shows a more significant ameliorative effect than curcumin, increasing the 17 β-estradiol and progesterone levels near normal levels in the serum of rats maintained under protein-restricted conditions.

Acknowledgement

The work was done with the support of the Biophysics Laboratory, Department of Physics, Jadavpur University, and Molecular and Cell Biology Laboratory, Department of Zoology, University of Kalyani. The UGC (NTA) NET JRF Fellowship support from UGC, Government of India (Ref. No. 210510536878, Dated: 12-03-2022) to Somashree Biswas is gratefully acknowledged.

References

1. Global Burden of Disease [database]. Washington, DC: Institute of Health Metrics; 2019. IHME, accessed 17 July 2021.
2. Bailador FD, Wonnacott. Nicotinic acetylcholine receptors and the regulation of neuronal signaling. *Trends Pharmacol Sci*. 2004;.25(6):317-24. DOI: 10.1016/j.tips.2004.04.006.
3. Cardinale A, Nastrucci C, Cesario A, *et al*. Nicotine: specific role in angiogenesis, proliferation and apoptosis. *Crit Rev Toxicol*. 2012;42:68–89. DOI: 10.3109/10408444.2011.623150.
4. Lee J, Cooke JP. Nicotine and pathological angiogenesis. *Life Sci*. 2012;91:1058–64. DOI: 10.1016/j.lfs.2012.06.032.
5. Ebrahimpour A, Shrestha S, Bonnen MD, et al. Nicotine modulates growth factors and microRNA to promote inflammatory and fibrotic processes. *J Pharmacol Exp Ther*. 2019;368(2):169–78. DOI: 10.1124/jpet.118.252650.
6. Solleti SK, Simon DM, Srisuma S, *et al*. Airway epithelial cell PPARγ modulates cigarette smoke-induced chemokine expression and emphysema susceptibility in mice. *Am J Physiol Lung Cell Mol Physiol*. 2015;309:L293–304. DOI: 10.1152/ajplung.00287.2014.
7. Bellaye PS, Kolb M. Why do patients get idiopathic pulmonary fibrosis? Current concepts in the pathogenesis of pulmonary fibrosis. *BMC Med*. 2015;13:176. DOI: 10.1186/s12916-015-0412-6.
8. Yang IV, Schwartz DA. Epigenetics of idiopathic pulmonary fibrosis. *Transl Res*. 2015;165:48–60. DOI:10.1016/j.trsl.2014.03.011.
9. Benowitz NL, Hukkanen J, Jacob P. Nicotine chemistry, metabolism, kinetics and biomarkers. *Handb Exp Pharmacol*. 2009;(192):29-60. DOI: 10.1007/978-3-540-69248-5_2.
10. Sanner T, Grimsrud TK. Nicotine: Carcinogenicity and Effects on Response to Cancer Exposure – A Review. *Front Oncol*. 2015;5:196. DOI: 10.3389/fonc.2015.00196.
11. Arabi M. Nicotinic infertility: assessing DNA and plasma membrane integrity of human spermatozoa. *Andrologia*. 2004;36:305-10. DOI: 10.1111/j.1439-0272.2004.00623.x.
12. Rosenblum MD, Remedios KA, Abbas AK. Mechanisms of human autoimmunity. *J Clin Invest*. 2015;125(6):2228-33. DOI: 10.1172/JCI78088.
13. Gour N, Karp MW. IL-4 and IL-13 Signaling in allergic airway disease. *Cytokine*. 2015;75(1):68-78. DOI: 10.1016/j.cyto.2015.05.014.
14. Rebe C, GhiringelliF. Interleukin-1β and Cancer. *Cancers (Basel)*. 2020;12(7):1791. DOI: 10.3390/cancers12071791.
15. Pelaia C, Paoletti G, Puggioni F, *et al*. Interleukin-5 in the Pathophysiology of Severe Asthma. *Front Physiol*. 2019;10:1514. DOI: 10.3389/fphys.2019.01514.
16. Tirado-Rodriguez B, Ortega E, Segura-Medina P, Huerta-Yepez S.

TGF-β: an important mediator of allergic disease and a molecule with dual activity in cancer development. *J Immunol Res*. 2014; 2014:318481. DOI: 10.1155/2014/318481.
17. Mendoza JL, Escalante NK, Jude KM *et al*. Structure of the IFNγ receptor complex guides design of biased agonists. *Nature*. 2019;567:56-60. DOI: 10.1038/s41586-019-0988-7.
18. Moghbeli M, Khedmatgozar H, Yadegari M. *et al*. Cytokines and the immune response in obesity-related disorders. *Adv Clin Chem*. 2021;101:135-68. DOI:10.1016/bs.acc.2020.06.004.
19. Comer DM, Elborn JS, Ennis M. Inflammatory and cytotoxic effects of acrolein, nicotine, acetaldehyde and cigarette smoke extract on human nasal epithelial cells. *BMC Pulm Med*. 2014;14:32. DOI: 10.1186/1471-2466-14-32.
20. Pace E, Ferraro M, Di Vincenzo S, *et al*. Oxidative stress and innate immunity responses in cigarette smoke stimulated nasal epithelial cells. *Toxicology*. 2014;28:292-9. DOI: 10.1016/j.tiv.2013.11.004.
21. Macnee W, Tuder RM. New paradigms in the pathogenesis of chronic obstructive pulmonary disease I. *Proc Am Thorac Soc*. 2009;6:527-31. DOI: 10.1513/pats.200905-025DS.
22. Rahman I. Oxidative stress, chromatin remodeling and gene transcription in inflammation and chronic lung diseases. *J Biochem Mol Biol*. 2003; 36:95-109. DOI: 10.5483/bmbrep.2003.36.1.095.
23. Wullaert A, Loo GV, Heyninick K, Beyaert R. Hepatic tumor necrosis factor signaling and nuclear factor-kappaB: effects on liver homeostasis and beyond. *Endocr Rev*. 2007; 28(4):365-86. DOI: 10.1210/er.2006-0031.
24. Peng WC, Logan CY, Fish M, *et al*. Inflammatory cytokine TNFα promotes the long-term expansion of primary hepatocytes in 3D culture. *Cell*. 2018;175(6):1607-19.e15. DOI: 10.1016/j.cell.2018.11.012.
25. Qian S, Wei Z, Yang W, *et al*. The role of BCL-2 family proteins in regulating apoptosis and cancer therapy. *Front Oncol*. 2022; 12:985363. DOI: 10.3389/fonc.2022.985363.
26. Ethier AR, Mckinney Ty, Tottenham LS, *et al*. The effect of reproductive hormones on women's daily smoking across the menstrual cycle. *Biol Sex Differ*. 2021;12(1):41. DOI: 10.1186/s13293-021-00384-1.
27. Okwu GN, Ukoha AL. Studies on the predisposing factors of protein energy malnutrition among pregnant women in a Nigerian community. *Online Journal of Health and Allied Sciences*. 2007;6(3):1-9. Available at https://web-archive.southampton.ac.uk/cogprints.org/5927/1/2007-3-1.pdf
28. Chattopadhyay K, Samanta A, Mukhopadhyay S, *et al*. Potential amelioration of nicotine-induced toxicity by nanocurcumin. *Drug Dev Res*. 2018; 79:119-28. DOI: 10.1002/ddr.21424.
29. Hawk PB, Oser BL, Summerson WH. Practical physiological chemistry. Mc Grew Hill Book Co.1954. New York, London.
30. Banerjee S, Bandyopadhyay G, Chattopadhyay K, Chattopadhyay BD. Amelioration of nicotine-induced damage of blood cells in protein-malnourished female rats by curcumin. *Int J Pharmacol*. 2010;6(4):444-55. DOI: 10.3923/ijp.2010.444.455.
31. Bandyopadhyay G, Sinha S, Chattopadhyay BD, Chakraborty A. Protective role of curcumin against nicotine-induced genotoxicity on rat liver under restricted dietary protein. *Eur J Phrmacol*. 2008;588:151-7. DOI: 10.1016/j.ejphar.2008.04.023
32. Chattopadhyay K, Chattopadhyay B. Effect of nicotine on lipid profile, peroxidation and antioxidant enzymes in female rats with restricted dietary protein. *Ind J Med Res*. 2008;127:571-6. PMID: 18765876.
33. Samanta A, Chowdhury T, Chattopadhyay B, Chattopadhyay K. Studies the therapeutic efficacy of nanocurcumin against nicotine-induced damage of blood cells. *Int J Pharmaceu Res Innovat*. 2021;14:12-26. Available at https://www.whitesscience.com/wp-content/uploads/woocommerce_uploads/2021/02/IJIPRI_14_12-24_2021-hodcbn.pdf.
34. Premkumar K, Kavitha S, Santhiya ST. Ramesh AR, Suwanteerangkul J. Interactive effects of saffron with garlic and curcumin against cyclophosphamide-induced genotoxicity in mice. *Asia Pac J Clin Nutr*. 2004;13(3):292-4. PMID: 15331343.
35. Ray S, Chattopadhyay N, Mitra A, Siddiqi M, Chatterjee A. Curcumin exhibits antimetastatic properties by modulating integrin receptors, collagenase activity, and expression of Nm23 and E-cadherin. *J Environ Pathol Toxicol Oncol*. 2003;22(1):49-58. PMID: 12678405.
36. Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. *Cold Spring Harb Perspect Biol*. 2014;6(10):a016295. DOI: 10.1101/cshperspect.a016295.
37. Luzina IG, Keegan AD, Heller NM, Rook GAW, Shea-Donohue T, Atamas SP. Regulation of inflammation by interleukin-4: a review of "alternatives". *J Leukoc Biol*. 2012;92(4):753–64. DOI: 10.1189/jlb.0412214.
38. Castro F, Cardoso AP, Gonçalves RM, Serre K, Oliveira MJ. Interferon-gamma at the crossroads of tumor immune surveillance or evasion. *Front Immunol*. 2018;9:847. DOI: 10.3389/fimmu.2018.00847.
39. Glantz LA, Gilmore JH, Lieberman JA. Jarskog LF. Apoptotic mechanisms and the synaptic pathology of schizophrenia. *Schizophrenia Res*. 2006;81:47-63. DOI: 10.1016/j.schres.2005.08.014.
40. Hardwick JM. Soane L. Multiple Functions of BCL-2 Family Proteins. *Cold Spring Harb Perspect Biol*. 2013;5(2):a008722. DOI: 10.1101/cshperspect.a008722.
41. Jandikova, H, Duskova M and Starka, L. The influence of smoking and cessation on the human reproductive hormonal balance. Physiol Res. 2017;66(Suppl 3): S323-s31.
42. Adesky ND, Vaccari JPDR, Bhattacharya, P. *et al*. Nicotine alters estrogen receptor-beta-regulated inflammasome activity and exacerbates ischemic brain damage in female rats. *Int J Mol Sci*. 2018;19(5):1330. DOI: 10.3390/ijms19051330.
43. Binion DG, Otterson MF, Rafiee P. Curcumin inhibits VEGF-mediated angiogenesis in human intestinal microvascular endothelial cells through COX-2 and MAPK inhibition. *Gut*. 2008;57(11):1509-17. DOI: 10.1136/gut.2008.152496.

PEER-REVIEWED CERTIFICATION

During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers' comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.

Journal of Xi'an University of Architecture & Technology ISSN No : 1006-7930

STUDY THE COMPARATIVE EFFICACIES OF CURCUMIN AND NANOCURCUMIN ON HUMAN CANCER CELLS

Somashree Biswas[1], Krishna Chattopadhyay[1], Solanki Sarkar[2], Arunima Biswas[2], Brajadulal Chattopadhyay[1*].

1. Department of Physics, Jadavpur University, Kolkata - 700032, India
2. Molecular and Cell Biology laboratory, Department of Zoology, University of Kalyani, Kalyani - 741235, India

*** Corresponding author**	**:**	**Dr. Brajadulal Chattopadhyay**
E. Mail	:	**bdc_physics@yahoo.co.in**
Somashree Biswas	**:**	**somashreebiswas92@gmail.com**
Krishna Chattopadhyay	**:**	**kris_ami@yahoo.co.in**
Solanki Sarkar	**:**	**sarkarsolan@gmail.com**
Arunima Biswa	**s:**	**arunima10@klyuniv.ac.in**

Journal of Xi'an University of Architecture & Technology ISSN No : 1006-7930

Abstract

The potential use of curcumin against diverse cancers is limited due to its poor bioavailability though curcumin possesses significant anti-invasion, anti-proliferation, anti-inflammatory and anti-angiogenesis activities. Nanocurcumin (NCur) having better bioavailability and bio-absorption properties, shows similar activities of curcumin. This work compares the anticancer efficacy of NCur and curcumin in terms of cell viability (MTT assay), apoptosis (DAPI staining) and proliferation (morphological studies) to human cervical cancer cells (SiHa) and lung cancer cells (A549). The MTT assay establishes the viability and cytotoxic effects of curcumin and NCur on cancerous cell lines *in vitro*. The human embryonic kidney cell line (HEK293) serves as a control. The study reveals that NCur is more efficacious in killing the cervical cancer cells SiHa (IC_{50} value 10.66 ± 1.04 μM) and the lung cancer cell A549 (IC_{50} value 7.65 ±1.35 μM) compared to curcumin (IC_{50} for SiHa is 14.57 ± 1.17 and for A549 is 12.17 ± 0.44) post 48 h of treatment. However, at 50% inhibitory concentrations of NCur towards the cancer cells, no noteworthy effect is observed in the normal HEK293 cells. DNA fragmentation assay indicates pronounced ability of NCur to induce apoptosis in cancerous cells compared to curcumin. These outcomes provide insights into understanding the inhibitory mechanism of NCur on the proliferation of SiHa and A549 cancer cells through ROS induced apoptosis. This study shows that NCur is more potent inhibitor than curcumin towards cancerous cells proliferation through apoptosis and reveals the importance of curcumin size in mounting potent anticancer activity.

Keywords: Apoptosis, Carcinogen, Inflammation, Nano-curcumin, ROS.

Journal of Xi'an University of Architecture & Technology ISSN No : 1006-7930

Introduction

Cancer is the foremost reason of fatality because of high occurrence and mortality[1]. Among different type of cancers cervical cancer and lung cancer are extremely common and deadly [2,3]. Cervical cancer is recognized among women globally and is related with Human papillomavirus (HPV) [4]. Conventional treatments (surgery, chemotherapy, radiotherapy etc.) are only efficient in the early stages of cervical cancer [5] as a result, it is imperative to find new methods for the treatment of cervical cancer. HPV-16 and HPV-18 are two ''high risk'' subgroup of HPVs [4] and encode viral proteins E6 and E7 respectively, which can interfere with cellular tumour suppressor proteins p53 and retinoblastoma susceptibility gene product Rb respectively[6]. HPV infections are typically transient and do not usually involve to clinically considerable lesions of the cervical mucosa apart from persistent infection over a significant period of time [5]. Higher number of HPV infection compared with the small incidence of cervical cancer cofactors like oral contraceptive use, smoking, infection with other sexually transmitted diseases such as Herpes simplex and host factors are involved in the malignant transformation of the cervical mucosa [7].

Non-small cell lung cancer (NSCLC) occurs in 80% of all lung cancer cases and small cell lung cancer (SCLC) occurs 20% among all lung cancer cases [8]. Due to the asymptomatic nature at early stage and limitations of effective screening of the disease, most lung cancer patients are diagnosed at an advanced stage [9]. Standard treatments until today including SCLC and NSCLC cancer patients are surgery, chemotherapy or radiation therapy [10]. Although the major advances in diagnostic and therapeutic approaches five-year survival rate for all stages collectively remain just about 16% among all the lung cancer patients [1]. Poor survival rate is associated with late diagnosis, drug resistance, and toxicity [11]. As a result, further research on lung cancer

mechanisms, non-toxic therapeutics drugs, and new intervention targets would prove useful in lung cancer therapy. In this context, we hypothesize the use of Phyto-chemicals or herbal drugs in treating lung cancer.

Plant extracts and natural Phyto-chemicals are widely studied anticancer agents because of low toxicity and minimal side effects than synthetic compounds. Over 35,000 plants species are being consumed around the world as they are thought to contain several healing properties with less side effects. *Curcuma longa*, well-known as turmeric has been used extensively since very old times as a spice in traditional cuisine as well as a traditional medicine in India and China [12,13]. Curcumin ($C_{21}H_{20}O_6$, MW 368.37) the natural poly-phenol compound, derived from the rhizome, is an orange-yellow crystalline powder with a mild bitter taste [14]. It has been used as an effective chemo preventive agent inhibiting tumour progression against liver cancer[15], gastric cancer [16], lung cancer[17], breastcancer[18], prostate cancer[19], and cervical cancer [20] etc. Curcumin is known for several therapeutic actions including anti-oxidant and anti-inflammatory [21] and anti-microbial, anti-viral and anti-fungal[22]. In addition, several *in vitro* and *in vivo* studies on curcumin demonstrate its anti-carcinogenic effects on lung cancer through inhibition of cell proliferation and induction of apoptosis [23,24]. In spite of all these valuable chemo protective properties of curcumin, its use in patients is hindered owing to various major problems like poor bioavailability, poor aqueous solubility, low absorption and rapid excretion from the body [25,26]. To resolve these issues, encapsulated nano-formulations of curcumin has been adopted[27, 28]. Here, we evaluated the anticancer properties of NCur and curcumin on lung cancer cells (A549) and cervical cancer cell SiHa cells. This study clearly showed that the anticancer properties of NCur was superior to that of conventional curcumin because of better absorption, higher aqueous solubility and greater cellular uptake of NCur than that of curcumin.

Journal of Xi'an University of Architecture & Technology ISSN No : 1006-7930

Materials& Methods

Nicotine hydrogen tartrate and curcumin were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). All other analytical grade and fine chemicals were supplied either by Spectro Chem Pvt. Ltd. (India) or Merck (India).

Preparation of and characterization of NCur

The preparation and characterization of NCur was done in our laboratory by a modified technique as follows[29]. Firstly, a 0.018 M curcumin solution was prepared by dissolving curcumin in dichloromethane (DCM) solvent, which was then added to warm (50ºC) Milli-Q ultrapure water drop slowly (with a flow rate of 0.2 mL/min) for 10 min with a continuous stirring by a ultrasonic-pulse sonicator (Hielscher Ultrasonic Processor- UP100H, Germany). The mixer solution turned into reddish-yellow colour. The coloured solution was separated by centrifugation technique, sterilized by autoclaving, and lyophilized to make powder form (Eyela-FDU- 2000, Japan). The orange coloured NCur powder was used the treatment of cancer cells.

The morphology of the as prepared NCur was observed by Field Emission Scanning Electron Microscopy (FE-SEM- FEI INSPECT F50, The Netherlands). DLS analysis was used to find out the size distribution profile of NCur. Fourier transform infrared spectroscopy (FTIR) (Perkin-Elmer FTIR-1600, USA) was used to characterize the characteristic functional groups. of the as prepared NCur. The crystalline nature of the NCur powder was determined by X-ray diffraction study (Bruker AXS, Inc., Model D8, WI).

Preparation of Cell lines and Cell Culture

Journal of Xi'an University of Architecture & Technology ISSN No : 1006-7930

The National Centre for Cell Science, Pune, India provided the HPV positive human cervical cancer cell line SiHa, human lung cancer cell line A549 and non-cancerous human embryonic kidney cell line, HEK293. Before initiating the experiments, the cell lines are tested for the presence of *Mycoplasma* as it escapes ultra-filtration and visualization under routine microscopic examination. DNA extracted from PBS washed cell lines is subjected to 16S rRNA detection.

The absence of 16S rRNA molecules ruled out the presence of Mycoplasma in cell line culture. The sequences of the forward and reverse primers adopted in this study are:

U1(forward) - 5′GTTTGATCCTGGCTCAGGAYDAAC3′;

U8(reverse) - 5′ GAAAGGAGGTRWTCCAYCCSCAC3′,

qPCR tests are performed to detect 16S rRNA by using the universal U1 and U8 set of degenerate primer pairs targeting 1.5 kb fragment. RT-PCR and microscopic DNA negative cultures are considered for downstream studies only. The cells are grown in Dulbecco's Modified Eagle Medium (North American Origin, Gibco) with 10% Fetal Bovine Serum (FBS, North American Origin, Gibco), and 1% Penicillin/Streptomycin solution (Gibco). The cells are incubated at 37 °C with 5% CO_2 and sub-cultured at regular intervals. The preparation and characterization of NCur is done according to the protocols reported elsewhere [27].

MTT assay

In 96 well plates, 0.2 ml of cell suspension (containing 2×10^4 cells) is seeded and cultured till ~ 90% confluence is achieved. Then, the culture medium is discarded and cells are treated with different concentrations (10-60 µM) of curcumin and NCur for 24 and 48 h, in triplicate in fresh phenol red free culture media and FCS. The culture medium is removed and the cells are washed with PBS. Then, 0.2 mL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium

bromide] reagent (0.5 mg/mL) is added to each well and incubated for 4 h, followed by addition of 0.2 mL DMSO to dissolve the purple-coloured crystals and absorbance measured at 590 nm with an ELISA plate reader (Bio Rad Mark TM Microplate Reader). The relative cell survival rate is calculated based on the ratio of the absorbance of the sample treatment relative to the absorbance of the control treatment.

Cell morphology assay

SiHa and A549 (2×10^5) cells are seeded in a 6 well plate and cultured overnight for adhesion of cells. After removing the culture medium, cells are treated with inhibitory concentrations of curcumin and NCur for 48 h, observed under a microscope (Olympus CKX53, Japan) and images are captured with Magvision software.

DAPI staining to Determine Apoptotic Nuclei

To analyse the morphological changes in the apoptotic cell nuclei, cells are stained with DAPI and observed under microscope. 2×10^5 cells of SiHa and A549 per well are seeded in 6-well plates and cultured overnight in DMEM medium supplemented with 10% FBS. The medium is then replaced with DMEM medium supplemented with 0.2% FBS and treated with curcumin and NCur for 48 h. Thereafter, the medium is completely removed and the cells are washed with ice cold PBS twice, fixed in 100% ethanol for 20 min at room temperature, and washed twice again with cold PBS. Then the cells are incubated with 100 nm of DAPI in PBST for 5 min in room temperature and observed under a fluorescence microscope (Zeiss).

Quantification of DNA fragmentation

DNA fragmentation is one of the hallmarks of late apoptosis in majority of cell types. DNA fragmentation is qualitatively analysed by agarose gel electrophoresis by observing the DNA ladder formation. Total DNA is isolated from cells according to the manufacturer's

protocol (ROCHE Apoptotic DNA-Ladder Kit, Germany). Samples are mixed with loading dye (0.25% bromophenol blue, 30% glycerol; v/v) and dispensed in wells of a 1% agarose gel. The gel is run at 5 V/cm for about 2 hr, and the DNA is detected by ethidium bromide under UV light (GelDoc; Bio-Rad XRS+).

H_2DCFDA staining

H_2DCFDA staining is a well-known technique for determining total intracellular Reactive Oxygen Species (ROS) levels. H_2DCFDAis a cell permeable dye which remains non-fluorescent till it reacts with ROS. SiHa and A549 cells are grown in 24 well plates and treated for 24 h with curcumin and NCur at IC_{50}. After removing the medium from the plate, the cells are rinsed with PBS and treated with 1mM H_2DCFDAdye for 20 min at 37 ^{0}C in dark. Then the cells are washed twice with PBS and examined under a microscope (Zeiss) for fluorescence emitting cells to determine ROS generation.

Statistical analysis

All experiments were done in triplicate and data were averaged over (N = 3) and presented mean ± S. D.

Results

Characterization and cytotoxic effects induced by curcumin and Nano-curcumin in cancer cell lines

The morphology of the as prepared NCur is shown in Fig. 1, where the average particle size of the NCur was 60 ± 10 nm (approximately). DLS analysis (Fig. 2) showed the size distribution profile of NCur, and averaged hydrodynamic diameter (65 nm) of the as prepared nanoparticles. The zeta potential of the as prepared NCur was less than - 25 mV as seen from Fig. 2b. The as prepared NCur shows a weak peak at 717 cm^{-1} allocated for the C–H vibrations of the

aromatic ring (Fig. 3). Another peak at 857 cm^{-1} correspond to the C-H bending and 958 cm^{-1}correspondsto benzoate trans-C−H vibration. Peak at 1149 cm^{-1} is assigned to the functional group of C−H stretching. Other peak at 1277 cm^{-1}represents the C−O stretching group, peak at 1425 cm^{-1} corresponds to phenolic C−O stretching and peak at 1504 cm^{-1} correspond to C=O. The strong absorption peak that arises from 1598 cm^{-1} can be denoted for the symmetric stretching vibrations about the aromatic ring (C=C ring). Another absorption peak noticed at 1626 cm^{-1} might be due to the (C=C) stretching. The peak at 3508 expresses that there is presence of OH stretching in NCur. For analyzing the crystalline nature and purity of NCur using XRD (fig.4). Very sharp intense and characteristic peaks of Ncur were observed at 2θ diffraction angle; 14.55°, 17.30°, 18.24°, 23.41°, 24.55°, 25.63°, 27.33°, and 31.63°.

The effect of different doses of curcumin and NCur on cervical cancer cell line SiHa and lung cancer cell line A549, as well as on normal epithelial cell line HEK293 are shown in Fig.5A and Fig 5B. The amount of solvent (DMSO) used in the culture of SiHa and A549 is 0.2%. The toxicity of DMSO is nullified by adding equal volume of solvent in the control group of cells HEK293. Notably, at this DMSO concentration no significant difference ($P > 0.05$) is observed on the cell viability of SiHa and A549 and HEK293. In Fig. 5B of cancerous cell line SiHa and A549, the increasing concentrations of NCur (10-60 μM) in the media decreases the percentage of viable cells significantly after 48h. The IC_{50} value of NCur for SiHa and A549 cells within 48 h is about 10.66 ± 1.04 and7.65 ± 1.35 μM (Table 1). In Fig. 5A, it is observed that with curcumin treatment at 10μM, the cell viability of SiHa and A549 declines to 60.22% and 54.21% respectively compared to normal cell line, HEK293. The morphological changes (indicated by arrows) of SiHa and A549 cells by curcumin and NCur are shown in Figs. 6A and 6B.

Effect of Curcumin and NCur on the level of intracellular ROS generation and in promoting apoptosis

It is seen that curcumin induced apoptosis in SiHa and A549 lung cancer cells when cells are exposed to 14 μM and 12 μM curcumin respectively while NCur induced apoptosis when cells are exposed to 10 μM and 7 μM respectively. The fluorescence microscopic images show an increased proportion of cells with higher fluorescence intensity when cells are treated with NCur for 48h (Figs. 7A and 7B). This is an indication of increased level of ROS in SiHa and A549 cancer cells by NCur. Curcumin can induce similar magnitude of ROS generation only at very higher doses in comparison to Ncur.

DNA damage assay

DNA fragmentation assay, showed distinctive DNA laddering indicating the ability of NCur to induce apoptosis in both SiHa and A549 cells (L2 and L4 respectively in Fig. 8). Lesser magnitude of DNA laddering is observed in the treatment of curcumin (L1 and L3 respectively) on these two cancer cell lines.

Discussion

Health risks from cancer is growing significantly worldwide and accordingly, researchers are putting their efforts in developing highly effective therapeutic processes for the disease. Here we demonstrate the use of safe herbal drugs (curcumin and NCur) in effective treatment of cancer using *in vitro* experiments. We further demonstrate that nano sized curcumin displays better anticancer property at a relatively smaller dose in comparison to standard curcumin powder. Therefore, we hypothesize that nano-sized curcumin may be considered for treating cervical and lung cancer.

Cytotoxicity is an important component of the immune system and is a highly regulated, multi-factorial process carried out by different cytotoxic cells of the immune system. Cytotoxic CD8+T cells, NK and NK-like T cells mount immune responses to cancer via pathways that

include the cytotoxic granzyme B/perforin pathway and by release of cytokines including IFN-γ and TNF-α[30]. The decreased IFN-γ is likely to contribute to the suppressive pro-tumour microenvironment and the survival of cancer cells, as this cytokine inhibits angiogenesis and cellular proliferation and promotes apoptosis of the cancer cells. In addition, it activates the adaptive immune system, and thus contributes to effective antigen processing and presentation [30]. Curcumin treatment for human cervical cancer and lung cancer come in this area. Curcumin has been considered as a promising approach for the treatment of multiple cancers, including gastric cancer, bladder cancer, renal cancer, etc. [31-33]. Curcumin, being a lipophilic molecule, interacts with the cellular membrane and is subsequently internalized, probably by diffusion. The higher curcumin uptake by tumour cells, against normal ones, has been assigned to various hypothetic factors, including the different membrane structure, protein composition and larger size [34]. The antioxidant activity of curcumin has been identified as the key mechanism by which this dietary phytochemical prevents cancer *in vivo*[35]. Some studies suggested that curcumin attenuates inflammation by modulation of antioxidant enzymes and down regulation of IL-1β, IL-6 and TNF-α [36]. Curcumin also suppresses the paclitaxel-induced NFκB pathway in cancer cells and shows anti-apoptotic properties[36]. The results of the present experiment confirmed the anti-inflammatory effects of curcumin and are in agreement with the previously published data in which curcumin led to a reduction in the formation of pro-inflammatory cytokines. The study similarly validates the significance of NCur in cancer treatment by using nano sized herbal powder for overcoming the limitations of poor solubility and absorption of curcumin for enhanced anti-inflammatory effects.

Bioavailability of a drug to the cells, whether *in vitro* or *in vivo*, is critical for its optimal efficacy. The IC_{50} values of NCur for SiHa and A549 cells within 48 h are about 10.66 ± 1.04

and7.65 ± 1.35 μM respectively (Table 1). From Fig. 5A, it is been observed that curcumin treatment at 10μM, the cell viability of SiHa and A549 declined to 60.22% and 54.21% respectively in 48 h. Whereas, NCur treatment at 10 μM, the cell viability of SiHa and A549 declined to 43.54% and 35.42% respectively in 48 h. This outcome implied that, NCur treatments possessed a higher impact toward the cervical cancer cell like SiHa and lung cancer cell line like A549 cells compared to curcumin. Interestingly, following an increase of curcumin and NCur concentration in the medium, the growth of normal cell line HEK293 was not affected significantly ($P<0.05$) (Figs. 5A & 5B), but at doses greater than 35μM concentration, the growth inhibition of HEK was also significantly affected ($P<0.05$). Moreover, at the 50% inhibitory concentrations of NCur towards the cancer cell lines, no significant effect was observed in the normal HEK293 cells.

Evaluation of apoptosis is crucial to differentiate it from necrosis. Nuclear fragmentation is one of the characteristic features of apoptotic mode of cell death. In view of the morphological features of the cells observed in response to using inverted microscopy, potent morphological changes in cancer cells (SiHa and A549) including cell shrinkage and star-shape with membrane blabbing which represent a typical apoptosis. When cells are treated with inhibitory concentration of curcumin and NCur cells become round in shape (Fig. 6A).

Morphological changes in cell nuclei are determined by fluorescence microscope. The blue fluorescent Hoechst 33342 is a cell permeable nucleic acid dye usually used to identify chromatin condensation and fragmentation by staining the condensed nuclei of apoptotic cells. Cell nuclear morphology is evaluated by fluorescence microscopy following DAPI staining. Apoptosis can be differentiated from necrosis by their characteristic nuclear changes. DAPI is a nuclear stain which is observed as blue fluorescence when excited under fluorescence

microscope. In our present study, DAPI staining revealed the changes associated with apoptosis in SiHa and A549 cells are both treated with curcumin and NCur which are shown in Fig. 6B. The treatment group showed fragmented apoptotic bodies, shrunken and emarginated nuclei in contrast to the control and large nucleus in the untreated cells, proving the apoptotic potential of the sample(drug). The morphological changes associated with apoptosis such as chromatin condensation, nuclear fragmentation (marked by arrows in Fig. 6B) are evident in both cancer cells upon treatment.

DNA laddering is one of the hallmark indications that helps in the differentiation of apoptosis from necrosis [37]. In the treatment group of our present study, the presence of the laddering (smear) pattern of DNA fragments and the absence of the necrotic streak further confirm apoptosis as noted in Fig. 8.. DNA fragmentation assay, which is an indicative of apoptosis, is used to define the mechanism of cell death. DNA is extracted from cell lines after the administration of various doses of curcumin and NCur and subjected to agarose gel electrophoresis for evaluation. The results showed distinctive DNA laddering indicating ability of NCur to cause apoptosis in both SiHa and A549 cells (L2 and L4 respectively in Fig. 8). Laddering is also observed in treatment of curcumin which was comparatively lesser (L1 and L3 respectively in Fig. 8) on these two cancer cell lines.

ROS generation usually associated with cell apoptosis. To compare the level of ROS generation in both cancer cell with or without the curcumin and NCur, we use an oxidation-sensitive fluorescent dye, 2′, 7′-dichlorofluoresceindiacetate. Fluorescent microscopy images revealed that the proportion of cells with higher fluorescence intensity is increased in cells treated with NCur for 48h (Fig. 7A and 7B), indicating that NCur significantly increased the level of ROS in SiHa and A549 cancer cells. Curcumin could only induce ROS generation at

very higher doses as compared to NCur. These results suggested that the elevation of intracellular ROS is perhaps the attributing factor for NCur dependent cell death which might be due to initiation of apoptosis in SiHa and A549 cells. Apoptotic induction can also be mediated through ROS intermediates [38]. It is reported that ROS could play a role in down regulating Bcl-2[39] as well as in triggering the release of cytochrome C from the mitochondria into the cytoplasm along with FAS associated proteins recruitment and finally leading to the activation of caspase 3 and apoptosis [40]. In our present study, increased levels of ROS in the treatment group indicate the apoptotic mode of cell death induced by NCur through the generation of ROS.

These observations clearly suggest that that NCur has better efficacy to increase the intracellular ROs supporting apoptosis of both SiHa (cervical cancer) and A549 (lung cancer) cells. Also, the NCur doses are found to be harmless and causes no-side effect to normal human body cells. On the other hand, curcumin needs higher doses to cause apoptosis of the same cancer cells and more time to initiate therapeutic activities.

Conclusion

This research establishes higher efficacy of nano sized curcumin in inhibiting the proliferation of both human SiHa (cervical cancer) and A549 (lung cancer) cells in comparison to normal sized curcumin. Natural curcumin exhibits slow and low therapeutic activity. Thus, the study re-establishes the scope of nanotechnology in developing alternate nature based herbal drugs in the treatment of cancer.

Funding

The work was done by the support of Biophysics Lab of Physics Department of Jadavpur University. The UGC (NTA) NET JRF Fellowship support from UGC, Government of India **(Ref. No.:210510536878, Dated: 12-03-2022)** to Somashree Biswas is gratefully acknowledged.

Acknowledgement

The authors are gratefully acknowledged the technical and experimental supports of Biophysics Laboratory of Physics Department of Jadavpur University and Molecular and Cell Biology laboratory, Department of Zoology, University of Kalyani, The Fellowship support from UGC to Somashree Biswas is also gratefully acknowledged.

Conflict of interest

There is no conflict of interest of any kind related to this study.

ORCID: 0000-0002-6072-9528

References

[1] **Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R.L., Torre, L.A. and, Jemal, A. (2018).** Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries.CA: A Cancer Journal for Clinicians. 68(6):394–424.

[2] **Arbyn, M., Weiderpass, E., Bruni, L., Sanjosé, S. de., Saraiya, M., Ferlay, J. and Bray, F. (2020).** Estimates of incidence and mortality of cervical cancer in 2018: a worldwide analysis. The Lancet Global Health. 8(2):191-203.

[3] **Torre, L.A., Siegel, R.L., Ward, E.M. and Jemal, A. (2016).** Global Cancer Incidence and Mortality Rates and Trends--An Update.Cancer Epidemiology, Biomarkers & Prevention. 25(1):16-27.

[4] **Wells, S.I., Aronow, B.J., Wise. T.M., William, S.S., Couge, J.A. and Howley, P.M. (2003).** Transcriptome signature of irreversible senescence in human papillomavirus-positive

cervical cancer cells.Proceedings of the National Academy of Sciences of the United States of America. 100 (12): 7093-7098.

[5] **Dehn, D., Torkko, K.C. and Shroyer, K.R. (2007).** Human papillomavirus testing and molecular markers of cervical dysplasia and carcinoma. Cancer. 111(1):1-14.

[6] **Chakrabarti, O. and Krishna, S. (2003).** Molecular interactions of "high risk" human papillomaviruses E6 and E7 oncoproteins: implications for tumour progression. Journal of Biosciences. 28(3):337-48.

[7] **Castellsagué, X., Diaz, M., Sanjosé, S.D., Muñoz, N., Herrero, R., Franceschi, S., Peeling ,R.W., Ashley, R., Smith, J.S., Snijders, P.J.F., Meijer, C.J.L.M., Bosch, F.X., Plummer, M., Moreno, V. P., Ruiz, Alonso de., Chichareon, S. C., J, Ngelangel., Eluf-Neto, Roló, A., Caceres, E., Santos, C., Chaouki, N., Gueddari, B. El., Hammouda, D. and Rajkumar, T. (2006).** Worldwide human papillomavirus etiology of cervical adenocarcinoma and its cofactors: implications for screening and prevention. Journal of the National Cancer Institute, 98(5):303-15.

[8] **Kwon, S.B., Kim, M.J., Ham, S.Y., Park, G.W., Choi, K.D., Jung, S.H. and Yoon, D.Y. (2015).**H9 induces apoptosis via the intrinsic pathway in non-small-cell lung cancer A549 cells.Journal of Microbiology and Biotechnology. 25(3):343-52.

[9] **Kogita, A., Togashi, Y., Hayashi, H., Sogabe, S., Terashima, M., Velasco, M.A. D., Sakai, K., Fujita, Y., Tomida, S., Takeyama, Y., Okuno, K., Nakagawa, K. and Nishio, K. (2014).** Hypoxia induces resistance to ALK inhibitors in the H3122 non-small cell lung cancer cell line with an ALK rearrangement via epithelial-mesenchymal transition. International Journal of Oncology.45(4): 1430–1436.

[10] **Miller, K.D., Siegel, R.L., Lin, C.C., Mariotto, A.B., Kramer, J.L., Rowland, J.H., Stein, K.D., Alteri, R. and Jemal, A. (2016).** Cancer treatment and survivorship statistics, 2016.CA: A Cancer Journal for Clinicians. 66:271-289.

[11] **Reck, M., Heigener, D.F., Mok, T., Soria, J.C. and Rabe, K.F.(2013).** Management of non-small-cell lung cancer: recent developments. Lancet. 382(9893):709-19.

[12] **Imran, M., Ullah, A., Saeed, F., Nadeem, M., Arshad, M.U. and Suleria, H.A.R. (2018).** Curcumin, anticancer, & antitumor perspectives: A comprehensive review. Critical Reviews in Food Science and Nutrition. 58(8):1271-1293.

[13] **Lestari, M.L.A.D. and Indrayanto, G. (2014).** Curcumin.Profiles of Drug Substances, Excipients and Related Methodology. 39:113-204.

[14] **Kotha, R.R. and Luthria, D.L. (2019).** Curcumin: Biological, Pharmaceutical, Nutraceutical, and Analytical Aspects. Molecules.24(16):2930.

[15] **Darvesh, A. S., Aggarwal, B. B. and Bishayee, A. (2012).** Curcumin and liver cancer: a review. Current Pharmaceutical Biotechnology.13(1):218-28.

[16] **Sintara, K. D., Thong-Ngam, D., Patumraj, S. and Klaikeaw, N. (2012).** Curcumin attenuates gastric cancer induced by N-methyl-N-nitrosourea and saturated sodium chloride in rats.Journal of Biomedicine and Biotechnology. 2012: 915380.

[17] **Yang, C.L., Ma, Y.G., Xue, Y.X., Liu, Y.Y., Xie, H. and Qiu, G.R. (2012).** Curcumin induces small cell lung cancer NCI-H446 cell apoptosis via the reactive oxygen species-mediated mitochondrial pathway and not the cell death receptor pathway.DNA and Cell Biology. 31(2):139-50.

[18] **Kim, S.R., Park, H.J., Bae, Y.H., Ahn, S.C., Wee, H.J., Yun, I., Jang, H.O., Bae, M.K. and Bae, S.K. (2012).** Curcumin down-regulates visfatin expression and inhibits breast cancer cell invasion. Endocrinology. 153(2):554-63.

[19] **Sundram, V., Chauhan, S.C., Ebeling, M. and Jaggi, M. (2012).** Curcumin Attenuates β-catenin Signaling in Prostate Cancer Cells through Activation of Protein Kinase D1. PLOS ONE. 7(4):35368.

[20] **Zhang, X., Zhu, L., Wang, X., Zhang, H., Wang, L. and Xia, L. (2023).** Basic research on curcumin in cervical cancer: Progress and perspectives. Biomedicine & Pharmacotherapy.162:114590.

[21] **Menon, V.P. and Sudheer, A.R. (2007).** Antioxidant and anti-inflammatory properties of curcumin.Advances in experimental medicine and biology. 595:105–125.

[22] **Moghadamtousi, S. Z., Kadir, H. A., Hassandarvish, P., Tajik, H., Abubakar, S. and Zandi, K. (2014).** A Review on Antibacterial, Antiviral, and Antifungal Activity of Curcumin.BioMed Research International. 2014:186864.

[23] **Tajuddin, W.N.B.W.M., Lajis, N.H., Abas, F., Othman, I. and Naidu, R. (2019).** Mechanistic Understanding of Curcumin's Therapeutic Effects in Lung Cancer. Nutrients, 11(12): 2989.

[24] **Kumar, G., Mittal, S., Sak, K. and Tuli, H.S. (2016).** Molecular mechanisms underlying chemopreventive potential of curcumin: Current challenges and future perspectives. Life Sciences.148:313–328.

[25] **Yallapu, M.M., Nagesh, P.K.B., Jaggi, M. and Chauhan, S.C. (2015).** Therapeutic Applications of Curcumin Nanoformulations. The American Association of Pharmaceutical Scientists.17(6): 1341–1356.

[26] **Gutierrez, J.K.T., Zanatta, G.C., Ortega, A.L.M., Balastegui, M.I.C., Sanitá, P.V., Pavarina, A.C., Barbugli, P.A. and Mima, E.G. D. O. (2017).** Encapsulation of curcumin in polymeric nanoparticles for antimicrobial Photodynamic Therapy. PLOS One. 12(11):0187418.

[27]. **Feltrin, F.S., Agner, T., Sayer, C., and Lona, L.M.F. (2022).** Curcumin encapsulation in functional PLGA nanoparticles: A promising strategy for cancer therapies. Advances in Colloid and Interface Science. 300: 102582. https://doi.org/10.1016/j.cis.2021.102582.

[28]. **Hu, Q., and Luo, Y. (2021).** Chitosan-based nanocarriers for encapsulation and delivery of curcumin: A review. International Journal of Biological Macromolecules. 179: 125 - 135. https://doi.org/10.1016/j.ijbiomac.2021.02.216.

[29] **Chattopadhyay, K., Samanta, A., Mukhopadhyay, S. and Chattopadhyay, B. (2018).** Potential amelioration of nicotine-induced toxicity by nanocurcumin. Drug Development Research. 79:119–128.

[30] **Hodge, G., Barnawi, J., Jurisevic, C., Moffat, D., Holmes, M., Reynolds, P.N., Jersmann, H. and Hodge, S. (2014).** Lung cancer is associated with decreased expression of perforin, granzyme B and interferon (IFN)-γ by infiltrating lung tissue T cells, natural killer (NK) T-like and NK cells.Clinical and Experimental Immunology.178(1):79–85.

[31] **Zhou, S., Yao, D., Guo, L. and Teng, L. (2017).** Curcumin suppresses gastric cancer by inhibiting gastrin-mediated acid secretion. Federation of European Biochemical Societies Open Biology.7(8):1078–1084.

[32] **Shi, J., Wang, Y., Jia, Z., Gao, Y., Zhao, C. and Yao, Y. (2017).** Curcumin inhibits bladder cancer progression via regulation of β-catenin expression. Tumour Biology.39(7):1-8.

[33] **Li, G., Wang, Z., Chong, T., Yang, J., Li,Hongliang. and Chen H. (2017).** Curcumin enhances the radiosensitivity of renal cancer cells by suppressing NF-κBsignaling pathway. Biomedicine & Pharmacotherapy.94:974-981.

[34] **Kunwar, A., Barik, A., Pandey, R. and Priyadarsini, K.I. (2006).** Transport of liposomal and albumin loaded curcumin to living cells: an absorption and fluorescence spectroscopic study. Biochimica et Biophysica Acta. 1760(10):1513-1520.

[35] **Nelson, K.M., Dahlin, J.L., Bisson, J., Graham, J., Pauli, G.F. and Walters, M.A. (2017).** The Essential Medicinal Chemistry of Curcumin. Journal of Medicinal Chemistry. 60:1620–1637.

[36] **Singh, A.K. and Vinayak, M. (2015).** Curcumin attenuates CFA induced thermal hyperalgesia by modulation of antioxidant enzymes and down regulation of TNF-α, IL-1β and IL-6.Neurochemical Research. 40(3):463–472.

[37] **Pajaniradje, S., Mohankumar, K., Subramanian, R. S. and Rajagopalan, R. (2014).** Antiproliferative and apoptotic effects of Sesbania grandiflora leaves in human cancer cells.BioMed Research International. vol. 2014: 474953.

[38] **Meshkini, A. and Yazdanparast, R. (2012).** Involvement of oxidative stress in taxol-induced apoptosis in chronic myelogenous leukemia K562 cells.Experimental and Toxicologic Pathology. 64(4):357-65.

[39] **Li, D., Ueta, E., Kimura, T., Yamamoto, T. and Osaki, T. (2004).** Reactive oxygen species (ROS) control the expression of Bcl-2 family proteins by regulating their phosphorylation and ubiquitination. Cancer Science, 95(8): 644–650.

[40] **Chen, K.C., Kao, P.H., Lin, S.R. and Chang, L.S. (2009).** Upregulation of Fas and FasL in Taiwan cobra phospholipase A2-treated human neuroblastoma SK-N-SH cells

through ROS- and Ca2+-mediated p38 MAPK activation. The Journal of Cellular Biochemistry.106(1): 93–102.

Table

Table 1: Inhibitory effects (IC_{50} in µM) of curcumin and NCur on cancer cells (SiHa and A549) and normal epithelial cell line (HEK293) after 24 h and 48 h incubation.

Cell Line	Curcumin IC_{50} values (µM), 24h	Nano-curcumin IC_{50} values (µM), 24h	Curcumin IC_{50}values (µM), 48h	Nano-curcumin IC_{50} value (µM), 48h
HEK293	57.36 ±2.08	51.31 ± 0.59	46.79 ± 1.14	36.10 ± 1.09
SiHa	25.64 ± 0.87	19.27 ± 1.34	14.57 ± 1.17	10.66 ± 1.04
A549	25.33 ± 0.74	17.80 ± 1.13	12.17 ± 0.44	7.65 ±1.35

Journal of Xi'an University of Architecture & Technology ISSN No : 1006-7930

Figures

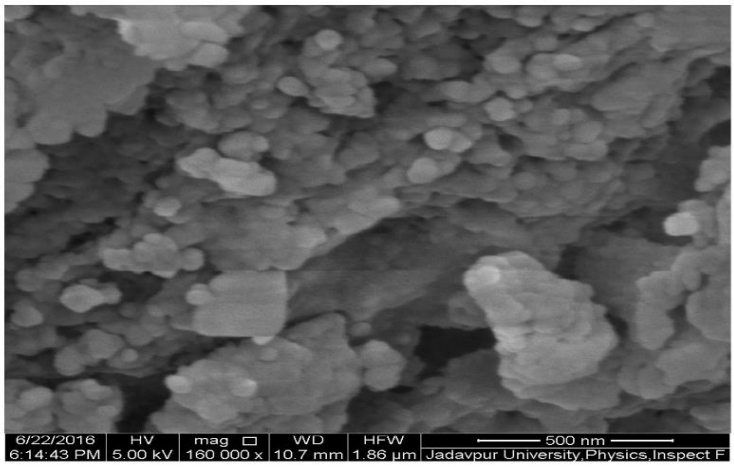


Fig. 1: SEM image of as prepared NCur

Journal of Xi'an University of Architecture & Technology ISSN No : 1006-7930

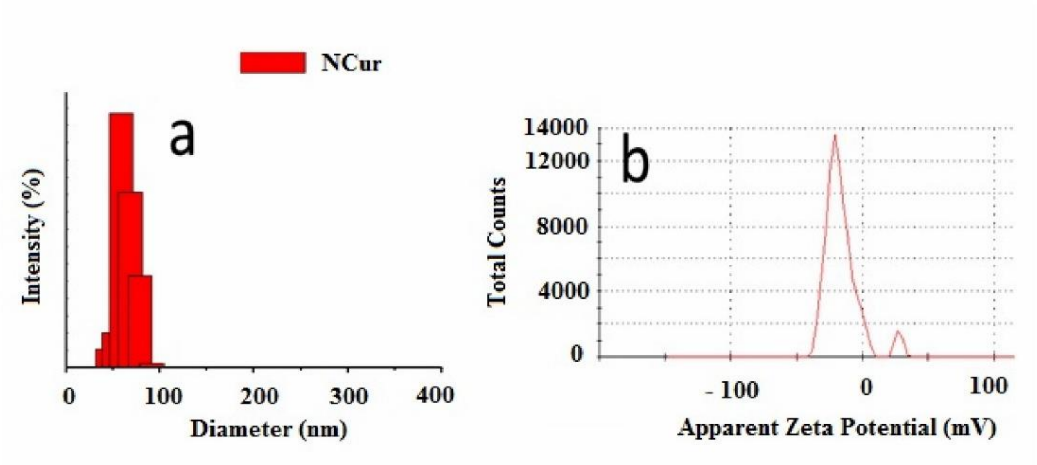


Fig. 2: DLS analysis of the as prepared NCur,

Journal of Xi'an University of Architecture & Technology ISSN No : 1006-7930

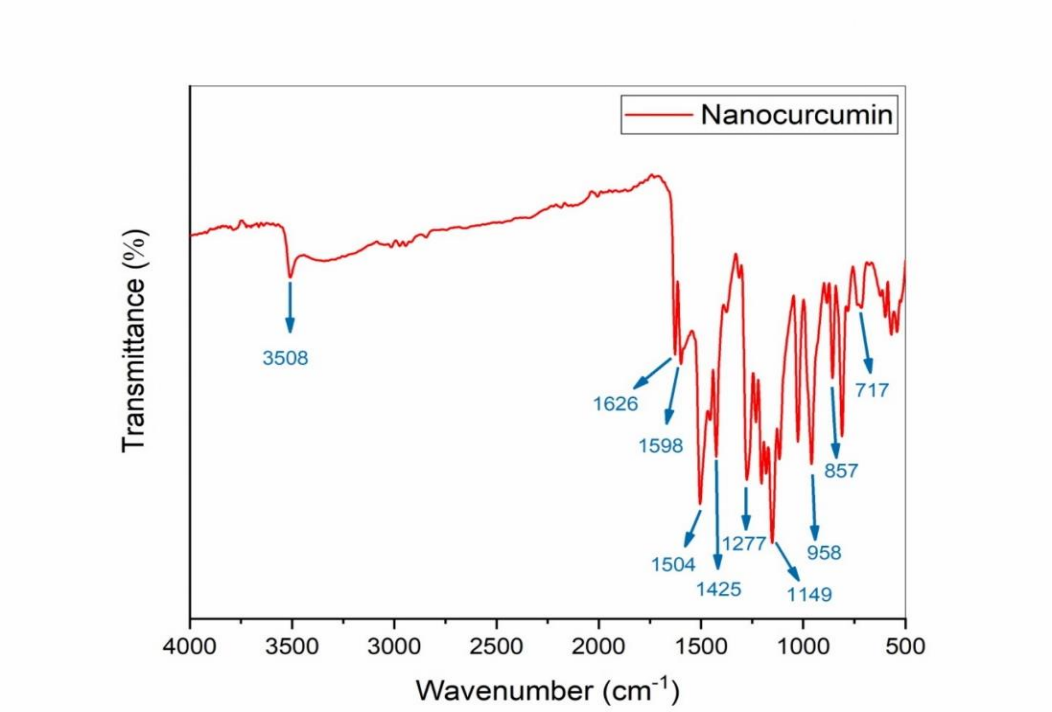


Fig. 3: Nanocurcumin was characterized by Fourier transform infrared spectroscopy (FTIR) (Perkin-Elmer FTIR-1600, USA) by mixing dried powder of nanoparticles with KBr. The data of FTIR revealed the information about the functional groups which were present in the nanocurcumin.

Journal of Xi'an University of Architecture & Technology ISSN No : 1006-7930

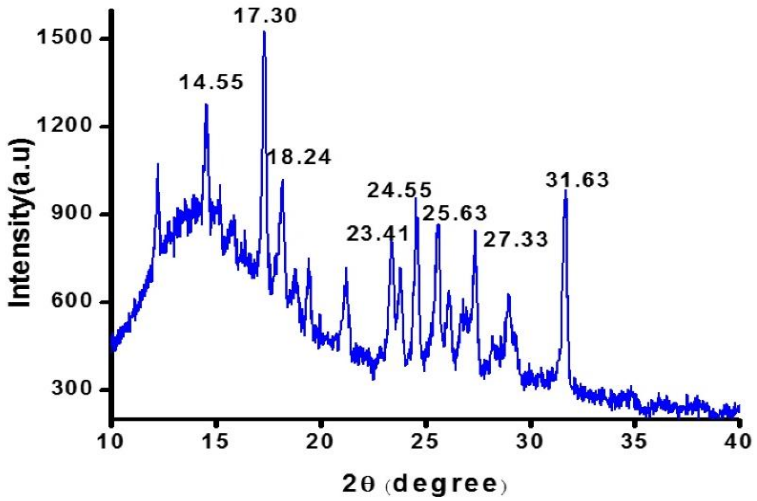


Fig. 4: The crystalline nature of the NCur powder was determined by X-ray diffraction study

Journal of Xi'an University of Architecture & Technology ISSN No : 1006-7930

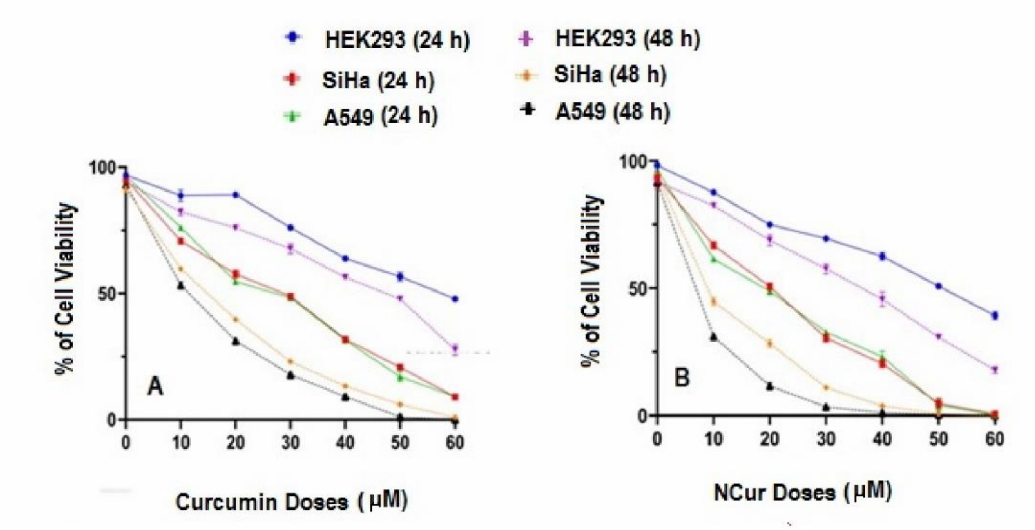


Fig. 5. Viability and morphological changes of SiHa, A549 and HEK293 cells, where (A) Viability of the cells after 48hrs of 10-60 µM of curcumin treatment. (B) Viability of the cells after 48hrs of 10-60 µM of NCur treatment.

Journal of Xi'an University of Architecture & Technology ISSN No : 1006-7930

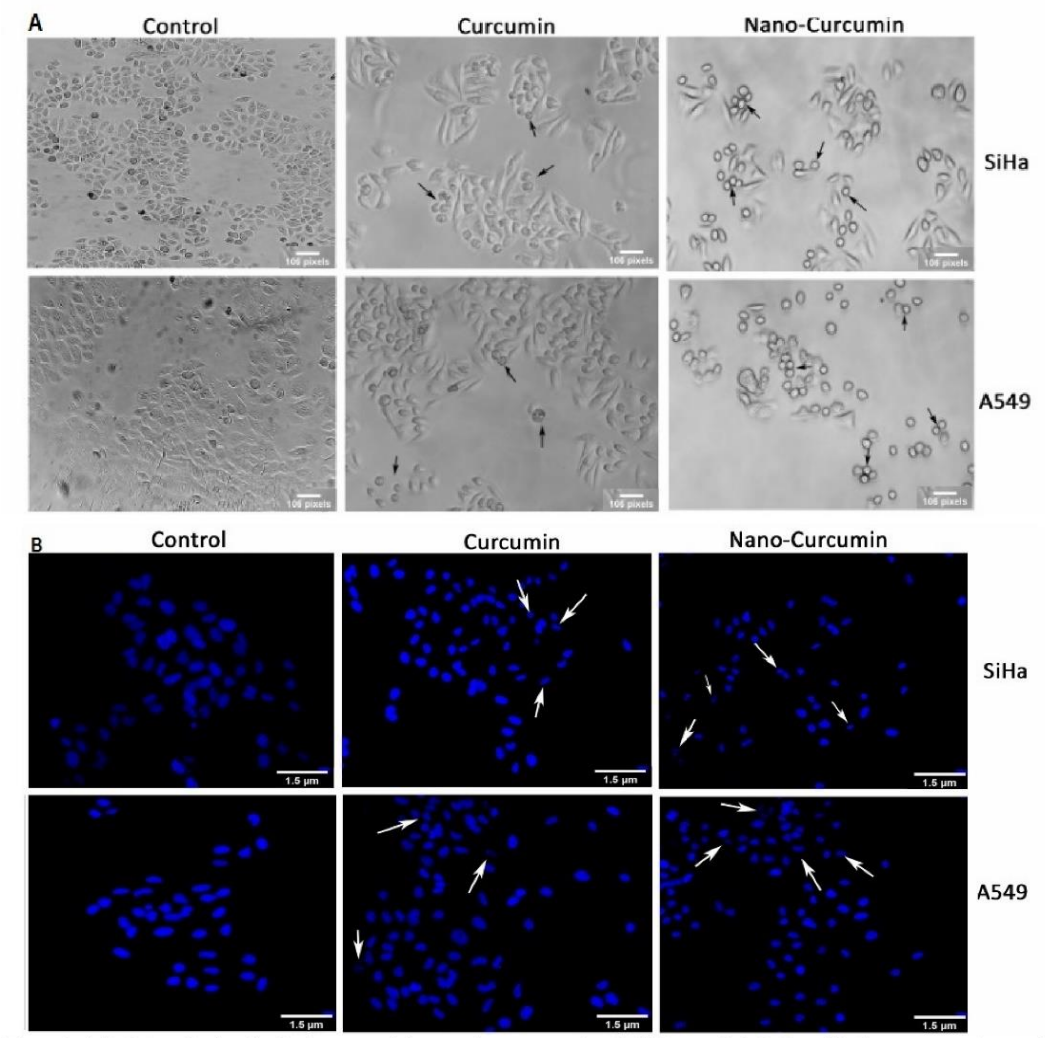


Fig. 6: (A) Morphological changes (shown by arrows) of SiHa and A549 cells by curcumin and NCur. (B) Fluorescence microscopic images of SiHa and A549 cells treated with respective IC_{50} concentrations of curcumin (14µM for SiHa and 12 µM for A549) and NCur (10 µM for SiHa and 7 µM for A549) and stained with 100 nM DAPI for 5 min. The curcumin and NCur induced DNA damage/ chromatin condensation are shown by arrows. Data represent mean ± SD of two experiments. $^{*}P < 0.05$.

Journal of Xi'an University of Architecture & Technology ISSN No : 1006-7930

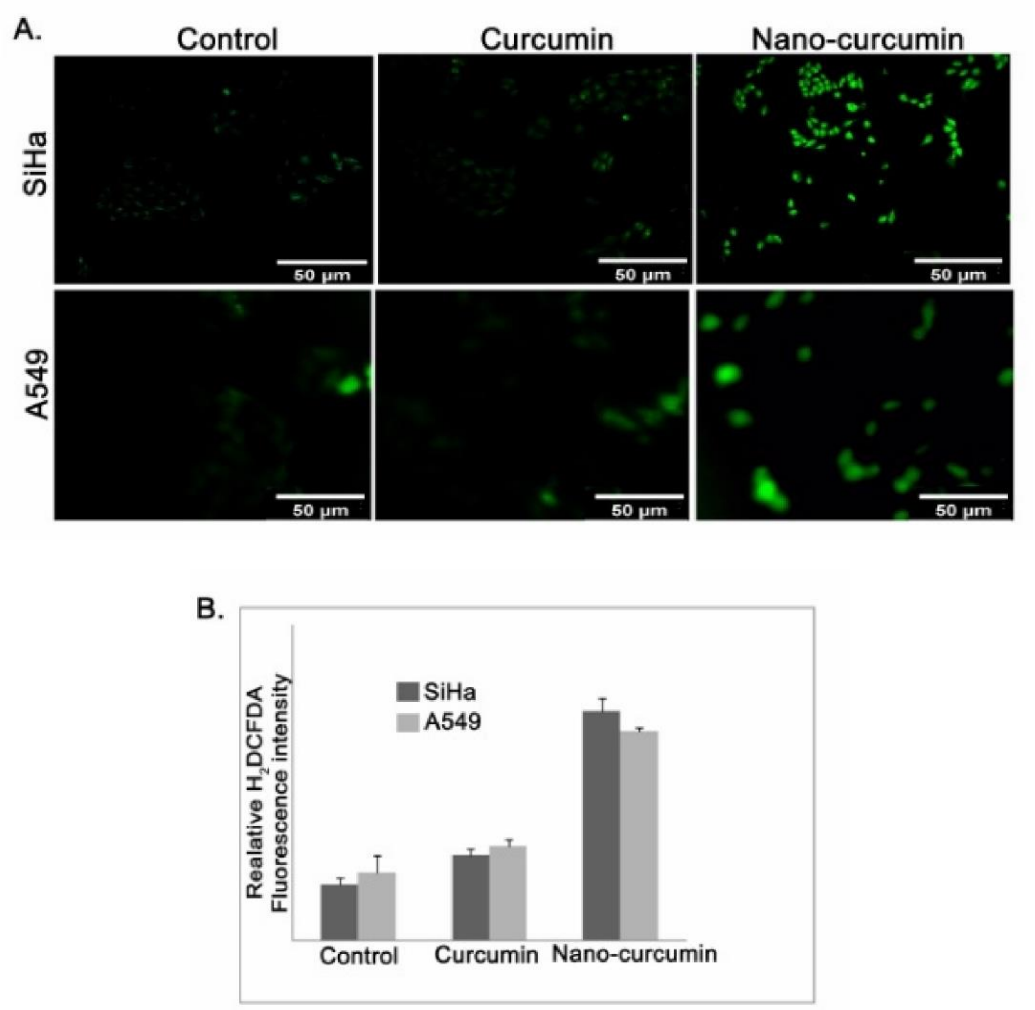


Fig. 7. (A)Determination of ROS generation and apoptotic cell death in SiHa and A549 cell lines. Where, SiHa and A549 cells treated with IC_{50} doses of curcumin and NCur and stained with H_2DCFDA. ROS generation is observed under fluorescence microscope. (B) Determination of ROS generation and apoptotic cell death in SiHa and A549 cell lines. Where, representative fluorescence intensities of H_2DCFDA which are plotted in the bar diagram after treatment with curcumin and NCur.

Journal of Xi'an University of Architecture & Technology ISSN No : 1006-7930

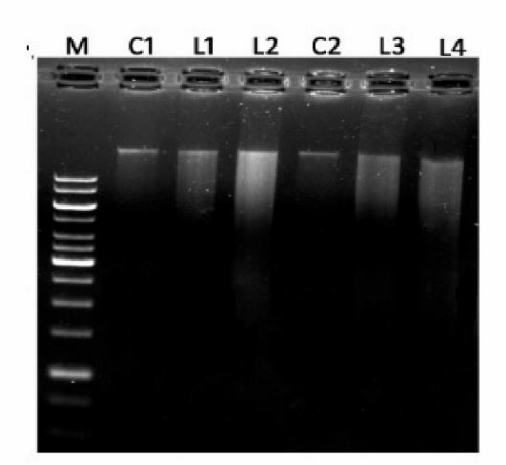
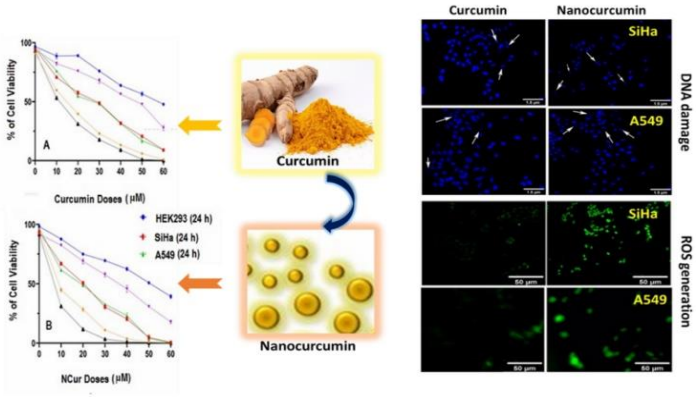


Fig. 8. DNA fragmentation is evaluated as described in Materials and Methods, were M - represents molecular weight marker (1Kb DNA ladder), C1 - SiHa cell control, (L1) - exposed with curcumin for 48h, (L2) - SiHa cell incubated for 48hrs with NCur, (C2) - A549 cell control, (L3) - incubated with curcumin for 48h, (L4) - A549 cells exposed to NCur for 48h.

Journal of Xi'an University of Architecture & Technology ISSN No : 1006-7930

Graphical Abstract



Scientific Journal of Biology

Research Article

Amelioration of Nicotine Induced Toxicity by Nanocurcumin in Protein Malnourished Condition -

Krishna Chattopadhyay[1]*, Somashree Biswas[2], Subrata Mukhopadhyay[1] and Brajadulal Chattopadhyay[2]

[1]*Department of Chemistry, Jadavpur University, Kolkata-700032, India*

[2]*Department of Physics, Jadavpur University, Kolkata-700032, India*

***Address for Correspondence:** Dr. Krishna Chattopadhyay, Department of Chemistry, Jadavpur University, Kolkata-700032, India, Tel: +919-433-122-559; E-mail: kris_ami@yahoo.co.in*

Submitted: 06 June 2018; **Approved:** 03 July 2018; **Published:** 07 July 2018

Cite this article: Krishna C, Biswas S, Mukhopadhyay S, Chattopadhyay B. Amelioration of Nicotine Induced Toxicity by Nanocurcumin in Protein Malnourished Condition. Sci J Biol. 2018;1(1): 001-008.

Scientific Journal of Biology

ABSTRACT

Topic: Nicotine, the core addictive component presents in a substantial quantity in tobacco. Addiction of nicotine in female populations especially who belong to lower socio-economic status causes many adverse effects, like inducing oxidative stress, genotoxicity and disrupting immune response in the body. The investigation was designed to determine first time the improved efficacy of nanocurcumin against aggravated nicotine-induced toxicity on female system.

Experiments: Experiments were conducted on female albino rats maintained under protein malnourished condition where animals were subcutaneously injected with effective dose of nicotine (2.5 mg kg-1 body weight day-1) and orally supplemented with effective dose of nanocurcumin (4 mg kg-1 body weight day-1) for 21 days. The animals were then sacrificed and various biochemical and histological experiments were performed on serum and different tissues.

Significant Findings: Disruption of liver and kidney functions, alteration of phosphatase enzymes activities, triglycerides and total cholesterol in serum and aggravated lipid peroxidation in serum and liver tissues were observed in nicotine-induced and protein malnourished condition. Nicotine also decreased the antioxidant enzymes activities and disrupted severely the structural integrity of liver, kidney and ovary tissues in such condition. Supplementation of nanocurcumin restored the normalcy of various biochemical functions and maintained the structural integrity of liver, kidney and ovarian tissues.

Conclusions: Nanocurcumin exhibited more effective ($P < 0.001$) amelioration than curcumin against the deleterious nicotine-induced toxicities and therefore, it can be used as a potential therapeutic blocker for protecting the health of malnourished female population against nicotine.

Keywords: Antioxidant; Nanocurcumin; Nicotine toxicity; Oxidative stress; Malnourished

INTRODUCTION

Increasing use of different forms of tobacco products is an alarming danger for human health worldwide. Along with the common tobacco forms like cigarettes and pipes consumed worldwide, the Indian populations are also habituated with smoking of Chutta and Beedi, (locally used handmade smoking elements) and chewing of tobacco leaves [1]. Consumption of different forms of tobacco is also increasing among the women and this has now become the biggest problems in India [2]. The route of administration for nicotine is through a variety of ways including smoking, insufflations, chewing, transdermal and vaporization etc. In addition, depending on the route of administration, the exposure time and dosage, the amount of nicotine enters in the body may vary.

Nicotine is well known to have serious systemic side effects in addition to being highly addictive [3]. It is the most acute acting pharmacological agent and major component of tobacco and plays a significant impact in the development of cardiovascular disorder [4], Chronic Obstructive Pulmonary Disease (COPD) and lung cancer [5] and many other non- communicable diseases [6]. It also aggravates Th1/Th2 cytokine imbalance, alters transcription factor, lipid peroxidation in the liver and other tissues and effects on the activities of antioxidant enzymes in rats [7,8]. Nicotine disrupts antioxidant mechanism by enhancing the production of Reactive Oxygen Species (ROS) and thereby decreases antioxidant level that causes peroxidative tissue damage [8-10]. The chemicals present in tobacco smoke alter the endocrine function, perhaps at the level of the ovary which in turn affects the release of female hormones. This endocrine disruption likely contributes to the reported associations of smoking with adverse reproductive outcomes, including menstrual dysfunction, infertility and early menopause [11].

Nutritional factors play an important role in the metabolism and pharmaco-toxicological activities of various drugs and xenobiotic [12]. The major nutritional disorders which are widely prevalent in all developing countries are due to Protein Energy Malnutrition (PEM) which affects all age groups and more so of poorer segments of the population. However, the repercussions are the strongest in children and women [12]. Low dietary protein possesses a constraint on the biosynthetic activity, disposition and toxicity. Report says that the protein content present in the nicotine induced hepatic cell decides either cell-survival pathway or cytotoxic pathway [13]. Though it is already established that nicotine causes various damage in our body in normal dietary condition but the toxic effects of nicotine particularly in protein restricted dietary situation are still cause of concern.

Curcumin is a natural diphenolic compound derived from turmeric Curcuma longa, and possesses many therapeutic properties like antihepatoxic, antioxidant, anti-inflammatory, antidiabetic, antitumour, hepato-protective, and anti-HIV activities [14,15]. It has proven to be a modulator of intracellular signaling pathways that control cellular inflammation, invasion and apoptosis [13]. Curcumin significantly ameliorates the nicotine-induced toxicity and regulates the imbalance between cell survival and death induced by nicotine [13,14]. Nanocurcumin is a nanoparticles form of curcumin in which the particles of curcumin are more soluble and deliverable in the body [16]. These particles have been shown to be more targeted to the tissue of interest that leads to better drug delivery and faster treatment without any wastage or side effects [17,18]. Our previous results revealed that nanocurcumin under normal dietary condition effectively ameliorated the nicotine-induced toxicities at much lower concentration due to its higher aqueous solubility and more bioavailability [19]. Curcumin in itself is an extremely effective and non-toxic compound for which it is preferred for therapy. But nanoparticles that are used to coat or carry curcumin molecules to the body might be toxic. Thus, recent research is now focusing not only on the formation of a better nanocurcumin medication but also on increasing the safety levels of nanoparticles and reducing the side effects of the drug to a minimum against the aggravated nicotine-induced toxicities under protein malnourished condition.

MATERIAL AND METHODS

Chemicals

Dichloromethane used as a solvent for the preparation of nanoparticles of curcumin was purchased from Merk, India. Nicotine hydrogen tartrate and curcumin were purchased from the Sigma Chemical Company, USA. All other required chemicals were

purchased from Spectrochem Pvt. Ltd. India. All the chemicals and reagents used were of analytical grade.

Animals and diet

Adult female albino rats of Wistar strain (weighing approximately 120-150 g) were taken from Animal Housing Facility of Jadavpur University, Kolkata, India. Prior to use, all animals were acclimatized under standard conditions of temperature and humidity with 12 h light/dark cycles. They were maintained in accordance with the guidelines of the rule of Instructional Animal Ethics Committee of Jadavpur University, Kolkata, India (Reference No. AEC/PHARM/1502/14/2015, Dated: 30th July, 2015). The animals were housed in polypropylene cages in an air-conditioned room. Animals were allowed to protein-restricted diet (5% casein, 83% carbohydrate, 7% fat, 4% salt mixture and 1% vitamin mixture) according to Hawk et al. [20] throughout the experiment and water adlibitum. Twenty four female albino rats were equally divided into four groups of six rats in each and treated as below for 21 days.

- Group-I: Served as Control group in which animals were received no treatment.
- Group-II: Nicotine treated group in which animals were injected with the effective dose of nicotine.
- Group-III: Nicotine and curcumin treated group in which animals were injected with the effective dose of nicotine and received effective dose of curcumin orally.
- Group-IV: Nicotine and nanocurcumin treated group in which animals were injected with the effective dose of nicotine and received effective dose of nanocurcumin orally.

Preparation of nanocurcumin

The preparation and characterization of nanocurcumin was done by modifying the method of Basniwal et al. [16] as already reported [19]. The as prepared nanocurcumin was used in this study.

Mode of treatment

The animals were maintained on their respective dietary regimen well before (1 week) the treatment start to till the completion of the treatment. The mode of treatment and effective doses of nicotine (2.5 mg kg-1 body weight day-1), curcumin (80 mg kg-1 body weight day-1) and nanocurcumin (4 mg kg-1 body weight day-1) were similar to the study of Chattopadhyay et al. [19]. The animals in control group received subcutaneous injection of 0.5 ml physiological saline only at the same time.

Collection of samples

After the last dose of injection received, animals were kept fasting overnight and sacrificed on the following morning after mild anesthesia. Blood was collected from the heart in sterilized with or without anticoagulant (heparin) containing tubes and serum was separated out after centrifugation and stored at -20° C prior to further analysis. Liver, kidney and ovary were dissected, cleaned properly and stored for further investigations. The tissues were kept under ice-cold conditions throughout the experiment.

Biochemical assays

Serum and tissues (liver, kidney and ovary) protein contents were determined by the method of Lowry et al. [21]. The Alkaline Phosphatase (ALP), Alanine-Transaminase (ALT), and Aspartase-Transaminase (AST) activities in the serum were measured by using the standard kit supplied by ARKRAY Healthcare Pvt. Ltd., Surat, India. The other liver function enzyme, Acid Phosphatase (ACP) in the serum was assayed according to the method illustrated by Bergmeyer and Bernt [22] by using para nitrophenyl-phosphate as the substrate. The lipid components such as TC: Total Cholesterol; HDL-C: High Density Lipoprotein-Cholesterol and triglyceride in serum were estimated by using standard kits supplied by Ranbaxy Diagnostic Ltd., Mumbai, India. VLDL-C and LDL-C: Low Density Lipoprotein-Cholesterol were calculated from the values of triglyceride, TC and HDL-C by using the Friedwald and Fredicksons formula [23]. Lipid Peroxidation (LPO) was measured in plasma and liver by the determination of Thiobarbituric Acid-Reactive Substances (TBARS) according to the standard protocol. The amount of MDA was calculated by taking the extinction coefficient of MDA as $1.56 \times 105\ M^{-1}\ cm^{-1}$. Antioxidant enzyme activities in liver such as Superoxide Dismutase (SOD) and Catalase (CAT) were determined from rats of all the groups [24]. The Glutathione-Reductase (GSH) and glutathione-peroxidase enzymes activities of the liver tissue were determined by the methods described by Griffith [25]. All the entire biochemical assays were repeated twice and data were averaged (n = 12).

Histological study

Cleaned tissues of liver and kidney were fixed by using Bouin's fluid. After fixation, the tissues were washed several times by different graded alcohol to remove excess fluid and then embedded in paraffin. Using of rotary microtome the embedded tissues were sliced. The paraffin sections were then attached on the slide and washed by xylol before staining. The tissue sections were then stained by using haematoxylin and eosin staining. Histological study of liver, kidney and ovary tissues were accomplished by using Leica DFC450C microscope at 20X by using CFP filter.

Statistical analysis

The experimental setup was repeated twice and all data were averaged over n = 12 animals, and given mean ± S.D. Significance levels were determined by using ANOVA, where * implied significant ($p < 0.01$) and ** implied more significant ($p < 0.001$) of the data when compared with the nicotine treatment. Similarly, $^{\#}$ implied significant ($p < 0.01$) and $^{\#\#}$ implied more significant ($p < 0.001$) of the data when compared with the nicotine plus curcumin treatments.

RESULTS

The as prepared nanocurcumin is shown in figure 1. The most sensitive and widely used liver enzymes i.e., AST and ALT activities in the serum were significantly increased due to nicotine treatment in protein malnourished condition (Table 1). The levels of activities of those two enzymes were decreased significantly ($P < 0.01$) in curcumin supplementation and more significantly ($P < 0.001$) in nanocurcumin supplementation as seen in table 1. Similar results were observed for ACP and ALP liver function enzymes activities (Table 1). Urea and creatinine levels in the serum of nicotine exposed animals were significantly elevated compared to the control group animals (Table 2). The supplementation of nanocurcumin showed much more ameliorative effects than curcumin by decreasing the levels of urea and creatinine respectively in the serum. Nicotine caused significant alterations of cholesterol, triglyceride, LDL-C, and VLDL-C concentrations and decreased HDL-C concentration in the serum under protein malnourished condition (Table 3). However, administration of nanocurcumin to the nicotine-induced animals

showed more significant ameliorative effects by normalizing almost the concentrations of those parameters in the serum of the rats. The results of lipid peroxidation both in the serum and liver tissue of rats as shown in table 4 similarly suggested that supplementation of nanocurcumin was more effective than curcumin in protein malnourished rats. The degenerative effects of the antioxidant enzymes (SOD, CAT, GSH and GPx) due to nicotinic stress and ameliorative effects caused by curcumin and nanocurcumin are shown in table 5. Nanocurcumin was found to be comparatively more active against nicotine-induced effect on antioxidant enzymes particularly in protein malnourished condition. Estimation of protein content in different tissues is given in table 6.

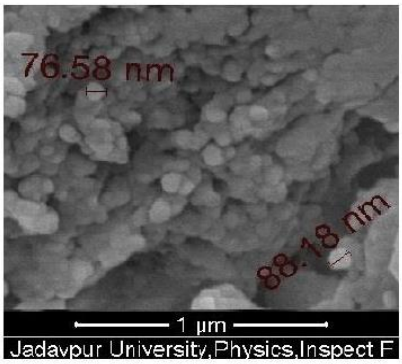


Figure 1: SEM picture of curcumin nanoparticles.

The histological study of the liver tissue of control group animals is shown in figure 2A. The distorted central vein (marked by arrow head) and irregular cell arrangement of the nicotine exposed liver are shown in figure 2B. The photographs of curcumin and nanocurcumin supplemented rat liver are shown in figure 2C and 2D respectively. The histological studies of kidney tissues of the control, nicotine treated, curcumin and nanocurcumin supplemented groups are presented in figure 3A to 3D respectively. Here destruction of Bowmann's capsule and cell walls are seen in nicotine treated condition (Figure 3B) which almost restored in nanocurcumin supplementation as seen in figure 3D. The toxic effect on ovary by nicotine (Figure 4A and 4B) and the corresponding protective effects of curcumin (Figure 4C) and nanocurcumin (Figure 4D) similarly shown in figure 4.

DISCUSSION

Though several studies in animal models has demonstrated the effective amelioration of curcumin against nicotine-induced toxicities, the undesirable pharmacokinetic properties restricts the clinical efficacy of curcumin in human subjects [26]. Nanocurcumin is aqueous soluble and more bio-available than curcumin [27]. Scientists are thus trying to use nanocurcumin against various

Table 1: Effect of nanocurcumin on hepatic enzymes in protein malnourished condition.

Enzymes	Control	Nicotine	Nicotine + Curcumin	Nicotine + Nanocurcumin
ACP (m mol/h/100 ml)	1.36 ± 0.10	1.94 ± 0.11(42.6↑)	1.52 ± 0.12"(11.8↑)	1.42 ± 0.10"## (4.4↑)
ALP (m mol/h/100 ml)	9.30 ± 0.60	19.20 ± 0.30(106.5↑)	12.95 ± 0.30"(39.2↑)	10.42 ± 0.23"## (12.0↑)
AST (IU/L)	9.80 ± 1.60	17.50 ± 4.00(78.6↑)	11.32 ± 0.70" (15.5↑)	10.35 ± 0.90"## (5.61↑)
ALT (IU/L)	37.40 ± 2.60	71.35 ± 1.61(90.77↑)	42.91 ± 3.49" (14.7↑)	39.99 ± 2.86"## (6.9↑)

The experimental setup was repeated twice and all data were averaged over n = 12 animals, and given mean ± S.D. Significance levels were determined by using ANOVA, where * implied significant ($P < 0.01$) and ** implied more significant ($P < 0.001$) of the data when compared with the data of nicotine treatment. Similarly, # implied significant ($P < 0.01$) and ## implied more significant ($P < 0.001$) of the data when compared with the data of nicotine + curcumin treatments. The data within the parenthesis represent the average percentage of increase (↑) or decrease (↓) relative to the control.

Table 2: Effect of nanocurcumin on renal function parameters in protein malnourished condition.

Parameter	Control	Nicotine	Nicotine + Curcumin	Nicotine + Nanocurcumin
Urea (mg/100 ml)	38.30 ± 2.15	56.80 ± 2.60(48.3↑)	47.00 ± 2.78'(22.7↑)	39.23 ± 2.06"## (2.4↓)
Creatinine (mg/100 ml)	1.35 ± 0.12	1.85 ± 0.21(37.0↑)	1.56 ± 0.50'(15.6↑)	1.30 ± 0.10"## (3.7↓)

The experimental setup was repeated twice and all data were averaged over n = 12 animals, and given mean ± S.D. Significance levels were determined by using ANOVA, where * implied significant ($P < 0.01$) and ** implied more significant ($P < 0.001$) of the data when compared with the data of nicotine treatment. Similarly, # implied significant ($P < 0.01$) and ## implied more significant ($P < 0.001$) of the data when compared with the data of nicotine + curcumin treatments. The data within the parenthesis represent the average percentage of increase (↑) or decrease (↓) relative to the control.

Table 3: Effect of nanocurcumin on Lipid profile in protein malnourished condition.

Parameters	Control	Nicotine	Nicotine + Curcumin	Nicotine + Nanocurcumin
Triglyceride(mg/dl)	105.6 ± 8.4	185.8 ± 9.1 (75.9↑)	109.5 ± 8.6" (3.7↑)	106.4 ± 2.5"## (0.8↑)
Cholesterol (mg/dl)	107.3 ± 7.9	138.7 ± 9.0 (29.3↑)	115.0 ± 5.6" (7.2↑)	109.3 ± 2.4"## (1.9↑)
HDL-C (mg/dl)	39.5 ± 7.0	29.8 ± 4.0 (24.6↓)	31.7 ± 1.0' (19.7↓)	33.5 ± 1.5"# (15.2 ↓)
VLDL-C (mg/dl)	21.1 ± 1.6	36.8 ± 0.7 (74.4↑)	30.7 ± 1.8' (45.5↑)	21.5 ± 0.4"## (1.03↑)
LDL-C (mg/dl)	46.1 ± 2.1	81.8 ± 4.1 (75.9↑)	64.79 ± 1.2' (40.5↑)	43.5 ± 1.5"## (5.6↓)

The experimental setup was repeated twice and all data were averaged over n = 12 animals, and given mean ± S.D. Significance levels were determined by using ANOVA, where * implied significant ($P < 0.01$) and ** implied more significant ($P < 0.001$) of the data when compared with the data of nicotine treatment. Similarly, # implied significant ($P < 0.01$) and ## implied more significant ($P < 0.001$) of the data when compared with the data of nicotine + curcumin treatments. The data within the parenthesis represent the average percentage of increase (↑) or decrease (↓) relative to the control.

Scientific Journal of Biology

Table 4: Effect of nanocurcumin on lipid peroxidation in protein malnourished condition.

MDA level	Control	Nicotine	Nicotine + Curcumin	Nicotine + Nanocurcumin
Serum (n mol/ml)	5.61 ± 0.6	7.98 ± 1.3(42.2↑)	6.30 ± 0.5"(12.3↑)	6.32 ± 1.1" (12.7↑)
Liver (n mol/mg protein)	14.98 ± 0.4	22.92 ± 0.5(53.0↑)	16.01 ± 2.96"(6.9↑)	15.50 ± 0.9"# (3.5↑)

The experimental setup was repeated twice and all data were averaged over n = 12 animals, and given mean ± S.D. Significance levels were determined by using ANOVA, where * implied significant ($P < 0.01$) and **implied more significant ($P < 0.001$) of the data when compared with the data of nicotine treatment. Similarly, # implied significant ($P < 0.01$) and ## implied more significant ($P < 0.001$) of the data when compared with the data of nicotine + curcumin treatments. The data within the parenthesis represent the average percentage of increase (↑) or decrease (↓) relative to the control.

Table 5: Effect of nanocurcumin on antioxidant enzymes in protein malnourished condition.

Enzymes	Control	Nicotine	Nicotine + Curcumin	Nicotine + Nanocurcumin
SOD(n mol/O_2 decomposed/ min/100 mg protein)	9.02 ± 0.2	4.09 ± 0.1(54.7↓)	6.72 ± 0.14'(25.5↓)	7.15 ± 0.5"#(20.7↓)
CAT\ (n mol/H_2O_2 decomposed/ min/mg protein)	40.21 ± 1.0	29.22 ± 1.0(27.3↓)	32.9 ± 0.5'(18.2↓)	35.50 ± 1.0"#(11.7↓)
GSH (µg/mg protein)	18.75 ± 1.76	12.02 ± 1.13	14.00 ± 1.41'	16.00 ± 1.41"#
GPx (n mol/min/mg protein)	150.52 ± 2.5	135.11 ± 2.9(10.2↓)	144.34 ± 2.2'(4.1↓)	149.50 ± 2.5"#(0.6↓)

The experimental setup was repeated twice and all data were averaged over n = 12 animals, and given mean ± S.D. Significance levels were determined by using ANOVA, where * implied significant ($P < 0.01$) and **implied more significant ($P < 0.001$) of the data when compared with the data of nicotine treatment. Similarly, # implied significant ($P < 0.01$) and ## implied more significant ($P < 0.001$) of the data when compared with the data of nicotine + curcumin treatments. The data within the parenthesis represent the average percentage of increase (↑) or decrease (↓) relative to the control.

Table 6: Protein content of the tissues in nicotine stress and nanocurcumin supplemented condition of rats under protein malnourished condition.

Protein content(mg/g wet tissue)	Control	Nicotine	Nicotine + Curcumin	Nicotine + Nanocurcumin
Liver	15.00 ± 0.70	7.12 ± 1.59	12. 85 ± 0.91'	13.77± 0.70"#
Kidney	15.5 ± 2.12	7.5 ± 1.41	11.50 ± 2.53'	14.00 ± 1.4"##
Ovary	18.75 ± 1.76	12.02 ± 1.13	14.00 ± 1.41'	16.00 ±1.41"#

The experimental setup was repeated twice and all data were averaged over n = 12 animals, and given mean ± S.D. Significance levels were determined by using ANOVA, where * implied significant ($P < 0.01$) and ** implied more significant ($P < 0.001$) of the data when compared with the data of nicotine treatment. Similarly, # implied significant ($P < 0.01$) and ## implied more significant ($P < 0.001$) of the data when compared with the data of nicotine + curcumin treatments. The data within the parenthesis represent the average percentage of increase (↑) or decrease (↓) relative to the control.

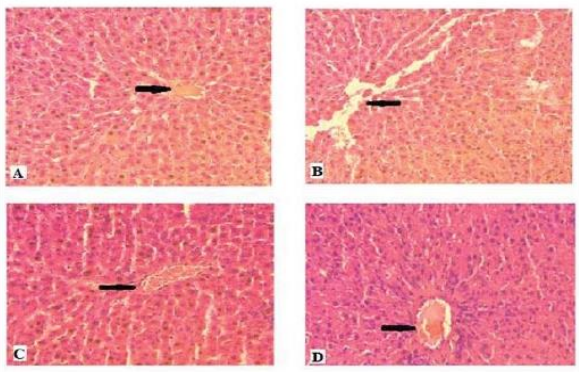


Figure 2: Histological section of Liver (20X)
A: Control Liver
B: Nicotine treated Liver
C: Nicotine and curcumin treated Liver
D: Nicotine and Nanocurcumin treated Liver

diseases to achieve its clinical benefits [17,18]. We have recently reported the effectiveness of orally supplemented nanocurcumin against the nicotine-induced toxicities in rats under normal protein-diet condition [19]. This study furnishes the useful application of nanocurcumin against the aggravated toxic effects that are induced by nicotine in rats under protein malnourished status.

The oxidative stress generated by nicotine increases the activities of ACP and ALP in the serum. The increased oxidative stress causes tissue injury which is related to the ROS generation [27]. In protein malnourished condition, the levels of ACP and ALP were elevated drastically showing more aggravated effect of nicotine on the tissues (Table 1). The supplementation of nanocurcumin showed more significant ($P < 0.001$) effect on the antioxidant status of protein malnourished female rats due to which the ACP and ALP activities were normalized. Nicotine also damages the cell membranes of liver tissues causing liver injury. The activities of AST and ALT are thus increased due to loss of functional integrity of liver cells [28]. It is already reported that curcumin can normalize the levels of the liver enzymes to some extent in normal protein condition [19,29]. The situation became more serious in protein malnourished condition because aggravated liver injury was observed in such condition. Our results suggest that nanocurcumin is better than curcumin to nullify the nicotinic toxicity in liver (Table 1). The smaller dimension of nanocurcumin is more bio-available than curcumin which may explain these findings.

Urea and creatinine levels were significantly elevated in the serum of nicotine exposed protein malnourished group compared to the control group (Table 2) which could be related to the progressive kidney failure as described by Addo et al. [30]. Usunobun et al. [31] have reported that some heavy metals in tobacco (e.g., Cadmium, Mercury, Lead etc.) may play some role in tobacco-induced renal damage. This finding suggests that nanocurcumin supplementation is a better restorative phenomenon compared to curcumin for the healing of kidney damage.

Scientific Journal of Biology

The increment of triglyceride (75.9%), VLDL-C (74.4%), VLDL-C (75.9%) and cholesterol (29.3%) levels and decrement of HDL-C (24.6%) level in the serum of rats under nicotine treatment clearly revealed that nicotine affected the lipid profile severely in protein malnourished condition (Table 3). This result is in agreement with the finding of Chattopadhyay et al. [10]. Balakrishnan and Menon [28] have shown that nicotine-stimulated catecholamine synthesis lipolysis adipose tissue which increased the serum cholesterol level. The absorption of total cholesterol and increase CYP7A1 gene expression (which is a rate limiting enzyme in the biosynthesis of bile acid from cholesterol) by curcumin helps to reduce the total cholesterol level in the serum [32]. The multiple inductions of fatty acid catabolism may lower the triglyceride level by curcumin. The increased lipid peroxidation in the plasma and liver tissues due to nicotinic stress in protein malnourished condition (Table 4) corroborates with the earlier findings [10,19]. The significant amelioration of lipid peroxidation by nanocurcumin was observed in this study. It may be inferred that due more bioavailability and higher intake of nanocurcumin into the cells, the above-mention beneficial effects of curcumin become more prominent for nanocurcumin in protein malnourished condition.

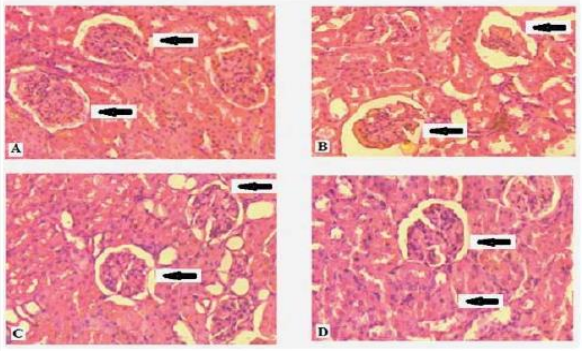


Figure 3: Histological section of Kidney (20X)
A: Control Kidney
B: Nicotine treated Kidney
C: Nicotine and curcumin treated Kidney
D: Nicotine and Nanocurcumin treated Kidney

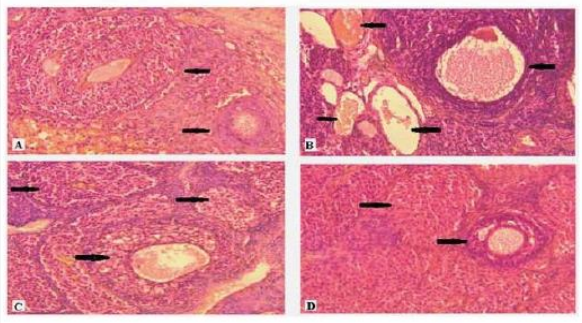


Figure 4: Histological section of Ovary (20X)
A: Control Ovary
B: Nicotine treated Ovary
C: Nicotine and curcumin treated Ovary
D: Nicotine and Nanocurcumin treated Ovary

Nicotine interrupts the mitochondrial respiratory chain and causes increased generations various free radical ions (e.g., super oxide ions, hydrogen peroxide ions etc.) and enhances lipid peroxidation on human circulating lymphocytes [33]. The primary role of antioxidant enzymes is to take part in defense mechanism for protecting the cells and cellular organelles from oxidative damage. It was noted that the activities of two main antioxidant enzymes (SOD and CAT) decreased significantly in the nicotine-treated rats compared to the control group (Table 5). Dispose of the free radicals, production of hydrogen peroxide or inactivation of the enzyme proteins by ROS generation and depletion of the enzyme substrates and/or down regulation of transcription and translation processes due to nicotine toxicity are the possible causes for the decreased activities of those scavenging enzymes [34]. Similarly, depletion of GSH and GPx levels not only weakens cell defense system but also enhances the oxidative stress that damages various tissues and organs [27]. The significant ameliorative effect of nanocurcumin proved its scavenging free radicals activity and maintained the cell membrane interiority and functions through inhibiting membrane lipid peroxidation. Nicotine also causes reduction in the protein concentration of liver, kidney and ovarian tissues due to its toxic effect. The increased concentration of the protein in different tissues suggests that nanocurcumin can protect the tissues in a better way against nicotine-induced damage as seen from table 6.

Significant histological changes were observed in hepatocytes of protein malnourished female rats under nicotine exposure. The regular hepatic cells arrangement and normal central vein (marked by arrow head) of the liver tissue of control group rats were noted in figure 2A. Nicotine abuse affected the liver tissues for which distorted cell arrangement and the enlarged central vein filled with sinusoidal fluid was observed in figure 2B under protein malnourished condition. This was in agreement with the observation of Bateman [35] who suggested that the histological changes might be preferred as standard for the assessment of liver cell damage. Nicotine stress caused damage of liver cells by enhancing the levels of liver enzymes (AST and ALT) into the blood which indicated the liver disease. Curcumin and nanocurcumin appeared to reduce the levels of those liver enzymes and thus prevented liver damages by maintaining the structural integrity of the liver cell membrane as seen from figures 2C and 2D respectively. Histological studies of kidney tissues (Figure 3A to 3D) of the protein malnourished animals were similarly revealed the effect of nicotine and corresponding amelioration of curcumin and nanocurcumin. Nicotine exposure distorted the normal arrangement of the glomerulus (marked by arrow head), the bowman space and the proximal/distal tubules of kidney tissue which were more significantly ($P < 0.001$) restored by supplementation of nanocurcumin (Figure 3D) in comparison to curcumin (Figure 3C). In the ovary of female rats maintained with protein malnourished diet, the regression of graafian follicles with disrupted cellular structures forming vacuoles were seen due to nicotine treatment (Figure 4B). This was probably due to the synergistic effect of nicotine toxicity and protein malnourished stress which decreased the production of ovarian hormones in such condition as reported by Sinha et al. [8]. Curcumin (Figure 4C) and nanocurcumin (Figure 4D) both restored the graafian follicular structures but the effect of nanocurcumin was more prominent in restoration of the normalcy of ovarian structure than curcumin.

CONCLUSION

The living organisms depend essentially on proteins, which

directly or indirectly regulate the biochemical processes. Protein malnutrition induces marked changes in the functioning form, leading to several biochemical defects, structural disruption and altered physiological functions. Thus the detrimental effects of nicotine are more pronounced in protein deficient condition. The undesirable complications of inorganic nanomaterial can be overcome by using the protein-based nanomaterial or herbal-nanomaterial as they exhibit less cytotoxicity [36,37]. Our study demands that nanocurcumin more effectively ameliorates nicotine-induced toxicities in protein malnourished condition by normalizing the hepato-enzymes activities, kidney function parameters, lipid profiles, anti-oxidant status and also maintaining the structural integrity of different tissues under nicotine stress condition. The safer and more effective nanocurcumin may be used as better therapeutic agent to protect the health of female population who are suffering both from protein malnutrition and nicotine-induced complications.

ACKNOWLEDGMENT

The financial support obtained from Women Scientist-B scheme, Department of Science and Technology (Sanctioned no. DST/Disha/SoRF-PM/013/2015(G), dated; 08/03/2016) is gratefully acknowledged. Authors are also grateful for the experimental and technical supports provided by the Biophysics Laboratory, Department of Physics, Jadavpur University. The permission of work on animal model provided by the Animal Ethics Committee of Jadavpur University is also gratefully acknowledged.

REFERENCES

1. Rahman M, Sakamoto J, Fukui T. Bidi smoking and oral cancer: A meta-analysis. Int J Cancer. 2003; 106: 600-604. https://goo.gl/Fn88wm
2. Mishra GA, Pimple SA, Shastri SS. An overview of the tobacco problem in India. Ind J Med Pediatr Oncol. 2012; 33: 139-145. https://goo.gl/wvsjrL
3. Mishra A, Chaturvedi P, Datta S, Sinukumar S, Joshi P, Garg A. Harmful effects of nicotine. Ind J Med Pediatr Oncol. 2015; 36: 24-31. https://goo.gl/bSpPRj
4. Benowitz NL, Burbank AD. Cardiovascular toxicity of nicotine: implications for electronic cigarette use. Trends Cardiovasc Med. 2016; 26: 515-523. https://goo.gl/HQ2bVE
5. Laniado Laborin R. Smoking and Chronic Obstructive Pulmonary Disease (COPD). Parallel epidemics of the 21st Century. Int J Environ Res Public Health. 2009; 6: 209-224. https://goo.gl/rv2R7w
6. Thakur JS, Garg R, Narain JP, Menabde N. Tobacco use: A major risk factor for non-communicable diseases in South-East Asia region. Ind J Pub Health. 2011; 55: 155-160. https://goo.gl/3KPPye
7. Maiti M, Chattopadhyay K, Verma M, Chattopadhyay BD. Curcumin protects against nicotine-induced stress during protein malnutrition in female rat through immunomodulation with cellular amelioration. Mol Biol Rep. 2015; 42: 1623-1637. https://goo.gl/J1AMTf
8. Sinha S, Maiti M, Chattopadhyay K, Chattopadhyay BD. Potential amelioration of curcumin against nicotine-induced toxicity of protein malnourished female rats. J Pharmacol Toxicol. 2012; 7: 166-180. https://goo.gl/X9WgRA
9. Chattopadhyay K, Chattopadhyay BD. Effect of nicotine on lipid profile, per oxidation and antioxidant enzymes in female rats with restricted dietary protein. Ind J Med Res. 2008; 127: 571-576. https://goo.gl/efwALn
10. Chattopadhyay K, Mondal S, Chattopadhyay BD. Ameliorative effect of sesame lignans on nicotine toxicity in rats. Food Chem Toxicol. 2010; 48: 3215-3220. https://goo.gl/61jRfw
11. Dechanet C, Anahory T, Mathieu Daude JC, Quantin X, Reyftmann L, Hamamah S, et al. Effects of cigarette smoking on reproduction. *Human Reprod Update. 2011;* 17: 76-95. https://goo.gl/1FiSA3
12. Krishnaswamy K. Drug/Xenobiotic-Metabolism, Disposition and Toxicity in Malnutrition. Def Sci J. 1987; 37: 133-142. https://goo.gl/2GPRSD
13. Banerjee S, Chattopadhyay K, Chhabra JK, Chattopadhyay B. Protein dependent fate of hepatic cells under nicotine induced stress and curcumin ameliorated condition. Eur J Pharmacol. 2012; 684: 132-145. https://goo.gl/6g53LM
14. Bandyopadhyaya G, Sinha S, Chattopadhyay BD, Chakraborty A. Protective role of curcumin against nicotine induced genotoxicity on rat liver under restricted dietary protein. Eur J Pharmacol. 2008; 588: 151-157. https://goo.gl/FWUrRV
15. Ruby AJ, Kuttan KD, Babu KN, Rajasekharan R, Kuttan R. Antitumour and antioxidant activity of natural curcuminoids. Cancer Lett. 1995; 94: 79-83. https://goo.gl/mokevu
16. Bhawana, Basniwal RK, Buttar HS, Jain VK, Jain N. Curcumin nanoparticles: preparation, characterization, and antimicrobial study. J Aggri Food Chem. 2011; 59: 2056-2061. https://goo.gl/a5s11s
17. Danafar H. Study of the Composition of Polycaprolactone/Poly (Ethylene Glycol)/Polycaprolactone Copolymer and Drug-to-Polymer ratio on drug loading efficiency of curcumin to nanoparticles. Jundishapur J Nat Pharm Prod. 2017; 12: 34179. https://goo.gl/KRGs4S
18. Nosrati H, Sefidi N, Sharafi A, Danafar H, Manjili HK. Bovine Serum Albumin (BSA) coated iron oxide magnetic nanoparticles as biocompatible carriers for curcumin-anticancer drug. Bioorg Chem. 2018; 76: 501-509. https://goo.gl/HXWqF4
19. Chattopadhyay K, Samanta A, Mukhopadhyay S, Chattopadhyay BD. Potential amelioration of nicotine-induced toxicity by nanocurcumin. Drug Dev Res. 2018; 79: 119-128. https://goo.gl/b8rBnn
20. Hawk PB, Oser BL, Summerson WH. Practical Physiological Chemistry. 13th ed. New York: Mc Graw Hill Book Comp; 1954. https://goo.gl/6SSF9E
21. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951; 193: 265-275. https://goo.gl/eEQA2K
22. Bergmeyer HU, Bernt E. Glutamate-pyruvate transaminase. In: HU Bergmeyer ed. Methods of enzymatic analysis. New York: Academic Press Inc; 1963.
23. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem. 1972; 18: 499-502. https://goo.gl/FxCkUA
24. Beauchamp C, Fridovich I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem. 1971; 44: 276-287. https://goo.gl/1zsLz3
25. Griffith OW. Determination of glutathione and glutathione sulfide using glutathione reductase and 2-vinyl pyridine. Anal Biochem. 1980; 106: 207-212. https://goo.gl/UTa8xg
26. Gupta SC, Sung B, Kim JH, Prasad S, Li S, Aggarwal BB. Multitargeting by turmeric, the golden spice: From kitchen to clinic. Mol Nutr Food Res. 2013; 57: 1510-1528. https://goo.gl/hR4SHS
27. Sreekala S, Indira M. Effects of exogenous selenium on nicotine-induced oxidative stress in rats. Biol Trace Element Res. 2009; 130: 62-71. https://goo.gl/fFZe7W
28. Balakrishnan A, Menon VP. Antioxidant properties of hesperidin in nicotine-induced lung toxicity. Fund Clin Pharmacol. 2007; 21: 535-544. https://goo.gl/fyUtQE
29. Salahshoor M, Mohamadian S, Kakabaraei S, Roshankhah S, Jalili C. Curcumin improves liver damage in male mice exposed to nicotine. J Tradit Comp Med. 2015; 6: 176-183. https://goo.gl/H4f6YU
30. Addo MA, Gbadago JK, Affum HA, Adom, T, Ahmed K, Okley GM. Mineral profile of ghanaian dried tobacco leaves and local snuff: A comparative study. J Rad Nucl Chem. 2008; 277: 517-524. https://goo.gl/XCrTjR
31. Usunobun U, Adegbeg J, Ademuyiwa O, Okugbo TF, Evuen U, Osibemhe M, et al. N-Nitrosodimethylamine (NDMA), liver function enzymes, renal function parameters and oxidative stress parameters: a Review. British J Pharm Toxicol. 2012; 3: 165-176.
32. Kim M, Kim Y. Hypocholesterolemic effects of curcumin via up-regulation of cholesterol 7a-hydroxylase in rats fed a high fat diet. Nutr Res Pract. 2010; 4: 191-195. https://goo.gl/3G3nwf
33. Miró O, Alonso JR, Jarreta D, Casademont J, Urbano-Márquez A, Cardellach

F. Smoking disturbs mitochondrial respiratory chain function and enhances lipid peroxidation on human circulating lymphocytes. Carcinogenesis. 1999; 20: 1331-1336. https://goo.gl/XoFWVh

34. Basha KK, Vani M, Poornima PS, Vijayudu B, Venkatramudu M, Ravi B, et al. Interaction of red grape extract and leaf extract on nicotine induced oxidative stress in the lung tissue of male albino rat. Int J Pharmaceut Sci Health. 2018; 2. https://goo.gl/URb6gA

35. Bateman AC. Patterns of histological change in liver disease: my approach to 'medical' liver biopsy reporting. Histopathol. 2007; 51: 585-596. https://goo.gl/kmqLp9

36. Milano F, Mari L, van de Luijtgaarden W, Parikh K, Calpe S, Krishnadath KK. Nano-curcumin inhibits proliferation of esophageal adenocarcinoma cells and enhances the T cell mediated immune response. Front Oncol. 2013; 3: 137. https://goo.gl/8Hckzw

37. Khosropanah MH, Dinarvand A, Nezhadhosseini A, Haghighi A, Hashemi S, Nirouzad F, et al. Analysis of the Antiproliferative Effects of Curcumin and Nanocurcumin in MDA-MB231 as a Breast Cancer Cell Line. Iran J Pharm Res. 2016; 15: 231-239. https://goo.gl/w24y3h

Journal of Biologically Active Products from Nature

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tbap20

Promising Efficacy of Nanocurcumin in Comparison to Curcumin Against Nicotine-induced Complications

Anwesha Samanta, Krishna Chattopadhyay, Somashree Biswas, Bhola Nath Paul & Brajadulal Chattopadhyay

To cite this article: Anwesha Samanta, Krishna Chattopadhyay, Somashree Biswas, Bhola Nath Paul & Brajadulal Chattopadhyay (2022) Promising Efficacy of Nanocurcumin in Comparison to Curcumin Against Nicotine-induced Complications, Journal of Biologically Active Products from Nature, 12:4, 366-377, DOI: 10.1080/22311866.2022.2120074

To link to this article: https://doi.org/10.1080/22311866.2022.2120074

Published online: 28 Sep 2022.

J. Biologically Act. Prod. Nat. 12 (4) 2022 pp 366 - 377 366

Taylor & Francis
Taylor & Francis Group

Journal of Biologically Active Products from Nature
https://www.tandfonline.com/loi/tbap

ISSN: (Print) 2231-1866; ISSN: (Online) 2231-1874

Original Article

Promising Efficacy of Nanocurcumin in Comparison to Curcumin Against Nicotine-induced Complications

Anwesha Samanta [1], Krishna Chattopadhyay [1], Somashree Biswas [1], Bhola Nath Paul [2] and Brajadulal Chattopadhyay [1]*

[1] Department of Physics, Jadavpur University, Kolkata - 700032, India
[2] Immunology Division, Indian Institute of Toxicology Research, Lucknow - 226001, India
* Corresponding Author: Brajadulal Chattopadhyay (bdc_physics@yahoo.co.in)

Received 09 April 2022; Received in revised form 24 August 2022; Accepted 25 August 2022

Abstract: Long-term exposure to nicotine severely affects the immune system. Nicotine-addicted women suffer more to diseases because of their history to several disorders or susceptibility to nicotine-related morbidity and mortality. The beneficial uses of nano-curcumin in nicotine-addicted women are still unknown, possibly due to its poor pharmacokinetics and limited bioavailability. Nanocurcumin is administered to study the immune-protective effects in nicotine exposed female rats. Female albino rats of Wistar strain (*Rattus norvegicus*) are nicotine injected (2.5 mg/kg bodyweights s.c., daily) for 21 days. Three hours later nanocurcumin is administered by oral gavages (4 mg/kg bodyweights/day). The dose and period of nanocurcumin is adopted from our earlier publications elsewhere. Animals are eradicated after the treatment period and immune-related cytokines, apoptotic markers, sex hormones and other parameters are measured from individual serum. Molecular docking, as well as, *in vitro* interactions of nanocurcumin with nicotine and some specific cellular proteins are measured for understanding the mechanism of action of nanocurcumin. Nanocurcumin reduced protein oxidation, augmented the immune system by regulating the expression of immunity-related cytokines and apoptotic markers. It helps maintaining the healthy levels of sex hormones and protein content of cells under nicotine-stressed condition. Molecular docking and *in vitro* interactions reveal that nanocurcumin possesses higher binding ability to nicotine and several cellular proteins. Cytokines, apoptotic markers and steroidogenic hormones are severely affected by nicotine exposure. Bio-soluble nanocurcumin acts as significant immune-modulator, which offers better protection than curcumin to female rats from nicotine-induced complications. Heightened therapeutic effects of nanocurcumin are discussed.

Keywords: Apoptotic molecule, Cytokines, Female hormones, Nicotine, Nanocurcumin.

Introduction

Intake of nicotine through tobacco (smoking or through any routes) generates oxidative stress [1]. It disrupts the immune system resulting cardiac disorders [2], and various cancers [3]. Tobacco consumptions in different forms like smoking of beedi, leaf tobacco chewing, consumption of zarda pellets are a common addiction in female population of India, particularly women who are socio-economically handicapped. Females are seen to differ in strength and nature of immune responses against various diseases [4]. Females are more susceptible to immunopathology, especially autoimmune disorders compared to males and smoking-related complications and mortality [5]. Nicotine-addicted women have greater increased risk of coronary heart disease [6].

Nicotine affects blood cells and hampers

the cellular and immune responses due to the generation of reactive oxygen species [7]. It reduces the haemoglobin concentration and therefore body loses its normal ability to supply oxygen in the vital organs leading to shortness of breath and others complications [8]. In serious cases, the symptoms like inflammation of arms and legs, excessive sweating, heartburn, vomiting etc. are noted in some individuals. Nicotine also induces DNA damage in the tissues [9].

Oxidative stress causes oxidative damage in the tissues (e.g., liver, kidney, ovary etc.) leading to several diseases. Nicotine abuse elicits acute phase response in liver by activating monocytes and macrophages and thereby causes acute liver injuries [10]. In addition, nicotine enhances the productions of interleukin-4 (IL-4) and interleukin-6 (IL-6) [11]. IL-4 induces differentiation of native helper Th0 cells to Th2 cells. Increased production of Th2 cells is associated with allergies and various types of cancers [12]. Higher expression of IL-6 is associated with the development of encephalitis in children and asthma [13]. Aberrant expression of IFN-γ, an important activator of macrophages associated with auto-inflammatory and autoimmune diseases, inhibits viral replication directly and possesses immune-stimulators and immune-modulators characters [14]. Exposure to tobacco smoke leads to the over-expression of tumour necrosis factor alpha (TNF-α) [15]. Nicotine also affects the Bcl-2 vs. Bax ratio [16]. These two molecules are important apoptotic proteins. The pro-apoptotic proteins in the Bcl-2 family (including Bax) normally act on the mitochondrial membrane to promote permeability. The activity of pro-apoptotic protein is inhibited by the action of Bcl-2 and its relative-protein Bcl-Xl [17].

In addition, nicotine alters the function of endocrine system and affects the release of female hormones, which are very important agents for the protection of women health [18]. Deficiency of estrogens results in the irregularity of periods, infertility, bone weakness, hot flashes, depression, and urinary tract infection [19]. Similarly, deficiency of progesterone affects the menstrual cycle, pregnancy, and embryogenesis of humans and other species [19].

Curcumin possesses a wide range of pharmacological properties [20]. It works against DNA damage also [9]. The promising therapeutic uses of curcumin are limited because of its poor water solubility and limited bioavailability [21]. Curcumin is well adapted by individuals but it exhibits toxicity in our body at high dosages [22]. Piperine has been used to enhance bioavailability of curcumin without altering the mechanism or magnitude of effect [23]. Several studies are designed to formulate nanocurcumin for enhancing its bioavailability [24]. The current work is an attempt to improve bio-distribution by increasing the bioavailability of curcumin which may help for better functioning of the immune system. We hypothesize that nanocurcumin may combat more effectively than curcumin against nicotine-induced immunological disruption and will lead to a healthy individual, particularly for nicotine-addicted female population.

Material and methods

Chemicals

Nicotine hydrogen tartrate, curcumin, α-Lactalbumin (α-LA), Cytochrome complex (Cyt-c) and Haemoglobin (Hb) proteins are purchased from Sigma Aldrich Co. (St. Louis, MO, USA). All other analytical grade chemicals are supplied either by SpectroChem Pvt. Ltd. (India) or Merck (India). PureGene AG (Zeiningen, Switzerland) made ELISA kits supplied by Genetix Biotech Asia Pvt. Ltd. (India) are used for the detection of cytokines, apoptotic protein and steroidogenic hormones. Nanocurcumin used in the study is prepared in our laboratory following the methodology published elsewhere [24].

Animals and treatments

Female albino rats of Wistar strain (*Rattus norvegicus*), 30 in number, 60-75 days old, weighing 140-150 g are procured from the Animal Housing Facility and maintained with normal protein diet according to the guidelines of the Institutional Animal Ethics Committee of the Jadavpur University, Kolkata, India (Ref. No.: AEC/PHARM/1502/14/2015, Dated:

30/07/2015) [24]. The animals were maintained with normal protein diet and divided into 6 groups, each containing 5 animals as described below -

Control group (C) - Animals injected with 0.2 ml physiological saline.

Nicotine treated group (NT) - Animal injected by effective dose of nicotine.

Nicotine treated and curcumin supplemented group (NTCS) - Animal injected by effective dose of nicotine followed by supplementation of effective dose of curcumin orally.

Nicotine treated and nanocurcumin supplemented group (NTNCS) - Animal injected with effective dose of nicotine followed by supplementation of effective dose of nanocurcumin orally.

Curcumin supplemented group (CS) - Animal supplemented of effective dose of curcumin orally.

Nanocurcumin supplemented group (NCS) - Animal supplemented of effective dose of nanocurcumin orally.

A solution of nicotine hydrogen tartrate salt (2.5 g of nicotine salt dissolved in per mL of normal saline water) is prepared as stock solution. The nicotine solution is diluted by normal saline water to make a final concentration 2.5 µg/µL. Before use, the required volume of nicotine solution (based on the bodyweights of the animal) is taken in a tube to which normal saline water is added to make a volume of ~ 0.2 ml, which contains effective dose of the drug and is injected subcutaneously daily to the respective animals. Both curcumin and nanocurcumin are separately dispersed in sterilized distilled water and administered by oral gavages after 3 h of nicotine treatment to the respective groups of animal. The effective doses of the drug i.e., nicotine (2.5 mg/kg bodyweights/day), curcumin (80 mg/kg bodyweights/day) and nanocurcumin (4 mg/kg bodyweights/day) are determined in our previous study [24]. Injection of effective dose of nicotine and oral supplementation of effective doses of curcumin and nanocurcumin respectively are continued for 21 days. After 21 days, animals are kept under fasting condition for 12 h and eradicated on the next day after mild anaesthesia. Blood samples are collected immediately from the heart and stored in without anticoagulant (heparin) containing containers. Sera are separated and stored at - 20ºC for future analysis. Vital organs are dissected out and stored for future studies in vacuum desiccators at -20ºC to prevent auto-oxidation.

Preparation and characterization of nanocurcumin

The synthesis of nanocurcumin is described earlier [24]. In brief, a solution of 0.018 M curcumin is prepared by dissolving curcumin in dichloromethane (DCM). The solution is added to warm (50ºC) Milli-Q ultrapure water with a flow rate of 0.2 mL/min for 10 min under ultrasonic-pulse sonication subjected to constant stirring (Hielscher Ultrasonic Processor- UP100H, Germany). The solution containing nanocurcumin turned yellow and was separated by centrifugation. It is then sterilized by autoclaving, freeze-dried at -80ºC followed by lyophilization (Eyela-FDU- 2000, Japan). A dry orange coloured powder of nanocurcumin is obtained. The details of characterization of the nanocurcumin are discussed earlier [24] where, the morphology of the nanoparticles is observed by Field Emission Scanning Electron Microscopy (FE-SEM, FEI INSPECT F50, The Netherlands), and characterized by Fourier transform infrared spectroscopy (FTIR) (Perkin-Elmer FTIR-1600, USA). The crystalline nature of the nanocurcumin powder is determined by X-ray diffractometer (Bruker AXS, Inc., Model D8, WI).

Total protein estimation from tissues and protein oxidation from serum

The total protein content of various tissues from different vital organs (liver, kidney and ovary) are estimated according to the method of Lowry *et al.* [25].

Protein oxidation is determined by measuring the carbonyl content of serum as described by Levine *et al.* [26]. Here, serum was first diluted with phosphate-buffered saline (10 mM sodium phosphate, pH 7.4, and 0.14 M NaCl) in the ratio 1 : 40, and centrifuged at 12,000 rpm for

10 min to remove all the particulate materials. Cold trichloroacetic acid (TCA, 20% final concentration) was added to the serum to precipitate the serum proteins by centrifugation similarly for 5 min. The precipitated protein was then dispersed to a solution containing 10 mM DNPH in 2 N HCl and kept in the dark at room temperature for 1 h with repeated vortexing in every 15 min. The protein solution was then mixed with cold TCA (final concentration 10%) and centrifuged at 12000 rpm for 5 min. After discarding the supernatants, the protein pellets were washed with 10% TCA through centrifugation similarly and 1 ml of ethanol/ethyl acetate (in the ratio of 1:1, v/v) was added to it to eliminate any free DNPH. Samples were then suspended in 6 M guanidine hydrochloride and kept at 37°C for 15 min with vortex mixing. Carbonyl contents present in the protein was estimated from the absorbance at 366 nm by using a molar absorption coefficient of 22,000 M^{-1} cm^{-1}.

ELISA of cytokines, apoptotic proteins and steroidogenic hormones

Nicotine, cucumin and nanocurcumin induced expression of different cytokines (IL-4, IL-6, TNF-α, and IFN-γ) and apoptotic regulating proteins (Bcl-2 and Bax) in the serum of rats under different groups are determined as per the protocols outlined in Quantikine Immunoassay Kits (PureGene).

PureGene ELISA Kits for Progesterone (PG-6770R) and for 17-β Estradiol (PG-5600) are used for detection of the total unconjugated form of steroidogenic hormones.

Molecular docking study

Human serum proteins, α-LA and Cyt-c are chosen for the Molecular docking studies. The docking experiments are performed by using GemDock, a program for computing a ligand conformation and orientation relative to active site of the receptor and Ucsf Chimera 1.13.1., which is an extensive molecular modeller algorithm. From protein data bank, α-LA protein (PDB ID: 1A4V) and Cyt-c protein (PDB ID: 5EXQ) are used as receptor molecules and nicotine (PubChem CID: 89594) and nanocurcumin (PubChem CID:969516) are taken as ligand molecules for docking. Human serum protein has 73% homology with the serum protein of *Rattus norvegicus* and thus adopted for molecular docking study. The chemical structure of nanocurcumin and curcumin being same, the PubChem ID of curcumin is considered for further analysis [27].

***In vitro* interaction study**

Earlier, it is observed that addition of 500 µM of nicotine completely suppressed the absorbance peaks of different cellular proteins or bio-molecules (50 µg/ml water) [8]. Therefore, the initial absorbance spectrum of nicotine hydrogen tartrate solution (500 µM) is recorded from 230 nm to 300 nm by using a UV-Visible spectrophotometer. Freshly synthesized nanocurcumin is then gradually added to the nicotine solution (final concentration of nanocurcumin to the mixture solution ranging from 10 µM to 100 µM), incubated for 15 minutes at ambient temperature and the absorbance spectrum of each addition is recorded from 230 to 300 nm. All the absorbance data of the interacting solutions are taken against its corresponding reference or control solutions.

In nicotine-treated α–LA protein vs. nano-curcumin interaction, at first absorbance spectrum of α–LA protein solutions (50 µg/ml water) is recorded from 230 nm to 500 nm. Nicotine (500 µM) is then added to the α-LA protein solution and absorbance spectrum of the mixture solution is recorded similarly from 230 nm to 500 nm. Various concentrations of nanocurcumin are added then gradually to the nicotine-treated protein solution so that the final concentration of nanocurcumin in the solution mixture ranges from 10 µM to 100 µM and the absorbance spectra are recorded similarly from 230 nm to 500 nm. Similar experiments are performed by using nicotine-treated Cyt-c protein vs. nanocurcumin.

The fluorescence spectra of α-LA (50 µg/ml water) or Cyt-c protein (50 µg/ml water) and nicotine (500 µM)-treated α-LA (50 µg/ml water) or Cyt-c protein (50 µg/ml water) are studied by intrinsic fluorescence spectroscopy. The excitation wavelengths are fixed at 255 nm

for α-LA protein and 420 nm for Cyt-c protein. The emission spectra for both are recorded from 300 to 600 nm. The spectra of nicotine (500 µM)-treated α-LA (50 µg/ml water) or Cyt-c protein (50 µg/ml water) vs. different concentrations of nanocurcumin are recorded similarly. The final concentrations of nanocurcumin are attained between 10 µM to 100 µM in the nicotine-treated protein solutions.

Statistical analysis

The experimental set up is repeated twice and the data (N = 10) are tabulated as a mean ± standard deviation (S.D.). The statistical analyses are done by one way analysis of variance (ANOVA), where * indicates $p < 0.01$ and ** indicates $p < 0.001$ of the data in comparison to nicotine treatment. Similarly, # is $p < 0.01$ and ## is $p < 0.001$ of the data compared to nicotine + curcumin treatment. The data within the parenthesis represent the average percentage of increase (↑) or decrease (↓) relative to the control.

Results

Nicotine treatment decreased the total protein contents of liver, kidney and ovary tissues (Table 1). The protein contents of those tissues are increased more significantly by supplementation of nanocurcumin ($p < 0.001$) than curcumin ($p < 0.01$). Almost 4 times more carbonyl contents are detected in the serum proteins of nicotine-treated rats. In contrast, both curcumin or nanocurcumin supplementation showed a reduction of carbonyl content in the serum protein of nicotine-treated animals. The effect of nanocurcumin against protein oxidation is more pronounced than curcumin (Table 1).

The levels of IL-4, IL-6, TNF-α and IFN-γ in the serum are increased in nicotine treated rats (Table 2), while a significant decrease of cytokine levels are observed in rats supplemented with either curcumin or nanocurcumin. The reduction is highly significant ($p < 0.001$) for nanocurcumin than that of curcumin ($p < 0.01$). The low level of apoptotic molecule Bcl-2 due to nicotine stress is increased effectively ($p < 0.01$) by curcumin and more effectively ($p < 0.001$) by nanocurcumin (Table 3). The increased concentration of Bax in the nicotine-treated serum of rats is decreased significantly ($p < 0.01$) by supplementation of curcumin and nanocurcumin both. Interestingly, nanocurcumin supplemented female rats restored effectively ($p < 0.001$) the steroidogenic hormonal levels (estrogen and progesterone) of nicotine-treated female rats (Table 4).

In-silico docking studies of nicotine vs. α-LA protein and nanocurcumin vs. α-LA protein clearly demonstrate the formation of complex structures between nicotine and α-LA protein (Fig. 1A) as well as between nanocurcumin and α-LA protein (Fig. 1B). Out of 10 ligand conformations, the best free binding energy between nanocurcumin

Table 1. Total protein content in wet tisssue (mg/g) of animals in different groups

Parameter	Groups					
	(C)	(NT)	(NTCS)	(NTNCS)	(CS)	(NCS)
Liver	23.9 ± 0.10	17.8 ± 1.0 (25.5↓)**	19.8 ± 1.8 (17.2↓)**	21.2 ± 1.6 (11.5↓)**#	24.1 ± 0.1 (1.0↑)	25.3 ± 0.1 (5.8↑)
Kidney	20.6 ± 3.2	16.2 ± 2.0 (21.5↓)**	17.9 ± 0.3 (13.2↓)**	19.1 ± 1.6 (7.3↓)*##	22.1 ± 0.5 (7.2↑)	22.7 ± 0.5 (10.2↑)
Ovary	20.9 ± 1.1	15.8 ± 1.6 (24.3↓)**	17.8 ± 1.9 (15.0↓) **	18.7 ± 1.8 (10.7↓)*#	21.1 ± 1.4 (1.0↑)	22.1 ± 1.4 (5.7↑)

The experimental setup was repeatd twice and all data were averaged over n =12 animals and given mean ± S.D. Significance levels were determined by usuing ANOVA, where * implied significant ($p < 0.01$) and ** implied more significant ($p < 0.001$) of the data when compared with the nicotine treatmentl. Similarly, # implied significant ($p < 0.1$) and ## implied more significant ($p < 0.001$) of the data when compared with nicotinxe + curcumin tratment. The data within the parenthesis represent the average percentage of increase (↑) or decrease (↓) relative to the control

Table 2. ELISA of cytokine molecules

Parameter	Groups					
	(C)	(NT)	(NTCS)	(NTNCS)	(CS)	(NCS)
IL-4 (pg/mL)	33.6 ± 1.1	51.9 ± 3.5 (54.5↑)**	41.7 ± 2.1 (24.1↑)*	36.5 ± 1.0 (8.6↑)**##	32.1 ± 1.2 (4.5↓)	32.8 ± 1.2 (2.4↓)
IL-6 (pg/mL)	136.8 ± 5.9	303.5 ± 3.5 (121.8↑)**	201.0 ± 1.7 (46.9↑)*	154.1 ± 7.1 (12.6↑)**##	146.4 ± 17.1 (7.0↑)	144.4 ± 17.1 (5.6↑)
TNF-α (pg/mL)	172.8 ± 6.0	403.4 ± 6.5 (133.4↑)**	217.4 ± 3.4 (25.6↑)*	188.5 ± 4.2 (9.1↑)**##	150.6 ± 5.6 (12.8↓)	156.4 ± 5.6 (9.5↓)
IFN-γ (pg/mL)	92.0 ± 6.4	136.0 ± 6.0 (47.8↑)**	110.2 ± 6.4 (19.8↑)**	97.0 ± 7.7 (5.2↑)*##	89.7 ± 10.3 (3.0↓)	90.3 ± 10.3 (1.8↓)

The experimental setup was repeatd twice and all data were averaged over n =12 animals and given mean ± S.D. Significance levels were determined by usuing ANOVA, where * implied significant ($p < 0.01$) and ** implied more significant ($p < 0.001$) of the data when compared with the nicotine treatment. Similarly, # implied significant ($p < 0.1$) and ## implied more significant ($p < 0.001$) of the data when compared with nicotine + curcumin tratment. The data within the parenthesis represent the average percentage of increase (↑) or decrease (↓) relative to the control

Table 3. Apoptosis regulator proteins

Parameter	Groups					
	(C)	(NT)	(NTCS)	(NTNCS)	(CS)	(NCS)
BCL-2 (ng/mL)	1.99 ± 0.01	0.56 ± 0.05 (71.8↓)**	0.93 ± 0.11 (53.3↓)**	1.42 ± 0.04 (28.6↓)**##	2.08 ± 0.20 (4.5↑)	2.14 ± 0.20 (7.5↑)
BAX (ng/mL)	1.02 ± 0.06	1.68 ± 0.21 (64.7↑)**	1.44 ± 0.02 (39.3↑)**	1.40 ± 0.02 (37.3↑)**#	0.98 (3.9↓)	0.94 ± 0.20 (1.1↓)

The experimental setup was repeatd twice and all data were averaged over n =12 animals and given mean ± S.D. Significance levels were determined by usuing ANOVA, where * implied significant ($p < 0.01$) and ** implied more significant ($p < 0.001$) of the data when compared with the nicotine treatment. Similarly, # implied significant ($p < 0.1$) and ## implied more significant ($p < 0.001$) of the data when compared with nicotine + curcumin tratment. The data within the parenthesis represent the average percentage of increase (↑) or decrease (↓) relative to the control

Table 4. ELISA of Steroidogenic hormonal levels

Parameter	Groups					
	(C)	(NT)	(NTCS)	(NTNCS)	(CS)	(NCS)
Steroidogenic hormones						
Estradiol (pg/mL)	81.1 ± 3.3	61.7 ± 2.4 (23.9↓)**	69.4 ± 2.4 (14.4↓)*	77.8 ± 4.7 (4.1↓)*##	85.0 ± 1.7 (1.0↑)	87.2 ± 1.7 (7.5↑)
Progesterone (ng/mL)	12.41 ± 0.05	8.98 ± 0.02 27.6↓)**	9.53 ± 0.09 (23.2↓)**	11.2 ± 0.22 (9.7↓)*##	12.87 ± 0.80 (5.7↑)	13.68 ± 0.80 (10.2↑)

The experimental setup was repeatd twice and all data were averaged over n =12 animals and given mean ± S.D. Significance levels were determined by usuing ANOVA, where * implied significant ($p < 0.01$) and ** implied more significant ($p < 0.001$) of the data when compared with the control. Similarly, # implied significant ($p < 0.1$) and ## implied more significant ($p < 0.001$) of the data when compared with nicotine tratment. The data within the parenthesis represent the average percentage of increase (↑) or decrease (↓) relative to the control

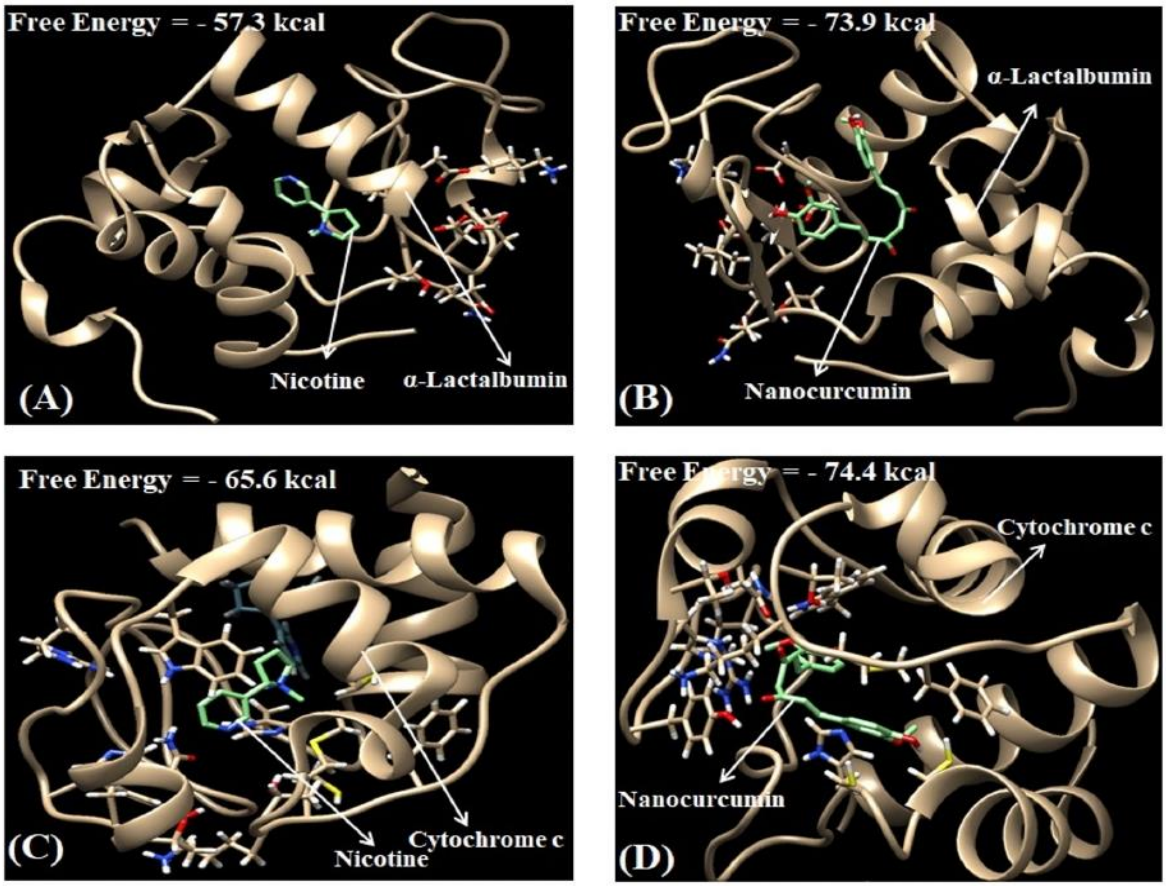


Figure 1. Molecular docking studty between nicotine/nanocurcumin vs. α-LA/Cyt-c protein. Inset A shows the docking interaction between nicotine vs. α-LA; Inset B shows the docking interaction between nanocurcumin vs. α-LA; Inset C shows the docking interaction between nicotine vs. Cys-c, and Inset D shows the docking interaction between nanocurcumin vs. Cyt-c protein

and α-LA protein is around -73.9 kcal. The free binding energy between α-LA vs. nicotine seems to be -57.3 kcal. Similar docking experiment on nicotine vs. Cyt-c and nanocurcumin vs. Cyt-c demonstrate that the probability of complex formation between nanocurcumin vs. Cyt-c is more favourable (free energy -74.4 kcal) than nicotine vs. Cytc complex formation (binding free energy - 65.6 kcal).

The UV-Visible spectral studies indicate that nanocurcumin causes significant suppression of the characteristic absorption peak (λ_{max} = 260 nm) of nicotine with the gradual addition of nanocurcumin (10 µM to 100 µM) to the nicotine solution (500 µM) (Fig. 2A). Similarly, the characteristic absorption peak of α-LA (λ_{max} = 280 nm) and Cyt-c (λ_{max} = 410 nm) proteins are suppressed substantially by nicotine (Figs. 2A and 2B). Gradual addiction of nanocurcumin reversed the quenching action of nicotine on these proteins.

The intrinsic fluorescence emission spectrum of α-LA with emission maxima at λ_{max} = 345 after excitation at 255 nm is shown in Fig. 3A. The blue shift of maxima (λ_{max} = 330 nm) of α-LA protein occurred due to the presence of nicotine. Gradual addition of nanocurcumin to the nicotine-treated protein solution, the fluorescence intensity of the treated protein is seen to increase and wavelength maxima overlap the native protein. Similar interaction is observed between nanocurcumin vs. nicotine-treated Cyt-c protein (Fig. 3B).

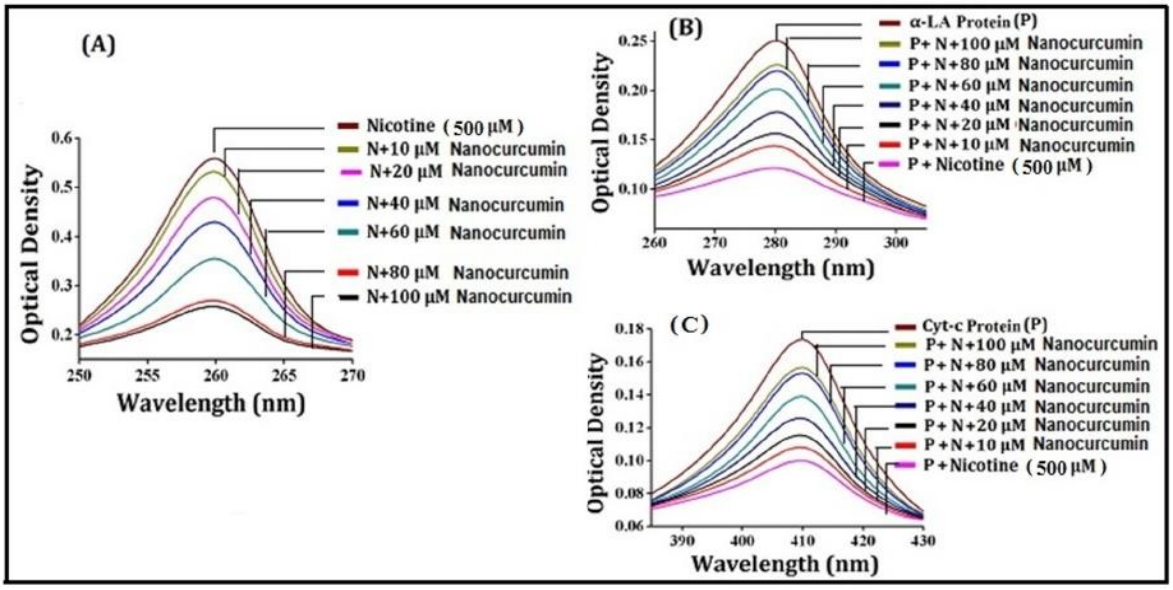


Figure 2. UV-Vis study of nicotine-nanocurcumin interaction. Inset A - Nicotine hydrogen tartrate solution (500 µM) was taken in a cuvette and its absorbance spectrum was recorded (230 nm - 300 nm) using UV-Visible spectrophotometer. Different concentration of freshly synthesized Cur-NPs was added to nicotine solution gradually (final concentration of Cur-NPs was 10 µM to 100 µM), incubated 15 min at ambient temperature and absorbance spectra of each interaction was recorded at 230 - 300 nm; Inset B shows the interaction absorption spectra of nanocurcumin vs. nicotine (500 µM) mediated pure protein of α-LA (50 µg/ml water); Inset C shows the interaction absorption spectra of nanocurcumin vs. nicotine (500 µM) mediated pure protein of Cyt-c (50 µg/ml water)

Discussion

Curcumin is a well-known potent anti-oxidative, anti-carcinogenic, and anti-inflammatory agent [22,24]. Inadequate bioavailability and adverse pharmacokinetics limit the use of curcumin against various complications [21]. Scientists working on curcumin are trying to increase its usage against several diseases through the formulation of nanocurcumin, because it is more water soluble and has higher physical stability [24].

It is known that the constituents of cigarette smoke (mainly nicotine) and the systemic inflammatory mediators enhance proteolysis and inhibit protein synthesis, leading to a loss of muscle mass. Nicotine causes oxidative damage in the tissues (e.g. liver, kidney, ovary tissues etc.) leading to several diseases. Our investigation shows that nicotine causes a decrement (20 to 25%) of tissue protein (liver, kidney and ovary), either by decreasing protein biosynthesis or increasing protein catabolism (Table 1). Depletion of tissue protein certainly declines the self-protective efficacy of the body due to the structural and functional deformity of the cells. Curcumin protects our body by regulating the action of other targets, such as apoptotic proteins, cell cycle regulators, growth factors, receptors, protein kinases, and transcription factors [9,28]. Thus, curcumin or nanocurcumin acts by blocking nicotine-induced stress in different tissues (Table 1). Increased oxidative stress by nicotine aggravates protein oxidation. It leads to increase in carbonyl contents of the tissues resulting dysfunction of biological enzymes, hormones, and immune system [7]. The anti-oxidative nature of curcumin reduces carbonyl content in the tissues by inhibiting the protein oxidation [26,29]. Our results show that nanocurcumin, being more bio-available molecule, has a better self-protective ability against protein oxidation than that of curcumin (Table 1).

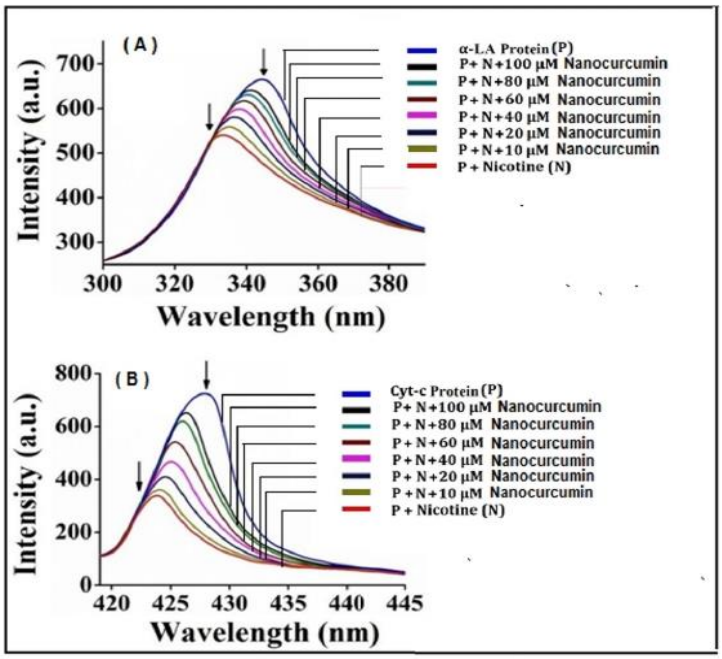


Figure 3. Fluorescence study of protein-nicotine-nanocurcumin interaction. The Fluorescence measurements of protein(s) (50 µg/ml) under different conditions of nicotine (500 µM) and nanocurcumin (10 µM to 100 µM) were studied by intrinsic fluorescence spectroscopy. The excitation wavelength was fixed at 255 nm for α-LA protein and 420 nm for Cyt-c protein respectively. The emission wavelength windows were recorded at 300-600 nm. At first nicotine was added to the protein solution and spectrum was taken similarly. Next, to the nicotine added protein solution, curcumin nanoparticles (final concentration 10 µM to 100 µM) was added gradually and absorption spectrum was recorded again

At the physiological level, IL-6 plays an important role in the cytokine network. However, elevated expression of IL-6 is related to fatigue scores. Higher magnitude of TNF-α can also be seen in fatigue condition. In particular, scientists have observed that childhood trauma and stress factors enhance the levels of pro-inflammatory cytokines (IL-6, TNF-α, IL-1β, INF-γ) and chemokines (MCP-1) in either patients or controls [30]. Our results thus corroborate with the earlier reports in which it is shown that nicotine augments the secretion of pro-inflammatory cytokines and generates excess amount of ROS within the cells [12,14]. Furthermore, it is known that curcumin supplementation leads to simultaneous co-expression of p53 and TGF-β along with down-regulation of Th1 & Th2 cytokines and increases immunity of cells. Here we find that the elevated levels of cytokines under nicotine exposure, are normalized by curcumin or nanocurcumin supplementation (Table 2). The immune response of nanocurcumin is more prominent than that of curcumin in terms of different cytokine levels under nicotine stressed condition (Table 2).

Bcl-2 plays an important role by promoting the action of cellular survival and inhibiting the function of pro-apoptotic proteins. Damaged Bcl-2 gene is identified as a cause of a number of cancers and a possible cause of schizophrenia and autoimmunity [16]. Hardwick *et al.* have shown that the damaged Bcl-2 gene is a cause of resistance to cancer treatments also [17]. They have also shown that the apoptosis regulator protein Bax forms a hetero-dimmer with Bcl-2 and functions as an apoptotic activator. This study shows that there is a decrement of anti-apoptotic molecule Bcl-2 and increment of pro-apoptotic

molecule Bax in nicotine treated rats (Table 3). Nanocurcumin works more effectively to restore the normalcy of the levels of apoptotic molecules and plays a promising beneficial immuno-modulatory role against apoptosis.

Nicotine is seen to cause a significant reduction of 17 β-estradiol and progesterone levels in normal protein fed condition which in turn affects the reproductive system (Table 4). This is in accordance with our other findings [18,24]. 17 β-estradiol levels are decreased in nicotine treated group due to the anti-estrogenic effect of nicotine. Nicotine also decreases progesterone level by increasing PGF2α and VEGF-mRNA expressions. Here, curcumin is seen to reduce the action of nicotine on estrogens metabolism due to its high estradiol binding capacity. Nanocurcumin is seen to have better effect on increasing of 17β-estradiol and progesterone levels of nicotine treated animals because of its higher bioavailability and greater binding ability to nicotine.

Molecular docking experiments provide a theoretical interpretation about the interactions amongst the nicotine, nanocurcumin and cellular proteins (α-LA and Cyt-c protein). The docking results indicate that nanocurcumin (curcumin) binds to α-LA protein (free energy -73.9 kcal) to form a nanocurcumin - α-LA protein complex. The formation of nanocurcumin - α-LA protein complex is more favourable than that of nicotine bound α-LA protein (free energy -57.3 kcal) complex. Similarly, nanocurcumin bound Cyt-c protein (free energy -74.4 kcal) complex is more favourable than the nicotine bound Cyt-c protein (free energy -65.6 kcal) complex (Fig. 1). This implies that nanocurcumin resists the nicotine to further interact with the protein moiety due to its higher binding probability (as revealed from free energy calculations) and thereby it provides extra protection against nicotine. Our previous study showed that curcumin had a strong interaction with nicotine (free energy -51.29 kcal) [23]. Strong binding ability of nanocurcumin (curcumin) may help the molecule to be a potent immune-modulator against nicotinic effects.

The interpretations of Molecular docking experiments have been substantiated by the wet-lab interaction studies amongst nanocurcumin, nicotine and cellular proteins. Nicotine has a tendency to bind with the tryptophan residue of proteins [8], due to which the absorption intensities of the two important mammalian proteins (α-LA and Cyt-c) are quenched by nicotine (Fig. 2A and 2B). Nanocurcumin also shows strong interaction with nicotine. Thus, in presence of nanocurcumin, availability of free nicotine molecules for interaction with the protein is less in the reaction medium. Nicotine, therefore, produces lesser damage to the proteins in the presence of nanocurcumin.

The emission maximum wavelength of α-LA protein (345 nm) is seen to shift towards 330 nm (blue shift) due to interaction with nicotine. It indicates increase in absorption energy due to the binding of nicotine with the internal sites of the protein leading to the conformational change of the protein (Fig. 3A). With the gradual addition of nanocurcumin to the nicotine bound protein solution, the intensity, as well as the emission maximum wavelength, is significantly overlap the emission maxima of the native protein. Nanocurcumin thus steadily assists the protein to restore its native state by lowering the energy due to the H-bonding or π-π conjugation. A similar phenomenon is observed in case of Cyt-c emission spectra (Fig. 3B).

The bioavailability of nanocurcumin is greater than that of curcumin due to its higher aqueous solubility. The higher binding ability to nicotine as well as to cellular proteins, it protects the cells from nicotinic effects. The enhanced self-protective efficacies of the tissues related to protein content, protein oxidation, cytokines and apoptotic markers expression and hormonal concentrations are more pronounced in case of nanocurcumin supplementation in rats than curcumin-treated group. Nanocurcumin thus may be used as a significant protective and therapeutic agent against nicotine-induced complications particularly in nicotine intoxicated female population.

Authors contribution

Anwesha: Methodology, Data curation, Investigation. Krishna Chattopadhyhay: Concep-

tualization, Visualization, Writing- Original draft preparation. Somashree Biswas: Data curation, Investigation. Bhola Nath Paul: Reviewing and Editing. Brajadulal Chattopadhyhay: Conceptualization, Validation, Writing- Original draft preparation, Supervision

Financial support
Jadavpur University, Kolkata, India

Acknowledgments
The financial support obtained from Jadavpur University is gratefully acknowledged. Dr. K. Chattopadhyay is a recipient of fellowship from Women Scientist-B scheme, Department of Science and Technology (Sanctioned no.: DST/Disha/SoRF-PM/013/2015(G), dated; 08/03/2016). Authors are also grateful for the experimental and technical supports provided by the Biophysics Laboratory, Department of Physics, Jadavpur University. The generous helps for executing ELISA from Dr. Hemanta Koley and Dr. Asish Mukhopadhyay, NICED, Kolkata are also gratefully acknowledged.

Conflict of interest
The authors declare no conflict of interest of any kind related to this work.

References

1. **Padmavathi, P., Raghu, P.S., Padmakanthan, S., Reddy, V.D., Bulle, S., Marthadu, S.B., Maturu, P. & Varadacharyulu, N.C. (2018).** Chronic cigarette smoking-induced oxidative/ nitrosative stress in human erythrocytes and platelets. Mol. Cellu. Toxicol. 14: 27-34.
2. **Rigotti, N.A., Pipe, A.L., Benowitz, N.L., Arteaga, C., Garza, D., Tonstad, S. (2010).** Efficacy and safety of varenicline for smoking cessation in patients with cardiovascular disease. Circulation. 121: 221-229.
3. **Jensen, K., Afroze, S., Munshi, M.K., Guerrier, M., Glaser, S.S. (2012).** Mechanism for nicotine in the development and progression of gastrointestinal cancers. Transl. Gastrointest. Cancer. 1: 81-87.
4. **Roved, J., Hansson, B., Tarka, M., Hasselquist, D., Westerdahl, H. (2018).** Evidence for sexual conflict over major histocompatibility complex diversity in a wild songbird. Proc. Royal Soc. B- Biol. Sci. 285(1884): 20180841.
5. **Allen, A.M., Oncken, C., Hatsukami, D. (2014).** Women and Smoking: The Effect of Gender on the Epidemiology, Health Effects, and Cessation of Smoking. Curr. Addict. Rep. 1(1): 53-60.
6. **Huxley, R.R., Woodward, M. (2011).** Cigarette smoking as a risk factor for coronary heart disease in women compared with men: a systematic review and meta-analysis of prospective cohort studies. Lancet. 378(9799): 1297-1305.
7. **Phaniendra, A., Jestadi, D.B., Periyasamy, L. (2015).** Free Radicals: Properties, Sources, Targets, and Their Implication in Various Diseases. Ind. J. Clin. Biochem. 30(1): 11-26.
8. **Zeng, Q., Shen, L.J., Li, S., Chen, L., Guo, X., Qian, C.N., Wu, P.H. (2016).** The effects of hemoglobin levels and their interactions with cigarette smoking on survival in nasopharyngeal carcinoma patients. Cancer Med. 5(5): 816-826.
9. **Banerjee, S., Chattopadhyay, K., Chhabra, J.K., Chattopadhyay, B.D. (2012).** Protein dependent fate of hepatic cells under nicotine induced stress and curcumin ameliorated condition. Eur. J. Pharmacol. 684: 132-145.
10. **Hoek, J.B., Pastorino, J.G. (2002).** Ethanol, oxidative stress, and cytokine-induced liver cell injury. Alcohol. 27: 63-68.
11. **Gour, N., Wills-Karp, M. (2015).** IL-4 and IL-13 Signalling in Allergic Airway Disease. Cytokine. 75(1): 68-78.
12. **Ul-Haq, Z., Naz, S., Mesaik, M.A. (2016).** Interleukin-4 receptor signalling and its binding mechanism: A therapeutic insight from inhibitors tool box. Cytokine Growth Factor Rev. 32: 3-15.
13. **Peters, M.C., McGrath, K.W., Hawkins, G.A., Hastie, A.T., Levy, B.D., Israel, E., Phillips, B.R., Mauger, D.T., Comhair, S.A., Erzurum, S.C., Johansson, M.W.,**

Jarjour, N.N., Coverstone, A.M., Castro, M., Holguin, F., Wenzel, S.E., Woodruff, P.G., Bleecker, E.R., Fahy, J.V. & National Heart, Lung, and Blood Institute Severe Asthma Research Program (2016). Plasma interleukin-6 concentrations, metabolic dysfunction, and asthma severity: a cross-sectional analysis of two cohorts. Lancet Respir. Med. 4(7): 574-584.

14. **Schoenborn, J.R. and Wilson, C.B. (2007).** Regulation of interferon-gamma during innate and adaptive immune responses. Adv. Immunol. 96: 41-101.
15. **Strzelak, A., Ratajczak, A., Adamiec, A., Feleszko, W. (2018).** Tobacco Smoke Induces and Alters Immune Responses in the Lung Triggering Inflammation, Allergy, Asthma and Other Lung Diseases: A Mechanistic Review. Int. J. Environ. Res. Public Health. 15(5): 1033.
16. **Jalili, C., Salahshoor, M.R., Moradi, M.T., Ahookhash, M., Taghadosi, M., Sohrabi, M. (2017).** Expression Changes of Apoptotic Genes in Tissues from Mice Exposed to Nicotine. Asian Pac. J. Cancer. Prev. 18(1): 239-244.
17. **Hardwick, J.M. and Soane, L. (2013).** Multiple functions of BCL-2 family proteins. Cold Spring Harb. Perspect. Biol. 5(2): a008722.
18. **Sinha, S., Maiti, M., Chattopadhyay, K., Chattopadhyay, B.D. (2012).** Potential amelioration of curcumin against nicotine-induced toxicity of protein malnourished female rats. J. Pharmacol. Toxicol. 7: 166-180.
19. **Jandikova, H., Duskova, M., Starka, L. (2017).** The Influence of Smoking and Cessation on the Human Reproductive Hormonal Balance. Physiol. Res. 66(3): S323-S331.
20. **Tung, B.T., Hai, N.T., Son, P.K. (2017).** Hepatoprotective effect of Phytosome Curcumin against paracetamol-induced liver toxicity in mice. Brazilian J. Pharma. Sci. 53(1): e16136.
21. **Anand, P., Kunnumakkara, A.B., Newman, R.A., Aggarwal, B.B. (2007).** Bioavailability of curcumin: problems and promises. Mol. Pharm. 4(6): 807-818.
22. **Lopez-Lazaro, M. (2008).** Anticancer and carcinogenic properties of curcumin: considerations for its clinical development as a cancer chemopreventive and chemotherapeutic agent. Mol. Nutrin. Food Res. 52(S1): S103-S127.
23. **Mhaske, D.B., Sreedharan, S., Mahadik, K.R. (2018).** Role of Piperine as an Effective Bioenhancer in Drug Absorption. Pharm. Anal. Acta. 9: 7.
24. **Chattopadhyay, K,, Samantam A., Mukhopadhyay, S., Chattopadhyay, B.D. (2018).** Potential amelioration of nicotine-induced toxicity by nanocurcumin. Drug. Dev. Res. 79: 119-128
25. **Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951).** Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193(1): 265-275.
26. **Levine, R.L., Williams, J.A., Stadtman, E.R., Shacter, E. (1994).** Carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol. 233: 346-357.
27. **Bhawana., Basniwal, R.K., Buttar, H.S., Jain, V.K., Jain, N. (2011).** Curcumin Nanoparticles: Preparation, Characterization, and Antimicrobial Study. J. Agricul. Food Chem. 59(5): 2056-2061.
28. **Sershen, H., Reith, M.E.A., Banay-Schwartz, M., Lajtha, A. (1982).** Effects of prenatal administration of nicotine on amino acid pools, protein metabolism, and nicotine binding in the brain. Neurochem. Res. 7: 1515-1522.
29. **Alisi, I.O., Uzairu, A., Abechi, S.E. (2020).** Molecular design of curcumin analogues with potent antioxidant properties and thermodynamic evaluation of their mechanism of free radical scavenge. Bulletin Natn. Res. Centre. 44: 137.
30. **Wang, M., Jiang, S., Zhou, L., Yu, F., Ding, H., Li, P., Zhou, M., Wang, K. (2019).** Potential Mechanisms of Action of Curcumin for Cancer Prevention: Focus on Cellular Signaling Pathways and miRNAs. Int. J. Biol. Sci. 15(6): 1200-1214.