Bioactivity guided isolation/ fractionation of anti-cancer principle from *Nyctanthes arbor-tristis* Linn

Thesis submitted

by

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CERTIFICATE

This is to certify that the thesis entitled ***"Bioactivity guided isolation/ fractionation of anti-cancer principle from Nyctanthes arbor-tristis Linn"*** *submitted by Shri. Birendra Nath Karan, who got his name registered on 29.05.2012 for the award of Ph.D. (Eng./ Pharmacy) degree of Jadavpur University is absolutely based upon his own work under the supervision of* ***Prof. Tapan Kumar Maity and Dr. Bikash Chandra Pal*** *and that neither his nor any part of the thesis has been submitted for any degree/ diploma or any other academic award anywhere before.*

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Declaration of originality and compliance of academic ethics

I hereby declare that the thesis contains literature survey and original research work by the undersigned candidate, as part of this Doctor of Philosophy (Ph.D.) course.

All information in this research work have been obtained and presented as per the academic and ethical rule.

I also declare that as per the rules and conduct, I have fully cited and referenced all materials and results those are not original of this work.

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Title of the Thesis: **"Bioactivity guided isolation/ fractionation of anti-cancer principle from *Nyctanthes arbor-tristis* Linn"**

Signature with Date

Dedicated to

My Late Parents

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PREFACE

According to the WHO, health is a state of "complete physical, mental, social well-being and not merely the absence of disease and infirmity." Various types of biochemical constituents are required and thousands of biochemical reactions need to take place within very short period in human body to maintain proper health. Abnormalities of the biochemical constituents manifest the cause of communicable and non-communicable diseases. Communicable diseases are the cause of various organisms and non-communicable diseases are the cause of abnormalities of biochemical constituents in the body. Drugs are required for the treatment of various diseases. According to Nobel laureate Paul Erhlich (1900) 'search of magic bullet (medicine) concept', Researchers also have to find out new compound (drug) from natural, semi-synthetic or synthetic sources for the mankind. Natural source is mainly plants. Phytochemical compounds are less toxic to the human body, cheap and moreover plants are easily available. For this reason this research work has been carried out based on the indigenous selected medicinal plant.

The thesis entitled, "Bioactivity guided Isolation/Fractionation of Anti-cancer Principle from *Nyctanthes arbor tristis* Linn." comprises the work done by the author in the Laboratory of Pharmaceutical Chemistry, Dept. of Pharmaceutical Technology, Jadavpur University, Kolkata, India for the degree of Doctor of Philosophy in Engineering /Pharmacy (Engg./Pharm.).

The various parts of the plants and their active compound(s) have been used for the treatment of many diseases in Indian traditional system of medicines. The sub Himalayan region including India, Nepal, Bangladesh, China etc. are the sources of medicinal plants. The demand of the phyto-medicines has been gradually increased throughout the world. So this research work has been performed to find out new drugs or lead compounds from the selected medicinal plant for the treatment of many ailments.

In the present study *Nyctanthes arbor-tristis* Linn. Family- Oleaceae, has been selected for bioactivity guided isolation, fractionation and characterization of isolated compound present in the leaf of the plant. The acute toxicity study has been performed to determine the dose levels for Pharmacological activities. The Pharmacological activities like anti-inflammatory,

anti-proliferative, antioxidant and anticancer etc. have been carried out (*in vitro* study) using isolated compound from leaf extract of *Nyctanthes arbor tristis* Linn. Similarly the antitumor, anticancer, antioxidant, histology activity index (HAI) of liver, histopathological architecture of liver of the mice have been investigated (*in vivo* study) using methanol leaf extract of *Nyctanthes arbor tristis* Linn.

In the conclusion of the thesis, various investigations have been performed in such a way that there are significant and justified relation between the Pharmacological activities and the research work.

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Chapter- 1

Introduction

1.1. Herbalism

According to WHO, herbalism is the use of various parts of the plants like leaves, fruits, seeds, stem, bark, wood, roots and rhizomes which may be entire, powdered or fragmented for the purpose of the healing of body function. The herbs and their preparations are effective and used as traditional medicine for diagnosis, mitigation, prevention and cure of diseases. As per the consideration of Food & Drug Administration (FDA), traditional herbal medicines are complementary with alternative medicines or whole medical system. Herbalism is the oldest medical practice using plants and foods for maintenance of good health including healing and building of the body. World herbalism includes Native American medicines, Western herbalism, traditional Chinese medicines, Ayurveda, traditional or folk medicines of the indigenous category. Various components of conventional medicines, Naturopathy, Homeopathy, are obtained from various parts of the plants and related with herbalism. Present concept of herbalism as per FDA includes dietary supplements like vitamins, minerals, glandular substances and unit of protein or amino acids. Herbalist takes some measures for the wellness and healing. The measures are detoxification of the herbal parts or their constituents, exercise, change of life style, hydration, fresh air including foods and herbs [1].

1.1.1. Herbalism in India

Herbalism in India consists of mainly four systems of medicines. They are namely:

1. Ayurveda system of medicines
2. Siddha system of medicines
3. Unani system of medicines
4. Homoeopathy system of medicines

1.1.2. Ayurveda system of medicines

This system is known as the mother of all systems of healing. Ayurveda system is considered as the science of life and it gives us knowledge of beneficial or toxic effect of herbal products for our health, diagnosis of the disease by the examination of pulse, urine, eye etc. Treatments of diseases are done by the drugs of herbal, mineral or animal origin. Generally

drugs are administered in the form of powder, infusion, decoction, fluid extract etc. Some identified plants having therapeutic activity are prescribed in Ayurveda Pharmacopoeia [2].

The ancient history of Ayurveda, the great physicians, namely Charaka, Sushruta, Madhva and Kashyapa were familiar personalities who were the pioneer of the development of this system of medicine or science of healing. Vagabhatta, Arundutta, Charapani, Dulhana were also famous for Ayurvedic activity [2].

For the evaluation of the Ayurveda drugs following parameters are to be considered:
Dravya (substance)
Guna (property)
Rasa (taste)
Virya (potency)
Vipka (post digestion effect)
Prabhva (therapeutics)
Karma (pharmacological action)

The famous Indian Ayurveda physician Charka described the method of collection of medicinal plants or the parts of plants in appropriate climatic condition. At present we extract the authenticated medicinal plants and isolate the compound(s). Isolated compounds are identified as alkaloid, glycoside, flavonoid etc. by the chemical test, melting point determination (solid compound), boiling point determination (Liquid compound), or by instrumental analysis methods (LC-MS, MASS SPECTROSCOPY, NMR, FTIR etc.). In Ayurveda, identification of the medicinal plants is a very tough task to maintain the Pharmacopoeial standard. A group of eight medicinal plants are known as **Astavarga** which have important role in Ayurveda formulation. Riddhi, Vriddhi, Jivaka, Rishava are the four herbal medicinal plants having importance in Ayurveda system of medicine [2].

The development of Ayurveda system of medicine is going on and plays a major role in the treatment of various diseases. Clinical trial of this dosage form must be done and documented the efficacy, pharmacological activity. Due to the demand of patient, efficacy, least toxicity, Ayurveda system of medicine will play an important activity in future of health care system [2].

Table- 1.1 : Herbal plants used in Ayurveda in various diseases due to presence of their active Phyto-chemical compound(s) [2]

Sl. No.	Name of the medicinal plants	Active constituent(s)	Clinical activity
1	*Pergularia pallida* Roxb.	Phenanthroindozolidine alkaloid,triphorinidine, Pergularinine	Anticancer
2	*Plumbago rosea* L	Naphthaquinone plumbagin	Anticancer
3	*Withania somnifera* Dunal	Withaferin A	Anticancer
4	*Rubia cordifolia*	Anthraquinone: rubidianin	Antioxidant
5	*Desmodium gangeticum*	Petrocarpenoid: gangetin	Anti-inflammatory
6	*Prema integrifolia* L.	Isoxazole Alkaloid: Premnazole	Anti-inflammatory
7	*Boswellia serrata*	Boswellic acid	Anti-inflammatory
8	*Tylophora asthmatica*	Phenanthroindalizine alkaloid: tylophorine	Anti-inflammatory, antispasmodic
9	*Commiphora mukul*	Sesquiterpene ketone: guggulsterones	Hypolipidemic
10	*Eclipta alba* L.	Apigenin, 4-hydroxy benzoic acid	Hepatoprotective
11	*Picrorhiza kurrooa*	Kutkins	Hepatoprotective
12	*Nadostachys jatamansi*	Sesquiterpene ketone: Jatamansone	Antiarrythmic, tranquilizer

1.1.3. Siddha system of medicines

Siddha system of medicine is a traditional system in India. It is Dravidian origin and mainly it is the ancient system of medicine for Tamilkalam (Tamil Nadu) in South India. There is a traditional concept that Lord Shiva transferred this knowledge of medicine to his wife Goddess *Parbati*. *Parbati* has transferred this knowledge to Nandi and ultimately it has been transferred to 18 Siddhas. Siddhas have extra ordinary powers. In Siddha medicine system metals, minerals, chemicals and herbs are used for cure of diseases. The herbal drug Cannabis is used first as a powerful painkiller in this system.

Siddha medicines were classified according to their properties:

i. Suvai (taste)
ii. Veerya (potency)
iii. Guna (chracteristic)
iv. Pirivu (class)
v. Mahimai (action)

Depending upon the mode of application, Siddha medicines are classified into two categories:

1) Internal medicines which are used for oral route.
2) External medicines which are used for application into the nasal cavity, eye, ear etc. [3,4] .

1.1.4. Unani system of medicines

Unani system of medicine is a traditional system of medicine or Unani Tibb. It is also known as Greco-Arab medicine. The Unani system of medicine has been developed in different periods and various geographical regions. The periods are Greek period, Arab-Persian period, Spanish period and Indian period. According to Unani system, health is considered as a state of body which consists of six elements like air; drinks and food; sleep and wakefulness; excretion and retention; physical activity; mental activity and rest. The diagnosis of disease based on this system is recording of pulse, examination of stool, urine and regular physical checkup.

Treatment procedures in the Unani systems are:

A) Regimental therapy by exercise, change of climate, massage, venesection, leaching, cupping, diet therapy etc.
B) Pharmacotherapy by the use of medicinal plants, minerals, animal derived compound alone or in combination.
C) Surgical measures are the last procedure of the treatment [5].

1.1.5. Homoeopathy system of medicines

Hahnemann was the pioneer of this system of medicine. He was born about 250 years ago in Germany. Homoeopathy is a system of National health care that has been worldwide used for over 200 years. This path treats each person as a unique individual with the aim of stimulating their own healing ability. A homoeopath selects the most appropriate medicine based on the specific symptoms of individual and personal status of health. Most of the medicines are extracted and prepared from medicinal herb.

As per the consideration of WHO, homoeopathy is the second largest medication system of treatment. This system is very much popular in India, Europe, South America and other countries of the world. Large number of people are treated and benefited by this system due to its low cost, negligible toxicity. Homoeopathy practice is **science** and application of it is **art** [6].

1.1.6. Amchi System of Medicine

Amchi System of Medicine (ASM) or Tibetan medical system is one of the Complementary and Alternative systems of Medicine (CAM). CAM is also known as SOWA RIGPA – means science of life according to the Tibetan language. ASM has been transmitted from India to Tibet between 7^{th} and 12^{th} centuries. It has no advancement than traditional Indian Medicine (TIM), Traditional Chinese Medicine (TCM), Homoeopathy, western medicinal herbalism. Medical practice has been started in the early age of this system. The expertization and skill have been shared from teacher to student and sometimes from parents to son. The young Amchi has also learnt the technique of identification, collection, preparation of medicines from plants after removing the poison principles. They also have acquired sufficient knowledge for diagnosis of disease by determining of pulse rate, analysis of urine, writing a

suitable prescription for healing. Simultaneously Amchi should have knowledge about Astrology.

It has been reported that 91 medicinal plants were used for the treatment of 93 ailments in the ASM. The most common diseases were jaundice, gastritis, dysentery, diarrhea, edema, rheumatism etc. The medicinal plants used in this system were - (i) *Sassurea obvalvata,*(ii) *Podophyllum hexandrum,*(iii) *Oxytropis microphylla,*(iv) *Gentiana aligida,*(v) *Artemisia spp.*etc. [7].

1.2. Phytochemistry and its medicinal aspects

Phytochemistry is the branch of chemistry which deals with chemical composition of the plants or phyto-drugs. It is also related with purification, isolation, bioactivity guided isolation, determination of physical and chemical properties of isolated compound, establishment of molecular formula by MASS SPECTROSCOPY, FTIR, ^{I}H NMR, ^{13}C NMR, LC-MS etc. and High Throughput Screening (HTS) of herbal extract or phytochemicals. Phytochemicals literally means chemical compounds obtained from plants.

Herbal or phyto-drugs were used in Ayurveda, Traditional Chinese Medicine, Amchi System of Medicine, Homoeopathy and western system of medicine.

Herbalism today has gained a tremendous progress due to the adequate knowledge of photochemistry in the subjects of Medicinal chemistry, Botany, Pharmacognosy, Anatomy & Physiology, Pathology, Pharmacology, Ethno Pharmacology, Chemical Ecology, Toxicology and traditional system of medicine. WHO also agrees with the popularity and demand of the phytomedicine or herbal medicine [8].

1.2.1. Phytochemical compounds

Phytochemicals are non-nutritive plant chemicals that contain protective or disease preventive compounds. These are defined as non-nutritive biologically active compounds. Phytochemicals have therapeutic and beneficial effect on health of the human. Approximately 25000 phytochemicals are identified in the nature of which 4,000 are Flavonoids and 10,000 are Alkaloids. Thousands of Phytochemicals are isolated, identified and categorized in specific classes depending upon the presence of their atoms and basic structural skeleton. At present most of the identified Phytochemicals are used commercially for the treatment of large number of diseases [9].

1.2.2. Major plant derived phytochemical compounds having anticancer property

Compounds are extracted, isolated, identified and evaluated their anticancer activity from the natural medicinal plants. Plant derived phytochemicals are Polyphenols, Brassinosteroids, Taxols etc.

1.2.2.1. Polyphenols

Flavonoids, Curcumin, Tannins, Gallacatechins and Resveratrol etc. are the examples of various plant derived phytochemicals having anticancer activity [10]. Gallacatechins is obtained from green tea, resveratrol can be found in specific food materials like grapes, peanut, wine. Major source of the Polyphenols is diet and they reduce the possibility of cancer due to their antioxidant activity [10,11].

Mechanism of action of Polyphenols

Polyphenols are act in the following mechanism:

(i) It initiates the apoptosis by regulation Cu^{2+} ions which are responsible for binding chromatin induced DNA fragmentation. DNA fragmentation takes place by resveratrol in presence of Cu^{2+} [10].

(ii) Polyphenol interferes the protein in cancer cells responsible for cellular growth.

(iii) It regulates acetylation, methylation and phosphorylation which alters the cancer agents by direct bonding. Curcumin is a phytochemical obtained from *Curcuma longa.* It is treated with various cancer cells and suppresses the tumor necrosis factor(TNFα) [12].

Example of Polyphenols - Flavonoids

Flavonoids are polyphenols. They belong to a big chemical group of secondary metabolites consisting of 10,000 known structural phytocompounds [13]. It has anticancer activity on various human cell lines like $HepG_2$ (Hepatic carcinoma), HeLa (cervical carcinoma), MCF-7(Adeno breast carcinoma) and HL-60(blood cancer/leukemia) [14]. Apoptotic proteins or genes are produced due to significant reduction of membrane potential of mitochondria. Thus, Acute Liver Failure (ALF) and Myeloid Leukemia Factor(MLF) induced apoptosis take place via intrinsic and extrinsic pathways. As a result cancer cells can't survive due to damage of mitochondria [15]. It has been reported that Flavonoids have more anticancer activity in low concentration [16].

1.2.2.2. Brassinosteroids (BRs)

Brassinosteroids are plant derived phytochemicals which have hormone signaling activity to control cell proliferation and change cellular architecture. It has action for resistance and tolerance against disease and stress [17]. BRs have significant anticancer effect. 28-homocastasterone (28-homos) and 24-epibrassinolide (24-epi BL) are the two brassinosteroids which have anticancer activity on various cell lines in micro molecular concentrations [18-20]. BRs induce apoptosis for inhibition of cell proliferation and cell cycle. BRs are used in various cell lines to evaluate the anticancer activity on Hela (cervical carcinoma), CEM (T-lymphoblastic leukemia), A-549 (lung cancer), HOS (osteosarcoma) and RPMI 5226 (Multiple myeloma) [18]. It binds with receptor of the cancer proteins and inhibits the growth hormone sensitive and hormone sensitive cancer cells [19,20]. BRs block the cell cycle G_1 phase and reduction in cyclin protein and act as anticancer drugs against breast cancer cell line. It is treated with BaX (Bcl-2 associated X, apoptosis regulator), pro-apoptotic protein and increases its level and treated with Bcl-2anti apoptotic protein and reduces its level [20]. So BRs have significant anticancer as well as the other therapeutic properties.

1.2.3. Other plant derived compounds possess anticancer activity

Plant derived compounds are administered orally, more tolerated and non-toxic to human cells [21]. Plant derived anticancer drugs are cytotoxic to the cancer cells but non-toxic to normal human cells.

Histone deacetylase inhibitors (HDAC) are sulforaphane, isothiocyanates, promiferin, isoflavones etc. They inhibit the activity of oncogenic proteins. Sulforaphane shows inhibitory effect of breast cancer cell proliferation [22].

Vinblastine, vincristine, vinorelbine, vinflunine, vindesine etc. are dimeric, terpenoid, indole group of alkaloids obtained from the plant *Catharanthus roseus*; which inhibit the microtubules by binding to β-tubilin. Microtubules disruptors are paclitaxel, docetaxel etc. The compounds block the cell cycle, metaphase to anaphase and induce apoptosis. Paclitaxel reduces the replication of cancer cells by stabilization and polymerization of the cells [23-25].

1.2.4. Anticancer plants and their phytochemical compounds

Acronychia bauer : It is an Australian plant. Bark extract contains triterpine, lupeol, alkaloids, acronycine, melicopidine and melicopine. The alkaloid acronycine has good antitumor activity [26].

***Allium sativum* :** This plant is known as Garlic. It contains 20 types of sulphide compounds containing amino acids. It has been reported that garlic has many medicinal activity depending on the phyto chemical compounds. Allicin has antibacterial, diallyl trisulphide has antithrombotic and methyl allyltrisulphide has anticancer activity [27]. It contains s-allyl-cysteine and s-allyl mercapto cysteine which have highest radical scavenging activity. It has been reported that garlic has hypoglycemic, antitumor, antioxidant, antiarthritic, hypolipidemic activity [28].

***Astragalus membranaceus* :** It is a Chinese herbal medicinal plant. Five bioactive fractions have been isolated from root extract. Both in-vitro and in-vivo study have been reported the antitumor activity [29].

Artemisia capillaries **:** It is a Korean medicinal plant having anticancer and antioxidant activity [30].

Camptotheca acuminata **:** It is a Chinese plant contains Camptothecin (CPT), an alkaloid which has anticancer and anti-viral activity. The bark and seed extracts of this plant contain Camptothecin and 10-hydroxycamptothecin. The compounds are more potent but less toxic in natural origin [31].

Beta vulgaris **:** It is also known as *Beet*. The modified root part is used to get medicinal activity. It has been reported that the *Beet* has significant tumor inhibitory effect [32].

Comelina sinensis **:** It is known as green tea. It has been reported that green tea has antioxidant, antitumor, cancer preventive, anti-mutagenic and hypocholesteromic activities. It contains catechin gallate, epigallocatechin gallate, caffeine and gallocatechin [33].

Curcuma longa **:** The vernacular name of *Curcuma longa* is turmeric and *Halud* (Bengali). The rhizome of this plant is used for extraction, isolation, purification and identification of active constituent 'curcumin'. It has been reported that curcumin has cytotoxicity in the lymphocytes against Dalton's lymphoma cells in the concentration 4mg/ml. The turmeric extract in the concentration 0.4mg/ml inhibits the tumor growth of Chinese Hamster Ovary (CHO) cells [34].

Inonotus obliques **:** It is known as chaga mushroom and used as folk medicine. The isolated and identified compounds from this plant are inotodiol and lanosterol. They have anticancer activity. It has been reported that the compounds have positive response in various cell lines in both *in-vivo* and *in-vitro* study. *Inonotus obliques* can be used as natural anticancer substance in the pharmaceutical and food industry [35].

Glycyrrhiza glabra **:** This plant is also known as licorice. Its root extract contains a novel estrogenic flavonoids, i.e. Licocalcone (LA). It has been reported that LA has chemopreventive and anticancer activity [36].

***Hydrastatis canadensis* :** It has been reported that *Hydrastatis canadensis* has anticancer activity on liver [37].

Podophyllum peltatum : A resin is produced from the whole plant, mainly from rhizome. In spite of the toxicity of the resin, it has been used as herbal medicine in the treatment of lymphadenopathy and certain types of tumors [38].

***Panax ginseng* :** This plant is known as *ginseng*. It has been reported that *ginseng* is a traditional medicine and used for the treatment of many diseases including breast cancer and other cancer [39].

***Solanum nigrum* :** This plant is the natural source of barley and wheat. This plant contains Lunacin which is a 43 amino acid having 4.8 kDa. It has been reported that Lunacin has cancer preventive activity in mammalian cells [40].

***Uncaria tomentosa* :** *Uncaria tomentosa* preparations have anticancer activity in both *in-vivo* and *in-vitro* study. It has main anticancer activity on drug resistant breast cancer [41].

Viscum album : This plant contains viscum album agglutinin-1(VAA1). It is used for adjuvant cancer therapy in combination with doxorubicin, cisplatin, taxol [42].

***Vitis vinifera* :** It has been reported that *Vitis vinifera* has significant antitumor activity on EAC cells [43].

1.3. Cancer

Cancer is the disease characterized by uncontrolled cell proliferation and second leading cause of death. It has been reported that cancer is the cause of more than 20% of all deaths. Chemical carcinogen, tobacco consuming, viral infections, radiation, environmental factor, rich lipid containing diet etc. are the possible cause of cancer [44]. There are three stages of carcinogenesis. They are Initiation, Promotion and Progression.

Initiation: This is the first stage of carcinogenesis where the cellular changes arise spontaneously or induced by exposure to a carcinogen. Here cellular genome undergoes mutations and neoplastic development.

Promotion: This is the second stage of carcinogenesis and the cells obtained after initiation are harmless. Then the cells are stimulated by promoting stimuli and cell proliferation takes place.

Progression: Progression is the third stage of carcinogenesis. Its mechanism is not fully established but it has been considered that mutation and aberration of chromosome are the possible cause of progression. Thus the process may be accelerated by repeated administration of carcinogenic stimuli [45].

1.3.1. History of Cancer

The word cancer obtained from the Greek word 'Karakinos' which means carcinoma. According to the history of cancer, Breast cancer was the oldest cancer and there was no treatment as per the record of Egypt in1500 BC. Human bone cancer, found in mummies was also recorded in Egypt manuscript in 1600 BC [46].

1.3.2. Theories of Cancer

There are many theories of cancers. (i) Humoral theory, (ii) Lymph theory, (iii) Blastema theory, (iv) Parasitic theory, (v) Chronic Irritation theory, (vi) Oncogenes and Tumor Suppressor genes theory.

Humoral theory: According to physician Hippocrates (460-370BC), Human body contains four types of fluids or Humors. They are blood, phlegm, black bile, yellow bile. According to the theory black bile is the cause of cancer.

Lymph theory: According to this theory tumors grow from the lymph.

Blastema theory: According to Muller, cancer is produced from cells not from lymph. According to Virchow, cancer cells are formed from other cells.

Parasitic theory: According to this consideration parasite is the cause of cancer.

Chronic Irritation theory: Virchow have considered that cancerous growth has produced due to chronic irritation.

Oncogenes and Tumor Suppressor genes theory: Mutated proto-oncogenes produce oncogenes which are the cause of cancer. Oncogenes are stimulated by chemical, viral or environmental change, wrong expression of gene, production of protein and abnormal cell proliferation. Genes which are opposite in function of oncogene have found to play active role in some type of cancers, are known as anti-oncogenes or tumor suppressor genes. These are responsible for regulation of unlimited cell division. Absence of tumor suppressor genes may be the cause of uncontrolled tissue growth. There are five types of tumor suppressor genes. The genes are namely Mutated Colon Cancer gene (MCC), Wilms Tumor gene (WT), P53 gene, Retinoblastoma susceptibility gene (RB), Deleted Colon Cancer gene (DCC). Some carcinogens damage the DNA and increase the level of P53 gene. P53 gene is not required for normal growth and development of the cell but its reduced level or absence has high possibility of tumors and genome instability [46].

Table- 1.2 : Types of cancer and common oncogene or tumor suppressor gene origin [47].

Type of cancer	Sources of tumor suppressor gene
Sporadic thyroid cancer	Rct mutation
Childhood neuroblastoma and lung cancer	N-myc amplification
Follicular lymphoma	Bcl-2 amplification, myc mutation
Colorectal and gastric cancer	BRCA 1, BRCA 2 mutation
Leukemia, colon, lung, breast, gastric cancer	C-myc amplification
Renal cell cancer	Von Hippel Lindan gene (VHL) dysfunction
Familial melanoma	P 16^{INK4A} mutation
Chronic myclogenous leukemia	Bcr-abl proto-oncogene translocation

1.4. Modern carcinogens

In 1911, Peyton Rous discovered a type of cancer in chicken that was caused by Rous sarcoma virus. More than 100 carcinogens have been identified which can be physical, chemical and biological. Cancer causing viruses are (i) Hepatitis B & C virus which produce liver cancer, (ii) Human papilloma virus (HPVs) produces cancer in penis, cervix and vulva, (iii) Epstein Barr Virus is the cause of nasopharyngeal and Hodgkin lymphoma [46].

1.5. Molecular mechanism of cancer

Major molecular mechanism of the cancer is the damage of DNA and production of abnormal DNA. Alteration of genetic materials proto-oncogenes are converted into oncogenes by mutation, translocations or amplification of gene. About 95% tumors are produced due to karyotype disorder. This abnormality occurs due to translocation of chromosomes, deletion or duplication of genetic materials and thus these abnormalities produce tumor. Chromosomal material loss is the cause of production of solid tumor. The deletions of chromosomal

material 3p produces lung cancer, 11p13 produces wilm's tumor, 13q14 produces retinoblastoma, 22 produces acoustic neuroma [48].

1.6. Cancer screening

The first cancer screening test was widely used **'Pap Test'**. This test was developed by George Papanicolaou. This method was related with menstrual cycle and was used to investigate the cervical cancer. In 1960 **mammography** was developed for identification of breast cancer. In the early 1970, **Ultrasound sonography**, **computed tomography** (CT scan), **magnetic resonance imaging** (MRI scans), **Positron Imaging tomography** (PET scans) etc. were used for cancer screening. Later cervix, breast, prostate, thyroid, oral, skin, lymph nodes, ovaries cancers were detected with the help of the above screening method [46].

Cancer screening is also performed by the following Laboratory Tests of Blood [49]:

1. **PSA (Prostate specific Antigen Test) :** Elevated level of PSA may be the cause of prostate cancer.
2. **CA-125 Test :** Elevated level of CA-125 is the cause of uterus, cervix, pancreas, liver, breast, lung, digestive tract cancer.
3. **AFP (Alpha fetoprotein) :** AFP is produced from fetus of pregnant mother. As a result AFP level is increased in pregnant mother. But high level of AFP in adult men and non-pregnant women may be diagnosed with liver, ovary or testicular cancer.
4. **CEA (Carcinoembryonic Antigen) Test :** Elevated level may cause the colorectal cancer.
5. **HCG (Human chorionic gonadotrophin) Test :** HCG is produced from placenta during pregnancy. Elevated level of HCG in non-pregnant female may be the cause of ovary, liver, stomach, pancreas, lung cancer and testis cancer in male.
6. **CA 19-9 Test :** This is the marker for screening of stomach, colon, and bile duct cancer. High level of CA-19-9 may indicate the pancreatic cancer. This test is also employed for detection of non-cancerous pancreatitis, cirrhosis of liver, cholecystitis.
7. **CA 15 -3 Test :** The elevated level of the biomarker is very much helpful for diagnosis of ovary, lung and prostate cancer.

8. **CA 27 – 29 Test :** The rising level of this cancer marker may be the cause of colon, stomach, kidney, lung, ovary, pancreas, uterus and liver cancer.
9. **LDH (Lactate dehydrogenase) Test :** LDH level is elevated in many cancers. LDH level is also raised in non-cancerous heart failures, hypothyroidism, anemia, lung and liver diseases.
10. **NSE (Neuron specific enolase) Test :** NSE is used to monitor and treatment in Neuroblastoma or small cell lung cancer.

1.7. Cancer statistics

1.7.1. Indian cancer statistics

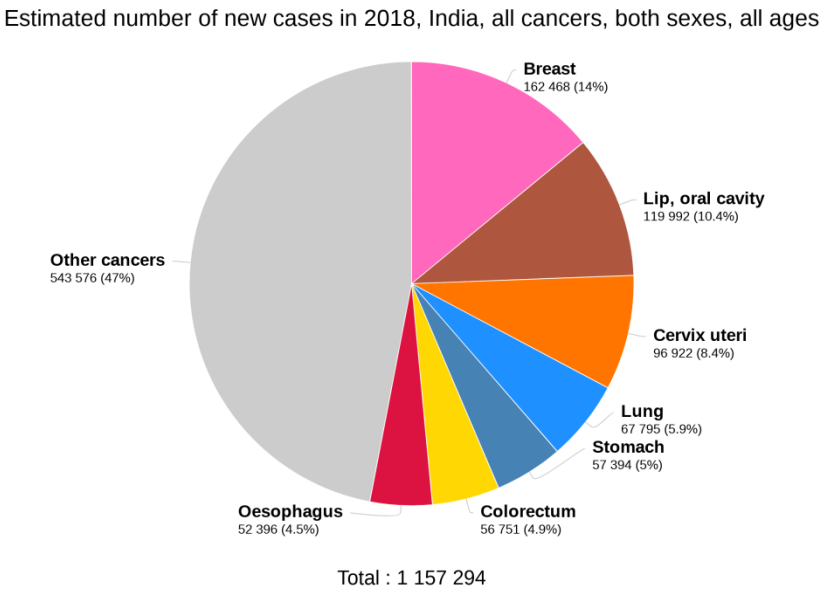
Cancer has become one of the major threats to human life. In a low socio-demographic index (SDI) country like India, the people are being increasingly diagnosed with this brutal disease.

According to the Indian Council of Medical Research (ICMR) report, about 14.5 lakh people have been affected by cancer in 2016 and 7.3 lakh people succumbed to the disease. In its projection, ICMR also said that the total number of new cases is expected to reach 17.3 lakh by 2020 and about 8.8 lakh death of cancer patients may occur. About 12.5% of the cancer affected patients attend for treatment in early stages, (i.e. in Stage I & II) and the remaining 87.5% patients use to come in advanced stages (Stage III & IV). The study said that among male, oral cancer topped the list and among females breast and cervix cancer. As per the report published with 2016 statistics, the number of the affected people in breast cancer are 1.5 lakh, lung cancer 1.14 lakh and cervix cancer 1 lakh. The report also predicted that the number may be increased up to 1.9 lakh, 1.4 lakh, and 1.04 lakh respectively by 2020. Among males, there was a significant increase in the cases colorectal cancer of male in Delhi, Bangalore, Chennai while among female there was a significant increase of breast, lung, ovary, uterus cancer in Bhopal and Barshi. It has also been predicted that one in eight Indians has likely to be affected by cancer during their life time [49].

The population in Indian subcontinent is around 1.35 billion as reported till 2018. As per the GLOBOCAN report (2018) the number of new cancer cases is 1,15,7294 among which 14% (1,62,468) cases are categorised as Breast Cancer, 10.4% (119 992) Lip, oral cavity cancer, 8.4% (96,922) Cervix uterine cancer, 5.9% (67,795) Lung Cancer and 5% (57,394) Stomach

cancer and rest of the people are affected with other type of cancers. The number of deaths, affected by cancer is 7,84,821 as reported in 2018. Breast Cancer is most common in females whereas Lip, Oral Cancer is very common in Indian males. The number of prevalent cancer cases in the last 5 years is 2,25,8208.

Figure - 1.1: GLOBOCAN Cancer statistics of India, 2018



1.7.2. Worldwide cancer statistics

According to the GLOBOCAN report 2018 (Figure: 1.2), the incidence of cancers in both sexes in various continents are:

Asia 48.4%, Africa 5.8%, Americas 21.0%, Europe 23.4%, Oceania 1.4% and total new cases are 18.1 million. Incidence of cancer in males are 49.2% in Asia, 4.7%in Africa, 20.7% in Americas, 23.8% in Europe, 1.6% in Oceania. The number of the new cases in Male are 9.5 million. Similarly incidence of cancers in females are 47.5% in Asia, 7.0% in Africa, 21.3% in America, 23.0% in Europe, 1.2% in Oceania and total number of new cases in females are 8.6 million.

Another GLOBOCAN,2018 report that has been published based on the mortality rate of cancer in the world describes that the mortality rate for both sexes in Asia is 57.3%, Africa 7.3%, Americas 14.4%, Europe 20.3%, Oceania 0.7% and the total number of deaths are 9.6 million. Mortality of males in Asia is 60.0%, Africa 5.9%, Americas 13.2%, Europe 20.2%, Oceania 0.7 % and the total number of deaths are 5.4 million. Similarly, mortality rates of female in Asia is 53.9%, Africa 9.0%, Americas 15.8%, Europe 20.6%, Oceania 0.7% and total number of deaths are 4.2 million.

Figure - 1.2: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries

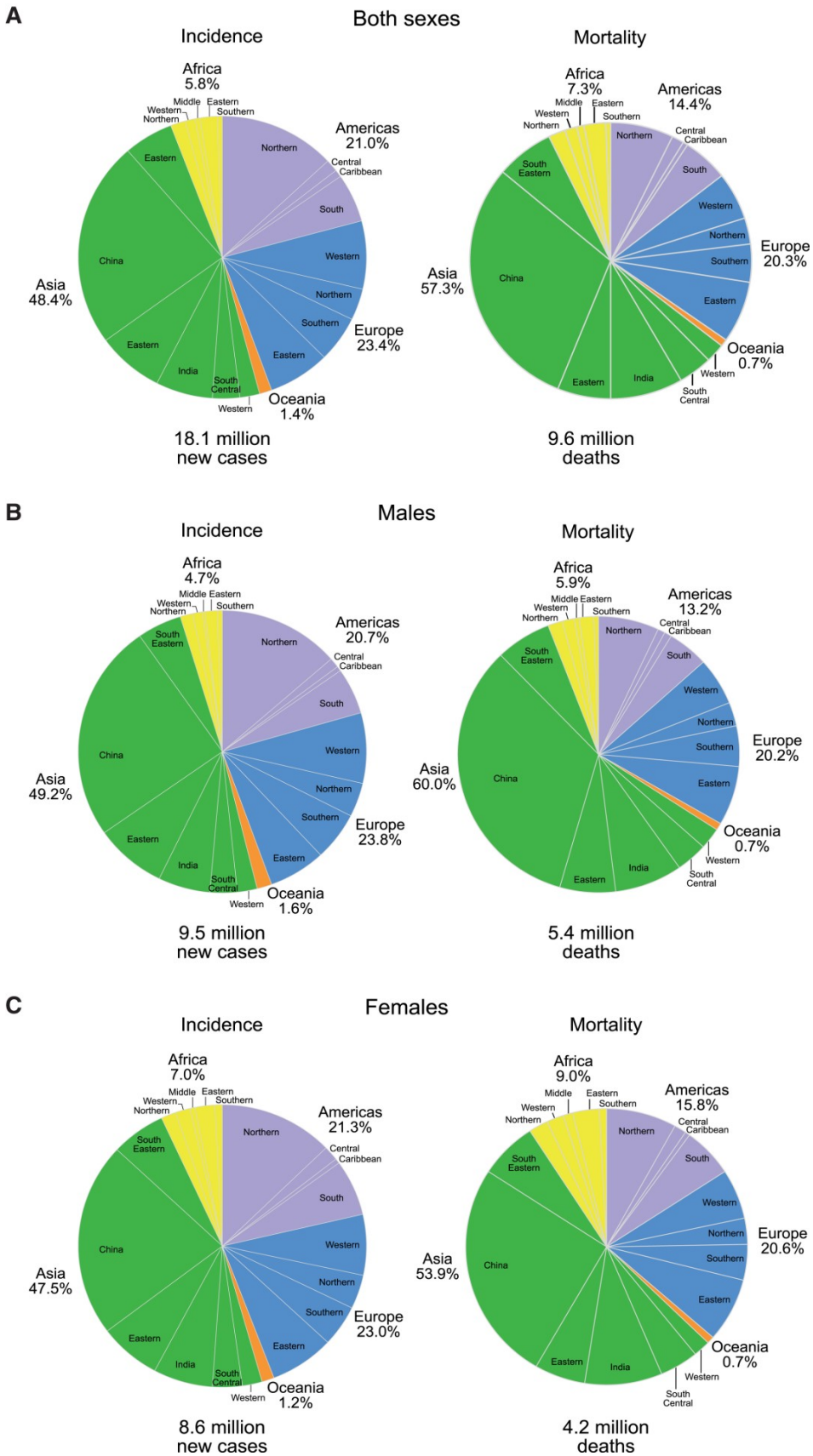


Table- 1.3 : New Cancer incidence, mortality for 36 Cancers and All Cancers in 2018 [50]

CANCER SITE	NO. OF NEW CASES (% OF ALL SITES)	NO. OF DEATHS (% OF ALL SITES)
Lung	2,093,876 (11.6)	1,761,007 (18.4)
Breast	2,088,849 (11.6)	626,679 (6.6)
Prostate	1,276,106 (7.1)	358,989 (3.8)
Colon	1,096,601 (6.1)	551,269 (5.8)
Nonmelanoma of skin	1,042,056 (5.8)	65,155 (0.7)
Stomach	1,033,701 (5.7)	782,685 (8.2)
Liver	841,080 (4.7)	781,631 (8.2)
Rectum	704,376 (3.9)	310,394 (3.2)
Esophagus	572,034 (3.2)	508,585 (5.3)
Cervix uteri	569,847 (3.2)	311,365 (3.3)
Thyroid	567,233 (3.1)	41,071 (0.4)
Bladder	549,393 (3.0)	199,922 (2.1)
Non-Hodgkin lymphoma	509,590 (2.8)	248,724 (2.6)
Pancreas	458,918 (2.5)	432,242 (4.5)
Leukemia	437,033 (2.4)	309,006 (3.2)
Kidney	403,262 (2.2)	175,098 (1.8)
Corpus uteri	382,069 (2.1)	89,929 (0.9)
Lip, oral cavity	354,864 (2.0)	177,384 (1.9)
Brain, nervous system	296,851 (1.6)	241,037 (2.5)
Ovary	295,414 (1.6)	184,799 (1.9)
Melanoma of skin	287,723 (1.6)	60,712 (0.6)
Gallbladder	219,420 (1.2)	165,087 (1.7)
Larynx	177,422 (1.0)	94,771 (1.0)
Multiple myeloma	159,985 (0.9)	106,105 (1.1)
Nasopharynx	129,079 (0.7)	72,987 (0.8)
Oropharynx	92,887 (0.5)	51,005 (0.5)
Hypopharynx	80,608 (0.4)	34,984 (0.4)
Hodgkin lymphoma	79,990 (0.4)	26,167 (0.3)
Testis	71,105 (0.4)	9,507 (0.1)
Salivary glands	52,799 (0.3)	22,176 (0.2)
Anus	48,541 (0.3)	19,129 (0.2)
Vulva	44,235 (0.2)	15,222 (0.2)
Kaposi sarcoma	41,799 (0.2)	19,902 (0.2)
Penis	34,475 (0.2)	15,138 (0.2)
Mesothelioma	30,443 (0.2)	25,576 (0.3)
Vagina	17,600 (0.1)	8,062 (0.1)
All sites excluding skin	17,036,901	9,489,872
All sites	18,078,957	9,555,027

1.8. Treatment of Cancer

Surgery, Radiation and chemotherapy are the three methods for treatment of cancer. General surgery method is followed to remove the cancerous tumor and cryogen surgery is followed to remove the tumor without operation by using cryogen or liquid nitrogen. Laser technology is also used to remove the tumor from cervix, larynx, liver, rectum, skin and other organs [46].

1.8.1. Radiation

Radiation technique was used for diagnosis and treatment of cancer since three years back of the discovery of X-ray (1896) by Roentgen. In the early 20^{th} century radiation therapy was followed for the treatment of cancer. Various radiation therapies are used to destroy the cancer cells. They are (i) conformal proton beam therapy-where proton can destroy the tumor cells.(ii) Stereotactic surgery and stereotactic therapy- where gamma knife is used to treat the common brain tumor. (iii) Intra-operative radiation therapy- where post-operative tumor adjacent abnormal DNA/abnormal cells are destroyed by radiation therapy.

1.8.2. Cancer Chemotherapy

There are more than 100 types of cancer and they are named depending on the organs or tissue where abnormal cell proliferation takes place. They are Acute Lymphoblastic Leukemia, Acute Myeloid Leukemia, Soft tissue Sarcoma**,** AIDS related Lymphoma, Anal cancer, Basal cell carcinoma (skin cancer), Bile duct cancer, Brain cancer, Breast cancer, Lung cancer, Cervical cancer, colorectal cancer, Uterine cancer, Esophageal cancer, Retinoblastoma, Fallopian tube cancer, Gallbladder cancer, Stomach cancer, Testicular cancer, Renal cancer, Laryngeal cancer, oral cavity cancer, Metastatic cancer, ovarian cancer, Pancreatic Neuroendocrine tumors, Thyroid cancer, Parathyroid cancer, Bone cancer, Intestinal cancer, Throat cancer, Urethral cancer, Prostate cancer, vaginal cancer, angiogenesis. These large numbers of cancers are treated with various types of chemical agents or chemotherapy and they may be obtained from natural or synthetic sources.

Table- 1.4 : Types of cancer chemotherapy and their clinical uses [51]

SlNo	Types of cancer chemotherapy	Name of the chemotherapy	Clinical uses
1	Alkylating Agent	chlorambucil, cyclophosphamide, lomustine, melphalen, carboplatin, cisplatin, oxaliplatin, dacarbazin, thiotepa	lung, ovary, leukemia, lymphoma, breast, multiple myeloma, Hodgkin disease, sarcoma cancer
2	Anti-metabolites	5-fluorouracil, cytarabine, 6-mercaptopurine, capecitarabine, methotrexate, gemcitarabine, hydroxyurea	breast, ovary, leukemia, intestinal and other types of cancer
3	Anti-tumor antibiotics (Anthracyclines group)	doxorubicin, daunorubicin, epirubicin, adriamycin, idarubicin	This group of chemotherapies are used for treatment of various types of cancer
4	Anti-tumor antibiotics (Excluding anthracyclines group)	mitomycin-c, bleomycin, actinomycin-D,	This group of chemotherapies are used for treatment of various types of cancer
5	Topoisomerase- I inhibitors	irinotecan, topotecan	Ovary, lung, g.i.t, leukemia and other cancers
6	Topoisomerase -II inhibitors	mitoxantrone, teniposide, etoposide	Acute myelogenous leukemia
7	Mitotic inhibitors	paclitaxel, docetaxel, estramustine, ixabepilone, vincristine, vinblastine, vinorelbine	myeloma, leukemia, lung, breast, lymphoma and other cancers
8	Hormones	testosterone, progesterone, estrogen	testosterone in cervix and breast cancer; estrogen & progesterone in prostate cancer, uterine cancer

1.9. Cancer Chemoprevention

It means inhibition, retardation, and reverse action of the tumerogenesis by using certain agents. Phytochemicals obtained from various medicinal plants interfere the carcinogenesis process. These agents modulate intracellular signaling Cascades mainly NFkB and mitogen activated protein kinase. These substances suppress cancer cell proliferation through apoptosis.

1.9.1. Mechanism of chemoprevention of cancer

1) Modulation of chemical carcinogen metabolism

Mainly two groups of enzymes have important role to control the toxic mutagenic and neoplastic effects of chemical carcinogen through biotransformation. The enzymes related to this activity are Phase I enzymes like cytochrome P_{450}, flavin dependent mono-oxygen and Phase II enzymes are glutathione, S-transferase, sulfotransferases, UDP glucoronosyltransferases.

2) Scavenging of free radicals

Superoxide, hydroxyl radicals, peroxy radicals etc. are the various reactive oxygen species (ROS) which have major role in initiation, promotion and progression stages of cancer. Free radicals scavengers have property to abolish the tumorigenesis [52].

3) Inhibition of enzymes

Inhibition of COX-1, COX-2, 5-LOX enzymes are inflammatory factors, responsible for cancer. Some chemical compounds present in plants having anti-inflammatory activity are responsible for antiproliferation of abnormal cells and prevention of cancer.

4) Caspase activation

Caspase-2,8,9,10 are **inhibitors** and caspase-3,6,7 are known as **effectors.** The effector caspases are responsible for activation of downstream of initiator caspases [53,54].

5) **Nuclear factor-KappaB (NFkB) and activator protein-1**

NFkB and AP-1, two major transcription factors, are liable for extracellular signals by inducing expression of particular early response downstream genes.

6) **Mitogen activated protein kinase pathways.**

7) **Antiproliferation and apoptosis inducing properties**

i. **Necrosis:** Necrosis is an accidental cell death occurring due to the uncontrolled external factors like toxins, trauma and infections in the external environment of the cell.

1.9.1.1. Apoptosis

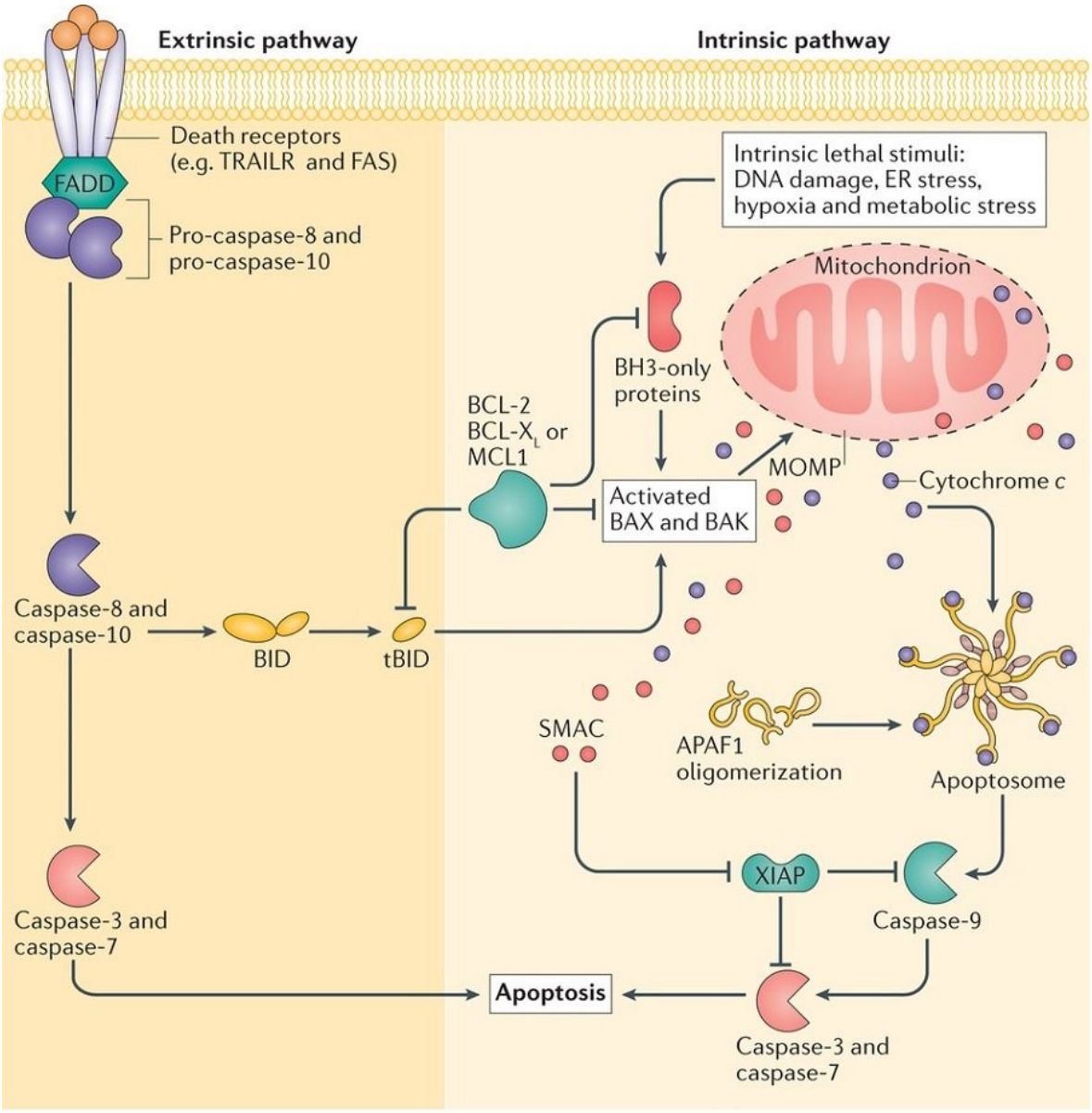
Apoptosis is a process of programmed cell death due to biochemical instructions of cellular DNA. It is a complex cellular process which is regulated by signal molecules. It is a natural physiological process where cell actively destroys itself for smooth functioning.

1.9.1.1.1. Mechanism of Apoptosis (Apoptosis Pathways)

The major apoptosis pathways are 'Extrinsic Pathways' and 'Intrinsic Pathways'. In case of 'Extrinsic Pathways' a cell receives a specific signal to initiate apoptosis from outside of cell or other cell and giving directions to commit programmed cell death. This pathway takes place in the cells which are diseased or there is no utility.

In the 'Intrinsic Pathways', a cell receives a specific signal to destroy its own gene or protein due to damage of cellular DNA and as a result cell is destroyed [55].

Figure - 1.3: Apoptosis Mechanism(Extrinsic and Intrinsic pathways)



i. Extrinsic pathway

This pathway consists of 4 steps.

Step i : The FAS (Facility associated signal) and TRAIL (TNF- related apoptosis inducing ligand) are two chemical messengers that trigger the extrinsic pathway of apoptosis. These two molecules are generally excreted by surrounding cells after the damage or having no importance. The receptors, when bind with FAS is known as FASR and when bind with TRAIL is known as TRAILR. The receptor proteins which encounter to the signal molecule are known as Ligand and they bind to it.

Step ii : Changes occur of TRAILR and FASR in intracellular domain. The cellular protein FADD is also changed and activated. The activated FADD interacts with two additional proteins which initiates the process of cell death.

Step iii : Two proteins, Pro-caspase-8 and pro caspase-10 are activated after interaction with FADD. Caspase-8 and Caspase-10 are dispersed through the cytoplasm and triggered to change other molecules of the cell including messengers that damage the DNA.

Step iv : Caspases-8 &10 transform the inactive molecule BID into tBID and moves to the mitochondria and activates the molecule BAX (BCL2 associated X, apoptosis regulator) and BAK (BCL2 antagonist/killer). BAX and BAK are liable for the 1st step of both extrinsic and intrinsic pathways of apoptosis.

ii. Intrinsic pathway

Step i : This step of apoptosis is triggered by cell damage or stress. This step causes the damage of DNA, oxygen insufficiency and hampers the normal function of cell. Then a set of proteins -"BH_3' are activated.

Step ii : BH3-is such type of proteins which consists of Pro and anti-apoptotic proteins. BH3 proteins are responsible for both encouraging and discouraging apoptosis.

Step iii : Activated BAX and BAK start MOMP(Mitochondrial outer membrane permeability). It is also known as 'Point of no Return' of apoptosis. MOMP is stopped by the inhibitor and cell will die. As a result Cytochrome C is released into cytoplasm.

Step iv : Cytochrome C is an ETC (Electron Transport Carrier) which after releasing from mitochondria acts as a signaling molecule in the cytoplasm. Then cytoplasm forms apoptosome which starts the cellular break down.

Step v : The apoptosome turns Procaspase-9 to Caspase-9. Caspase-9 is responsible for further change of cell.

Step vi : Caspase-9 then activates Caspase-3 and Caspase-7

Step vii : After activation of Caspase-3&7, break down of the cellular materials are initiated, especially by caspase-3. Caspases-3 condenses and damages the DNA of the cell [56].

1.9.1.2. Necrosis

Necrosis is an accidental cell death occurring due to the uncontrolled external factors like toxins, trauma, lack of oxygen and infections in the external environment of the cell.

There are six types of necrosis. They are:

1. Liquefactive necrosis
2. Coagulative nerosis
3. Caseous necrosis
4. Fibrinoid necrosis
5. Fat necrosis
6. Gangrene necrosis

Liquefactive necrosis: In this type of necrosis, digestive enzymes have important activity to liquefy the mass of dissolved tissue. This necrosis occurs at any part of the body excluding brain. Anoxia and hypoxia are the cause of necrosis in the brain.

Coagulative necrosis: This type of necrosis occurs due to lack of oxygen or mechanical injury. The cells are died without losing their cellular structure or architecture.

Caseous necrosis: This type of necrosis occurs due to tuberculosis and fungal infections. The structure of necrosis is known as granuloma.

Fibrinoid necrosis: In this type of necrosis fibrin is deposited. This necrosis occurs in blood vessels.

Fat necrosis: In this type of necrosis enzymes break down the adipocytes and release free fatty acids. This necrosis occurs in pancreatitis and swelling of breast tissue.

Gangrene necrosis: In this type of necrosis tissue death occurs in the lower extremities or lower limbs. This type of necrosis is known as gangrene [56].

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Chapter- 2

Literature Review of *Nyctanthes arbor-tristis* Linn

Nyctanthes arbor-tristis Linn. is a popular and well documented plant. It has various vernacular names in different languages and various regions. It is a native of India, distributed widely in the Sub-Himalayan region and also found in Indian gardens as an ornamental plant. The indigenous people of India traditionally use various parts of *Nyctanthes arbor-tristis* (NA) for the treatment of various diseases. Due to the presence of medicinal value in various parts of the plant, it has been used as traditional medicine in Ayurveda, Siddha and Unani systems since ancient times [1]. The whole plant is used for the treatment of cancer. The root is used for treating fever, sciatica, and anorexia; bark as an expectorant, leaf to control diabetes and as a cholagogue, diaphoretic and anthelmintic [2]. The liquid extract produced by decoction of the bark is used to treat arthritis and malaria [3]. It has been reported that leaf extract of NA has tranquilizing, laxative and antihistamine activities [4]. NA has some biological activities like antioxidant [5], anti-inflammatory [6], antileishmanial [7]. Seed extract of NA contains various glycosides which have anti-inflammatory and immunomodulatory activities [8]. The first anticancer activity of NA was reported in a cytotoxicity study from Petroleum ether, Chloroform, ethyl acetate extract of the flower in 2001 [9]. Earlier researchers have reported the isolation of polysaccharides, iridoid glycosides, phenylpropanoid glycoside, ß-sitosterol, ß-amyrin, hentriacontane, benzoic acid, glycosides, nyctanthoside, iridoid glucoside, nyctanthic acid, friedelin, lupeol, oleanolic acid, 6ß-hydroxylonganin, alkaloids, tannins, terpenoids, glycosides, and arbortristoside A, B, C from this plant [10-13]. Intensive pharmacological evaluation revealed its significant biological activities like anti-diabetes [14], anti-allergy [15], anti-oxidant [16] and anti-inflammatory activities [17].

2.1. Plant Identity

Nyctanthes arbor-tristis belongs to [18]:

Class : Eudicots
Division : Angiosperm
Family : Oleaceae
Genus : Nyctanthes
Kingdom : Plantae
Order : Lamiales
Species : *Nyctanthes arbor-tristis*

Figure - 2.1: Twig having leaves and flower of *Nyctanthes arbor-tristis*

2.1.1. Vernacular Names of Nyctanthes arbor-tristis L.

1. Sephalika (Bengali)
2. Harisingar (Hindi)
3. Parijatha (Sanskrit)
4. Night Jasmine (English)
5. Parijatakam (Malayalam)
6. Parijathak (Marathi)
7. Gangasiuli (Oriya)

2.2. Morphological description of the plant

Nyctanthes arbor-tristis L.(NA) is a shrub or small tree with approximately 10 meter height. Life span of the plant is about 5-20 years. Bark is grey and rough; Leaf is simple, ovate, hairy, both the surfaces are rough, 5-14 cm long, 2.5-7.5 cm wide, dorsal surface with deep green color, ventral surface with light green color; Base is round or cuneate; dorsoventral Lamina is present; Phyllotaxy is opposite decussate; entire margin is present; Venation is reticulate, unicostate, 3-6 pairs of lateral veins are present; Flower is white, fragrant with 5 to 8 lobed; Corolla contains centrally orange color; Corolla Tube has bright orange color; Odor is indistinct or faint; it tastes bitter or astringent [19-21].

2.3. Geographical Source

The plant grows in India, Bangladesh, Nepal, Thailand and Pakistan [22]. This plant is widely grown in all over the world mainly tropical and subtropical regions.

2.4. Identification and Authentication of the plant

A twig containing leaves and flowers of *Nyctanthes arbor-tristis* L. (NA) was collected from the herbal garden of Institute of Pharmacy, Kalyani, Govt. of W.B. India. Then a herbarium was appropriately prepared for identification and authentication. The herbarium of this plant was identified by the Central Herbarium, Shibpur, Howrah, India (BSI, Howrah) as *Nyctanthes arbor-tristis* Linn. Family Oleaceae and has been issued a certificate, Number CNH/31/2013/Tech/1065 for authentication.

2.5. Reported phytochemical compounds and their Biological activities

There are large numbers of medicinal plants present in the nature. Some are used in traditional system of medication and some are used scientifically in modern system of medication for cure, mitigation or prevention of diseases. Most of the herbal medicinal plants are safe and contain pharmacologically active chemical compounds. *Nyctanthes arbor-tristis* L. (NA) is a traditionally used herbal medicinal plant which contains various reported phytochemical compounds in its various parts. The compounds mentioned in the following table are generally isolated from Ethanol, methanol, chloroform, ethyl acetate, n-butanol, aqueous extract.

Table- 2.1 : Reported phytochemical compounds and their Biological activities

Parts of plants	Chemical compounds	Biological activity	References
Flower	nyctanthin, essential oils, d-mannitol, glucose, tannin, carotenoid, glycosides (β-monogentiobioside ester of α-crocetin), β-monogentiobioside β-D monoglucoside ester of α-crocetin	Anti-leishmanial, diuretic, antioxidant, anti-bilious, anti-inflammatory, sedative, cytoprotective	[23-27]
Bark	glycoside and alkaloids	Antimicrobial	[28]
Stem	β-sitostrol, glycoside naringenin-4'-o-β- glucapyranosyl-α-xylonopyranoside and other glycosides	Ani-oxidant & antipyretic	[29]
Seeds	Arbortristoside (A,B,C); 3,4-secotriterpine acid; glycerides of linoleic acid; myristic acid; nyctanthic acid; stearic acid; palmitic acid; oleic acid; D-glucose; D-mannose	Anti-leishmanial, immunomodulatory, antifungal, antibacterial, hepatoprotective, antiarthritic	[24,26,30-35]
Leaves	Nyctanthic acid, oleanolic acid, Flavanol glycosides, nicotiflorin tannic acid, ascorbic acid, methyl salicylate, aspragaline, β-sitosterol, carotene, mannitol, lupeol, irridoid glycosides, glucose and fructose, volatile oil, friedeline.	Anti-inflammatory, antibacterial, anthelmintic, hepatoprotective, immunopotential, antispasmodic, antioxidant, anidiabetic, anti-leishmanial, CNS depressant case	[25,36-40]
Entire plant	Arbortrisitoside A,B,C; Iridoid glycoside; 2,3,4,6-tetra-o-methyl-D-glucose; 2,3,6-tri-o-methyl-D-glucose; 2,3,6-tri-o-methyl-D-mannose; 2,3-di-o-methyl-D-mannose	Antidiabetic, Immunno stimulant, anti-arthritic, anti-viral	[35,41]

2.6. Structures of the reported chemical compounds present in *Nyctanthes arbor-tristis*

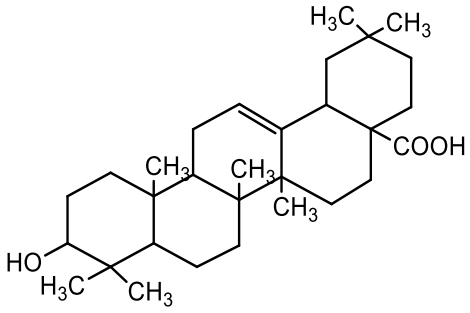

Figure - 2.2: Oleanolic acid

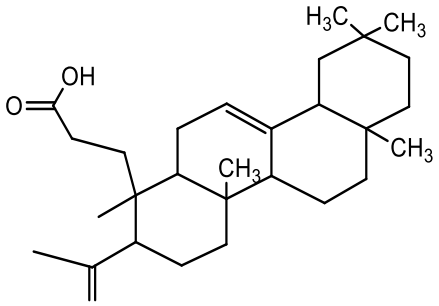

Figure - 2.3: Nyctanthetic acid

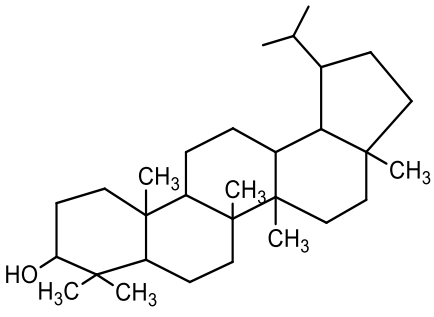

Figure - 2.4: Lupeol

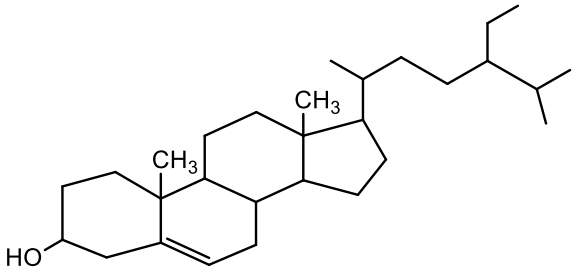

Figure - 2.5: Beta-sitosterol

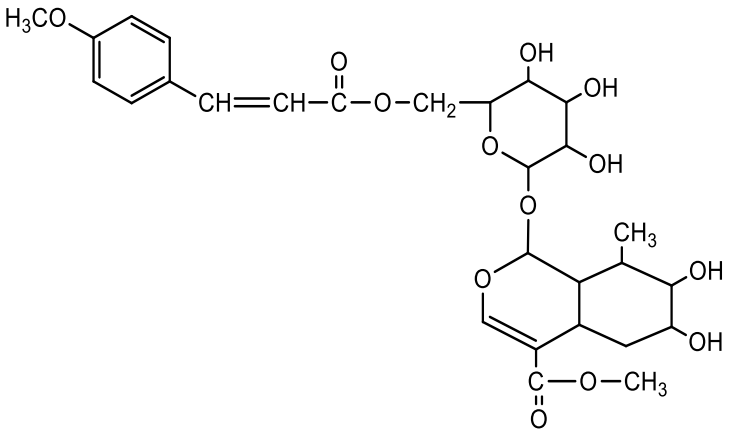

Figure - 2.6: Arbortristoside A

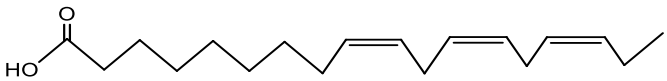

Figure - 2.7: Alpha linoleic acid

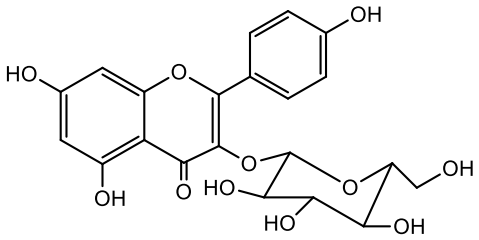

Figure - 2.8: Astragaline

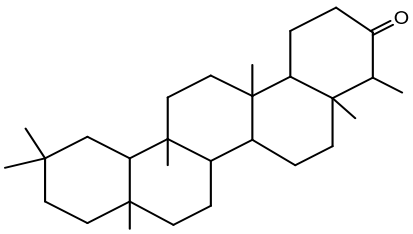

Figure - 2.9: Friedelin

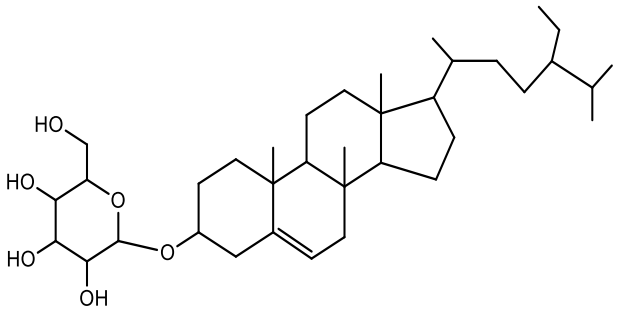

Figure - 2.10: Beta sitosterol-D-glucoside

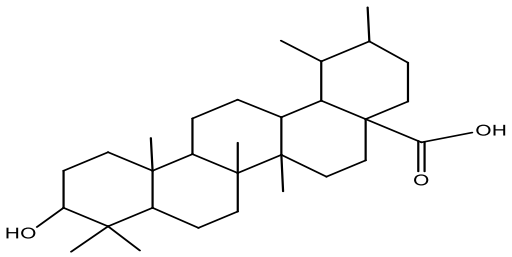

Figure - 2.11: Ursolic acid

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Chapter- 3

Aim and objective of the study

It has been observed through the literature survey that *Nyctanthes arbor-tristis* Linn. has significant anti-allergy, antidiabetic, antioxidant, anti-inflammatory activities including other pharmacological activities. But no in-depth studies are present on characterization and identification of bioactive phytochemical compound(s) which are present in the leaf extract of *Nyctanthes arbor-tristis* Linn and pharmacologically responsible for their antioxidant, inflammatory, anticancer activities. There were many scopes for extraction, fractionation, characterization (Mass, NMR spectroscopy, FT-IR), biological activity studies (*in vitro & in vivo*) in our departmental laboratory and near the well-equipped laboratory of IICB (Kolkata), IACS (Kolkata). As this plant is easily available and many traditional uses have been reported, our aim was to explore

- Bioactivity guided Isolation and Characterization of the phytocompound(s) obtained from methanol extract of the leaf and identification by spectroscopic analysis from *Nyctanthes arbor-tristis* Linn.

- Evaluation of anti-cancer activity of the isolated compound against various human cancer cell lines - HepG2 (liver), A549 (lung), HL-60 (Leukemia), MCF-7 (Breast), HCT-116 (Colorectal), PC-3 (Prostate), HeLa (Cervix) - *In-vitro* study.

- Evaluation of anti-cancer activity of methanol extract of the leaves of *Nyctanthes arbor-tristis* Linn on EAC cells-*in-vivo study.*

- Investigation of in vivo antioxidant activity of the leaf extract of *Nyctanthes arbor-tristis* Linn on EAC cell-bearing Swiss Albino mice.

- Study of histopathological architecture and determination of Histology Activity Index (HAI) from liver of methanol leaf extract of *Nyctanthes arbor-tristis* (MENA) treated and EAC cells bearing Swiss Albino mice.

Chapter- 4

Bioactivity guided Isolation, Fractionation, Characterization and Investigation of Antioxidant and Anticancer activity

4.1. Materials and Methods

4.1.1. Chemicals and reagents

Methanol, Ethyl Acetate and n-Butanol solvents were used for extraction and fractionation as analytical grade. Other required solvents were HPLC-grade and analytical grade reagents. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), MTT (3-(4,5-dimethylthiazol- 2-yl)-2,5 diphenyl tetrazolium bromide), phosphate-buffered saline (PBS), DMSO (dimethyl sulfoxide) which were purchased from Sigma-Aldrich, Munich, Germany. Penicillin/streptomycin (PS) and trypsin were purchased from Gibco, Life technology, Grand Island, NY, USA. RPMI 1640 medium, trypsin, and heat inactivated fetal bovine serum (HIFBS) were obtained from Gibco, UK. Boswellic acid, ascorbic acid (vitamin C), dexamethasone, and doxorubicin were procured from Sigma-Aldrich, Bangalore, India. Dulbecco's modified Eagle's medium and L-glutamate were purchased from Gibco BRL (Grand Island, USA). The incubator was procured from Corning (Rochester, USA). The COX-1 and COX-2 (human ovine) inhibitor Screening assay kit [Catalog No. 760111] was procured from Cayman, U.S.A. N, N, N, N'-tetramethyl-pphenylenediamine (TMPD) and lipopolysaccharide were purchased from Sigma (St Louis, USA) and the ELISA kits for mouse TNF-α were purchased from R&D Systems (Minneapolis, MN, USA). All other chemicals used in the experiment were of analytical grade available in India.

4.1.2. Extraction, Isolation and bioactivity guided fractionation of Compound

3.5 kg leaves of *Nyctanthes arbor-tristis* L. (NA) were collected from the medicinal garden of Institute of Pharmacy, Kalyani, Nadia and thoroughly washed with tap water followed by distilled water and dried under shade at room temperature for two weeks. The dried leaves weighed 1.5 kg was grinded into coarse powder with the help of mixer grinder. The powder of plant material was extracted at room temperature by multiple maceration using 95% methanol as menstruum. The extract was evaporated under reduced pressure using Rotary Evaporator. The concentrated extract was lyophilized and 80 gm methanol extract (MENA) was obtained. A little amount of MENA was taken to determine the presence of chemical constituents. Then polarity based fractionation of MENA was carried out using Ethyl Acetate (EtOAc) followed by n-Butanol (n-Bu) and finally water as solvent. The Ethyl Acetate extract was dried by rotary evaporator at 40^0C, n-Butanol extract was concentrated at 55^0C and aqueous extract was dried using a freeze dryer. The Ethyl Acetate Fraction (EtOAcF, 63% w/w); n-Butanol Fraction (n-BuF, 23%w/w) and Aqueous Fraction (AqF, 10.22%w/w)

were evaporated to dry and yield 50gm, 20gm and 10 gm respectively. These four fractions like MENA, EtOAcF, n-BuF and AqF were subjected to anti-inflammatory, anticancer and anti-oxidant activity. The part of the EtOAcF weighed 25gm was selected based on the result of bioactivity . It was extended for further separation using column chromatography (4.5cm i.d. x 45 cm long; silica gel: 70-230 mesh size, central Drug house, New Delhi) and eluted in a gradient manner with chloroform-ethyl acetate-methanol, increasing polarity to collect fourteen fractions (labeled as sub fractions (sub of EtOAc): 1to 14). These 14 fractions were used to test for evaluation of anti-inflammatory activity *in-vitro* assays. The sub-fraction numbers 6 to 9 (900 mg) were showed more or less similar and best anti-inflammatory activity compared to sub-fractions numbers 1 to 5 and 10-14. Therefore, the sub fractions 6-9 were pooled and further purified in a similar process using silica gel column chromatography (100-250 mesh) with stepwise gradient elution with chloroform-methanol (60:40 v/v) and purified by crystallization in methanol, yielding the compound (70 mg, 2.8%). The isolated compound was obtained as a white powder. The melting point of the isolated compound was determined by open capillary method [1].

Table- 4.1 : Chemical test performed from the leaf extract of *Nyctanthes arbor tristis* L. (MENA) for detection of various phytochemical compounds

Experiment	Observation	Inference
1. 1ml methanol extract of *Nyctanthes arbor tristis (*MENA) was added with 1ml Hager's reagent (1% picric acid solution).	Yellow color precipitate was observed	Alkaloid was present
2. 0.5 g MENA was diluted with 5ml water. It was treated with ½ ml of glacial acetic acid and few drops of $FeCl_3$ solution. About 1ml conc. H_2SO_4 was added to it slowly.	A brown ring was formed at the junction of the two liquids.	Glycoside was present.
3. 4-5 ml MENA was treated with 2ml $CHCl_3$. Then conc. H_2SO_4 was added slowly.	A reddish brown coloration was formed at the interface.	Tarpenoid was present
4. MENA was treated with dil. NH_4OH and conc. H_2SO_4	A yellow coloration was produced.	Flavonoid was present.
5. Molish's Test: MENA solution was treated with 1ml. of ethanolic α napthol solution. Then conc. H_2SO_4 was added slowly.	A purple or reddish violet color ring was formed.	Carbohydrate was present.
6. Fehiling's Test: MENA solution was reacted with mixture of equal amount of Fehling's A & B and heated for few minutes.	Formation of brick red color.	Reducing sugar was present.
7. Benedict's Test: 1-2ml. of MENA solution was boiled with 4-5 ml of Benedict's reagent.	A red precipitate was produced.	Reducing sugar was present.
8. Few mg of MENA was mixed with H_2SO_4 and boiled. It was cooled and filtered. The filtrate was mixed with Benzene and 2ml of NH_4OH soln. was added.	Ammoniacal layer was not produced	Anthraquinonne was absent
9. 1 ml. MENA solution was treated with blue color mixture of 40% NaOH &1% $CuSO_4$ solutions.	A pinkish or purple violet color was produced	Protein was present.
10. 5 g MENA was mixed with 4-5 ml water and shaken vigorously. Then the content was mixed with olive oil and shaken.	Stable froth was produced and then an emulsion was formed after addition of oil	Saponin was present
11. MENA was boiled with water and filtered after cooling. Then treated with 0.1% $FeCl_3$ solution.	Blue black coloration was produced.	Tannin was present.
12. Few ml MENA solution was treated with 1-2ml Folin's reagent	A violet or brown color was produced.	Phenol/Phenolic compound was present

4.2. Cell Culture and Cell Lines

RAW 264.7 (Mouse Macrophage cell line) was collected from National Centre for Cell Sciences (NCCS), Pune, India. RAW 264.7 macrophage cell was purchased from the American Type Culture Collection (USA) and cultured in suitable medium, i.e. high-glucose Dulbecco's modified Eagle's medium (4500 mg/L D-glucose; Invitrogen, Carlsbad, CA) supplemented with 100 IU/mL penicillin/streptomycin (PS) and 10% fetal bovine serum. Cells were maintained under the specific conditions like 75 cm^2 flasks, under 5% CO2 and 70% humidity at 37°C until 70% confluent. Human lung carcinoma (A549), Hepatocellular carcinoma (HepG2), human leukemia (HL-60), human breast adenocarcinoma (MCF-7), human prostate carcinoma (PC-3), human colorectal carcinoma (HCT 116) and human cervical carcinoma (HeLa), were obtained from American Type Culture Collection (ATCC), Rockville, MD, USA. Cells were cultured in RPMI-1640 (Invitrogen, Gibco, Waltham, MA, USA) medium which consist of L-glutamine at 5% CO_2-humidifed atmosphere at 37 °C growth medium supplemented with 10% heat-inactivated fetal bovine serum (HIFBS) and 1% PS.

4.3. Structure elucidation of isolated compound

The structure of most bioactive isolated compound was identified by the structural characterization using FT-IR, ^{1}H-NMR, ^{13}C-NMR, mass spectroscopy, melting point (m.p.). The results were compared with spectral data obtained from the literature [2].

4.3.1. Fourier Transform Infrared (FTIR) spectroscopy

The isolated compound was a powder and characterized by FTIR spectrophotometer (Perkin Elmer Spectrum one FTIR spectrometer, USA) using potassium bromide (KBr) disc method. The IR spectrum was scanned at infrared region of 400–4000 cm^{-1}.

4.3.2. Nuclear Magnetic Resonance (NMR) spectroscopy

^{1}H-NMR and ^{13}C-NMR spectra of isolated compound in $CDCl_3$ were acquired on a Bruker Avance -400 (400- MHz) spectrometer, using tetramethylsilane (TMS), as an internal standard (chemical shift values were quoted in ppm, δ). The NMR peaks were labelled as

singlet (s), doublet (d), triplet (t) and multiplet (m) chemical shifts were referenced with respect to solvent signals.

4.3.3. Liquid chromatography Mass Spectrometry (LC-MS) Analysis

Mass spectra of the isolated compound was determined using liquid chromatography-mass spectrometry. MS experiments were performed on Agilent 6540 LC-Q-T of Mass spectrometer. Positive mode ESI was used for the ionization of the compound. Agilent 6540 UHD Accurate-Mass detector was used with AJS ESI (Agilent Jet Stream) as source. Agilent Mass Hunter Qualitative analysis (B.04.00) software was used for data analysis and Agilent Mass Hunter workstation software was used for data acquisition. The Chromatography was performed using a Zorbax reverse-phase column (RRHD SB-C18 3 × 150 mm, 1.8 μm) (Agilent Technologies, Santa Clara, CA). The compound was prepared in MS grade methanol and 5 μL of the compound was injected through UPLC into ESI probe. The LC-MS conditions: mobile phase; double distilled deionized water and acetonitrile both with 0.1% formic acid (100 μL·100 mL−1) at a flow rate 0.5 mL·min−1 positive ion mode; gas (N_2); temperature, 350°C; nebulizer pressure, 15 psi; nebulizer pressure 60 psig, nozzle voltage 1 kV and capillary voltage 3.5 kV. Signals in the m/z 100–1000 units range were recorded.

4.4. Enzyme Inhibition Assay

4.4.1. Cycloxygenase-1(COX-1) and Cycloxygenase-2 (COX-2) inhibition assay

The assay was performed by using Colorimetric COX (human ovine) inhibitor screening assay kit [3]. Briefly, the reaction mixture contains, 150 μL of assay buffer, 10 μl of heme, 10 μL of enzyme (either COX-1 or COX-2), and 10 μL of crude extracts (10 mg/mL) and isolated compound (1.0 mg/mL). The assay utilizes the peroxidase component of the COX catalytic domain. The peroxidase activity was assayed colorimetrically by monitoring the appearance of oxidized N, N, N, N'-tetramethyl-p-phenylenediamine (TMPD) at 590 nm. Boswellic acid was used as a standard drug. The percent COX inhibition was calculated using following equation:

COX inhibition activity (%) = (1-T/C) X 100, where T = Absorbance of the inhibitor well at 590 nm, and C = Absorbance of the 100 % initial activity without inhibitor well at 590 nm.

4.4.2. 5-Lipoxygenase (5-LOX) assay

In 5-lipoxygenase assay, the mixture contained 25 µL of each sample dissolved in dimethylsulfoxide, 50 µL of linoleic acid (0.003 g/10 mL) and made up to 1 mL with 0.1 M borate buffer with Tween 80- 0.005%. The reaction was initiated with the addition of 1.5 µL 5-lipoxygenase (0.054 g/mL) [4]. The increase in absorbance at 234 nm was recorded for 5 min. The percentage inhibition of enzyme activity was calculated by comparison with the negative control: % = $[(A_0 - A_1)/A_0] \times 100$, where A_0 was the absorbance of the blank sample and A1was the absorbance of the sample. Tests were carried out in triplicate. Sample concentration providing 50% inhibition (IC_{50}) was obtained plotting the inhibition percentage against sample concentrations. Boswellic acid was used as a positive control.

4.4.3. Nitrite assay

The nitrite concentration was measured as an indicator of NO production, according to the Griess reaction [5]. Nitric oxide levels were determined by measuring the amount of nitrite in the cell culture supernatant using Griess reagent according to the manufacturer's protocol. The RAW 264.7 cells were stimulated with LPS (1 µg/ mL) and samples for 24 h followed by centrifugation at 1500 rpm for 10 min. Next, 50 µL of cell culture supernatant was mixed with 50 µL of Griess reagent (consisting of 1 % sulfanilamide, 0.1 % N-(1-naphthylethylenediamine) dihydrochloride and 2.5 % ortho-phosphoric acid) and was incubated for 15 min at room temperature. The absorbance was measured using an ELISA(Enzyme Linked Immunosorbent Assay) reader at 540 nm and compared with a standard calibration curve prepared with sodium nitrite solution. Dexamethasone was used as a positive control.

4.4.4. Measurement of cytokine TNF-α (Tumor Necrosis – α) level

The RAW 264.7 cells were seeded at 5 × 104 cells/swell in flat-bottomed 96-well plates. Samples and LPS (1 µg/mL) were added to the culture medium and were incubated at 37 °C for 24 h. The medium was collected in a microcentrifuge tube and was centrifuged at 1500 rpm for 10 min. The supernatant was decanted into new microcentrifuge tubes, and the level of TNF-α, was determined using the commercial mouse ELISA kit. Dexamethasone was used as a positive control.

4.4.5. In Vitro antioxidant activity (DPPH Assay)

The method of Padmanabhan and Jangle [6], with slight modification, was followed to determine the radical scavenging capacity (RSC) of the crude extracts and the isolated pure compound. The free radical scavenging activity of the herbal extracts/pure compound and positive control (ascorbic acid) was determined using the stable free radical DPPH (1,1-Diphenyl-2-picrylhydrazyl). In this method, 96 well plates were used. Briefly, a 10 μL aliquot of each extract and isolated pure compound, which was added to 100 μL of 60 µM DPPH solution (prepared in ethanol) in a 96-well microtiter plate and mixed thoroughly. The reaction mixture was incubated in the dark for 30 min at room temperature. The absorbance of the wells was then measured at 540 nm on a microplate reader (Spectramax Plus, Molecular Devices, CA, USA). The radical scavenging activity of the samples was expressed as % inhibition of DPPH absorbance using the following equation:

Inhibition (%) = [1 − (A sample – A sample blank) A control] × 100

Where A control was the absorbance of the control (DPPH solution without test sample), A sample was the absorbance of the test sample (DPPH solution plus test sample), and A sample blank was the absorbance of the sample only (sample without DPPH solution).

4.4.6. Cell proliferation assay using MTT (3–(4,5-dimethyltiazol-yl)-2,5-diphenyl tetrazolium bromide) Bioassay

Antiproliferation screening activity of crude extracts and sub fractions of ethyl acetate extract was determined against one human cancer cell, HepG2 (human hepato carcinoma cell line) using the MTT assay (Promega, Fitchburg, WI, USA) and the antiproliferative activity of isolated compound, betulinic acid was investigated with the MTT bioassay using various human cancer cell lines such as liver-HepG2, lung-A549, Leukaemia-HL-60, breast-MCF-7, colon-HCT-116, prostrate-PC-3 and cervix-HeLa. Briefly, each cell line was cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100 U/mL of penicillin and 100 µg/mL of streptomycin). This assay is based on the cleavage of the MTT to purple formazan crystals by metabolically active cells. MTT assay was done as described previously [7]. The cancer cells were inoculated into a 96-well culture plate (1 × 104 cells/well) and treated with tested compounds in different concentrations at 37^{o}C for 24 h. After removing the medium from each well, 100 µL of MTT (500 µg/mL) was added to each well, and the plate was incubated at 37°C for 1 h. When purple precipitate was clearly visible under the microscope, 80 µL of DMSO was added to each well. The plate was incubated in the dark for 1 h at room temperature. The

spectrophotometric absorbance of the samples was detected by using an ELISA reader (SpectraMax M5e, Molecular Devices LLC, Sunnyvale, CA, USA) at 570 nm treatments. All measurements were performed in triplicate. The percentage of inhibition was calculated according to the equation:

Cell inhibition % = (1 − (A Sample − A Blank)/(A Control – A Blank)) × 100.

4.5. Statistical Analysis

All the data analysis was performed with Sigma Plot Statistical Software (Version 11.0) and Graphpad Prism (version 5.0). The differences between the mean values (in triplicate) were assessed for statistical difference using one-way analysis of variance (ANOVA) if any. $p<0.05$ was considered as statistically significant. The results were shown as the mean ± standard deviation (SD).

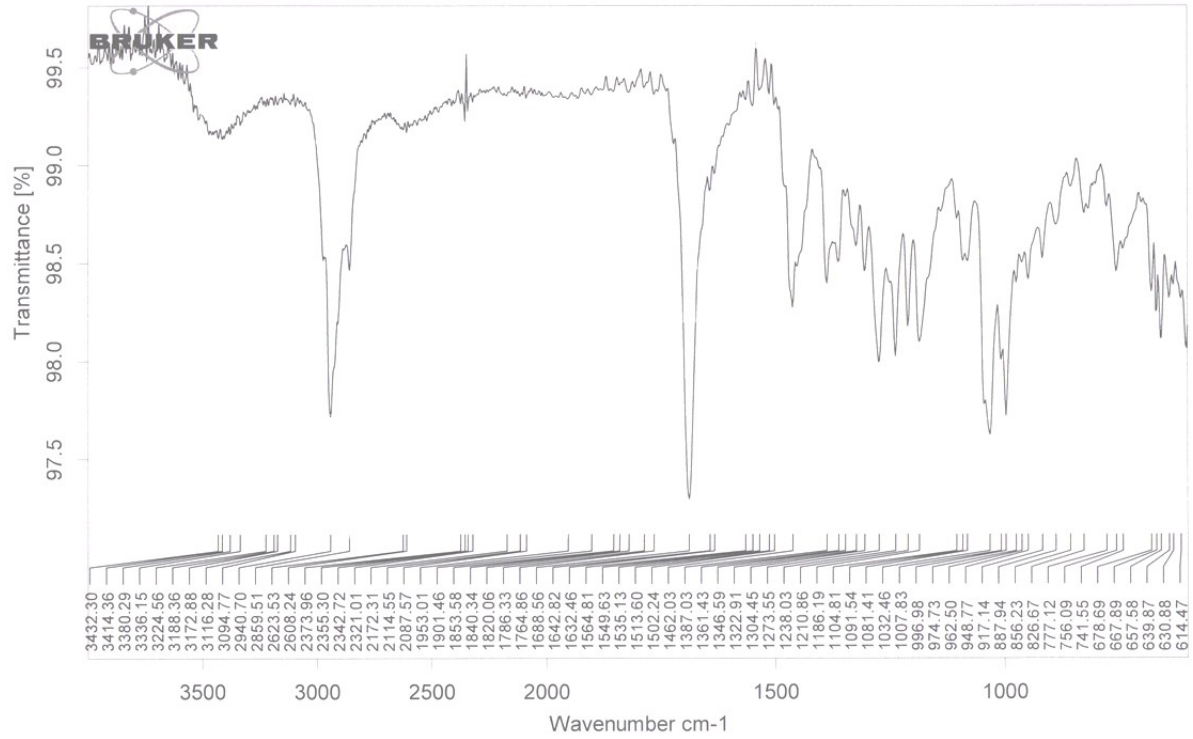
4.6. Results

4.6.1. Spectral analysis

4.6.1.1. FT-IR Spectroscopy

The FT-IR spectrum of the isolated compound from the methanol extract of *Nyctanthes arbor-tristis* L. leaves showed a clear and distinguished peak in the finger print and functional group region (Figure 4.1). A broad peak near 3432.3 cm^{-1} represented the aromatic alcoholic functional group (-OH) present in the structure. The stretching vibrational frequency (C-H str.) at 2940.7 cm^{-1} and 2342.7 cm^{-1} were found and proposed to be the aliphatic $–CH_3$ and $–CH_2$ groups present in the structure. The C=O and C-O stretching vibration was observed at 1688.6 cm^{-1} and 1032.5 cm^{-1} respectively. These frequencies evident the presence of carboxylic acid group (-COOH) in the structure. Similarly, C-H deformation at 1387.0 cm^{-1} indicated the gem dimethyl group (two methyl groups of the same carbon atom) present in the isolated structure. As per the literature such type of functional groups closely match with the structure of triterpenoids *i.e.* betulinic acid (lupane), oleanolic acid (oleanane), and ursolic acid (ursane) [8-10]. The presence of $–CH_2$ completely justifying the presence of lupane triterpene “betulinic acid” from the other triterpenoids identified and reported in the literature [8-10].

Figure - 4.1: FT-IR of the isolated compound from the methanol extract of *Nyctanthes arbor-tristis* leaves.



4.6.1.2. Nuclear Magnetic Resonance Spectroscopy

The ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of lupane triterpene displayed resonances at δ 0.92 (3H, m, *J* = 29.5, H-30), 1.25 (6H, s, H-23 & H-24), 1.70 (9H, br, CH_3 × 3, H-25, H-26, & H-27), 3.21 (1H, OH × 1, H-3), 5.29 (2H, br, *J* = 5, H-29) (Figure 4.2). The resonance at δ 5.29 confirms the two hydrogen atoms at C-29 position which is very specific to the structure of betulinic acid. Similarly, ^{13}C-NMR spectrum (300 MHz, $CDCl_3$) of lupane triterpene showed resonances for 30 carbons at δ 184.34 (C-28), 124.03 (C-20), 80.44 (C-29), 56.62 (C-3), 49.03 (C-17), 47.91 (C-5), 47.28 (C-9), 43.01 (C-19), 42.40 (C-18), 40.67 (C-14), 40.16 (C-8), 39.80 (C-4), 38.48 (C-1), 35.20 (C-13), 34.47 (C-10), 34.03 (C-22), 33.84 (C-7), 32.07 (C-16), 31.10 (C-15), 29.50 (C-21), 29.09 (C-23), 28.58 (C-2), 27.32 (C-12), 24.98 (C-11), 24.80 (C-30), 24.33 (C-6), 19.69 (C-25), 18.52 (C-26), 16.95 (C-24), 16.72 (C-27). The 30 carbon resonance represented the structure contains the 30 carbon atoms which favor the structure of lupane triterpene "betulinic acid". The resonances at δ 42.40 (C-18), 43.01 (C-19), 124.03 (C-20), 29.50 (C-21), 34.03 (C-22), and 29.09 (C-23) confirmed the five membered group in betulinic acid (Figure 4.3). Similarly, ^{13}C resonances at δ 80.44 (C-29) confirmed the C-29 which is critical to the structure of betulinic acid. ^{1}H and ^{13}C NMR data

obtained were in agreement with those reported in literature [9,11] for betulinic acid. Hence ^{1}H and ^{13}C NMR helpful to confirm the presence of betulinic acid in leaves of *Nyctanthes arbor-tristis* L.

Note: br- broad, s- singlet, and m- multiplet

Figure - 4.2: 1H-NMR of the isolated compound from the methanol extract of *Nyctanthes arbor-tristis* leaves.

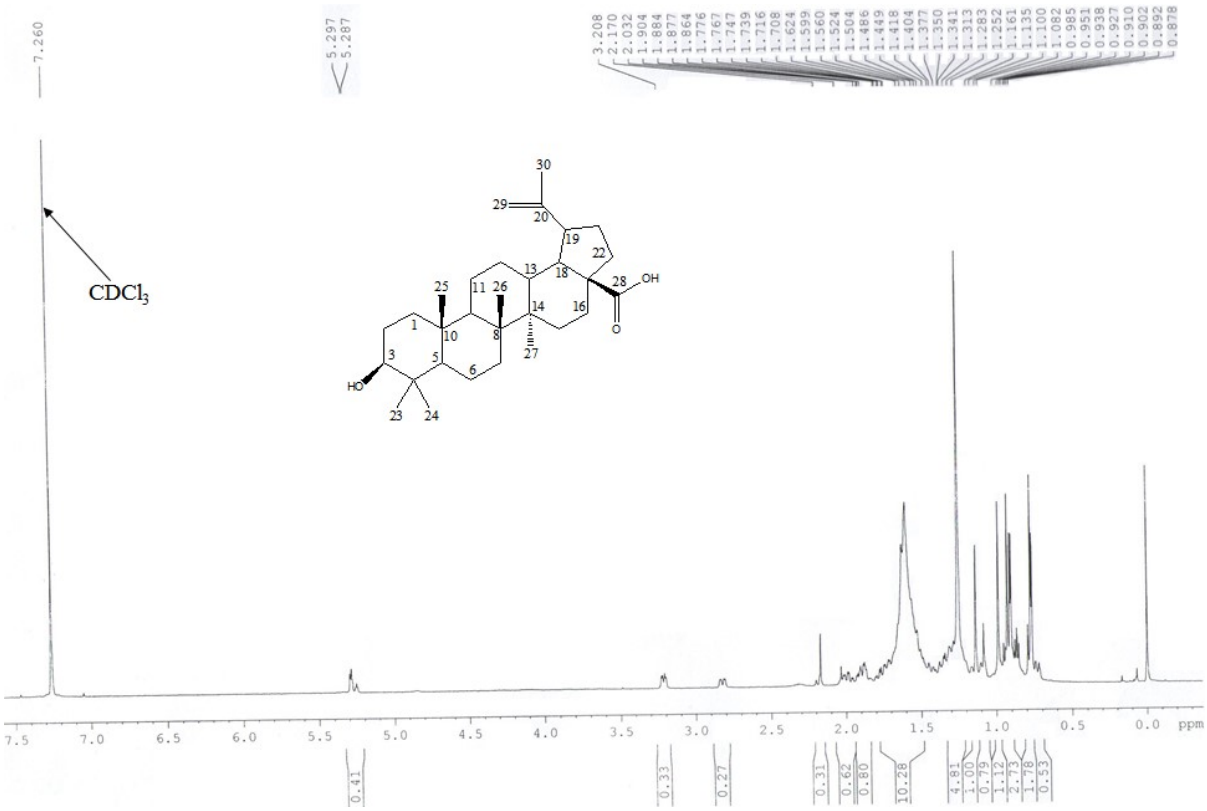
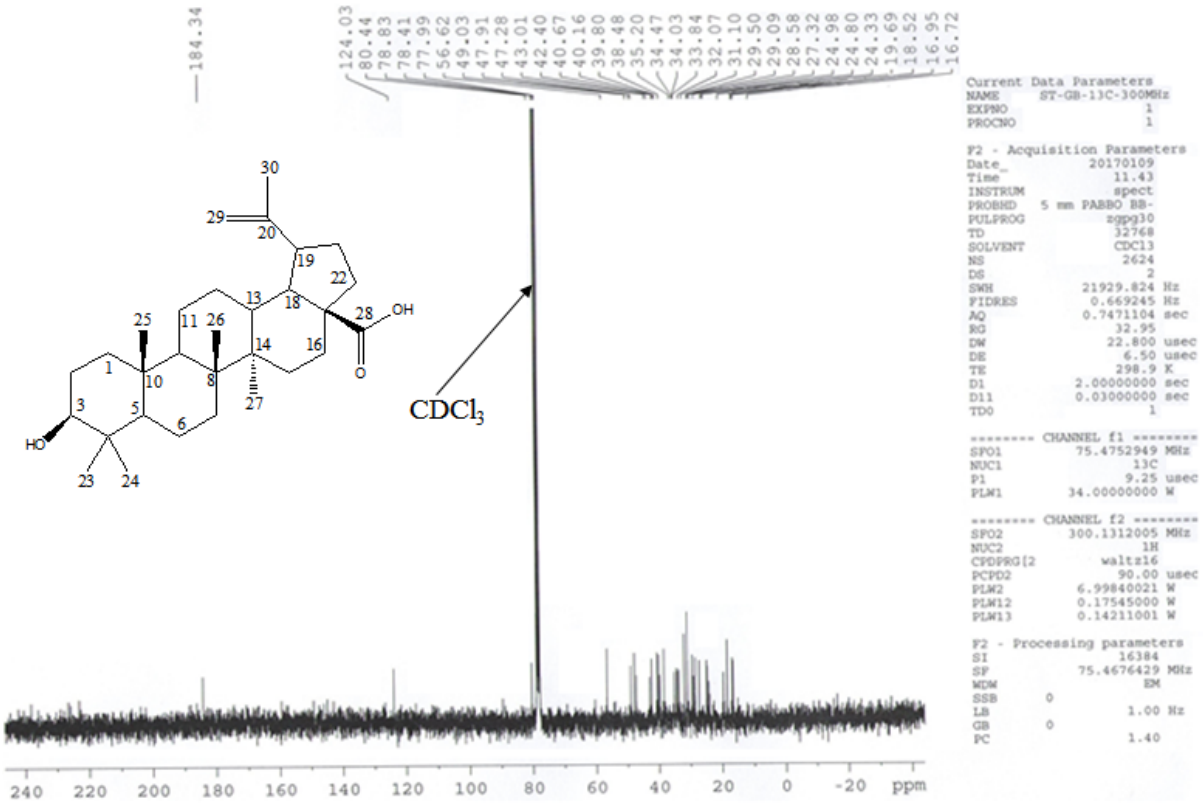


Figure - 4.3: 13C-NMR of the isolated compound from the methanol extract of *Nyctanthes arbor-tristis* leaves.



4.6.1.3. Mass Spectroscopy

The ESI-MS (+ve mode) spectrum of the isolated compound from the methanolic extract of *Nyctanthes abor-tristis* leaves (Figure 4.4) exhibited the presence of the molecular ion adduct along with the parent molecule at *m/z* 457.172, proposed to be betulinic acid (calcd. for $C_{30}H_{48}O_3^+$, 457.37) for the first time. Similarly, betulinic acid sodium adduct $[M+Na]^+$ and dimer of betulinic acid sodium adduct $[2M+Na]^+$ were also observed in the mass spectrum at *m/z* 479.056 (calcd. for $C_{30}H_{48}NaO_3^+$, 479.35) and 935.16 (calcd for $C_{60}H_{96}NaO_6^+$, 935.71), respectively (Figures 4&5). Monoisotopic adducts formation, either with solvents, alkali or other metal ions (Na, K, etc.) or with other contaminating components which are frequently observed in ESI-MS analysis [12,13]. Other lower fragmentations at *m/z* 413.04, 300.98, and 148.93 were found to be $C_{29}H_{42}Na^+$ or $C_{29}H_{49}O^+$, $C_{22}H_{14}Na^+$, and $C_{10}H_6Na^{\cdot+}$ or $C_9H_9O_2^+$, respectively in the mass spectra (Figures 4.4 & 4.5). The mass spectral data were in agreement with those previously reported in the literature for the same compound [9,11] and the molecular mass fragmentation pattern helped to determine the comprehensive structural

information of the isolated compound from the methanol crude extracts of the leaves of *Nyctanthes arbor-tristis* L. Finally, it can be concluded that the isolated compound could be the lupane triterpene *i.e.* betulinic acid, which was further corroborated and confirmed by the FT-IR (Figure 4.1), ^{1}H-NMR (Figure 4.2) and ^{13}C-NMR (Figure 4.3) spectral information.

Figure - 4.4: Mass spectrum of isolated compound from the methanol extract of *Nyctanthes arbor-tristis* leaves showing the proposed structure with mass fragmentation peaks.

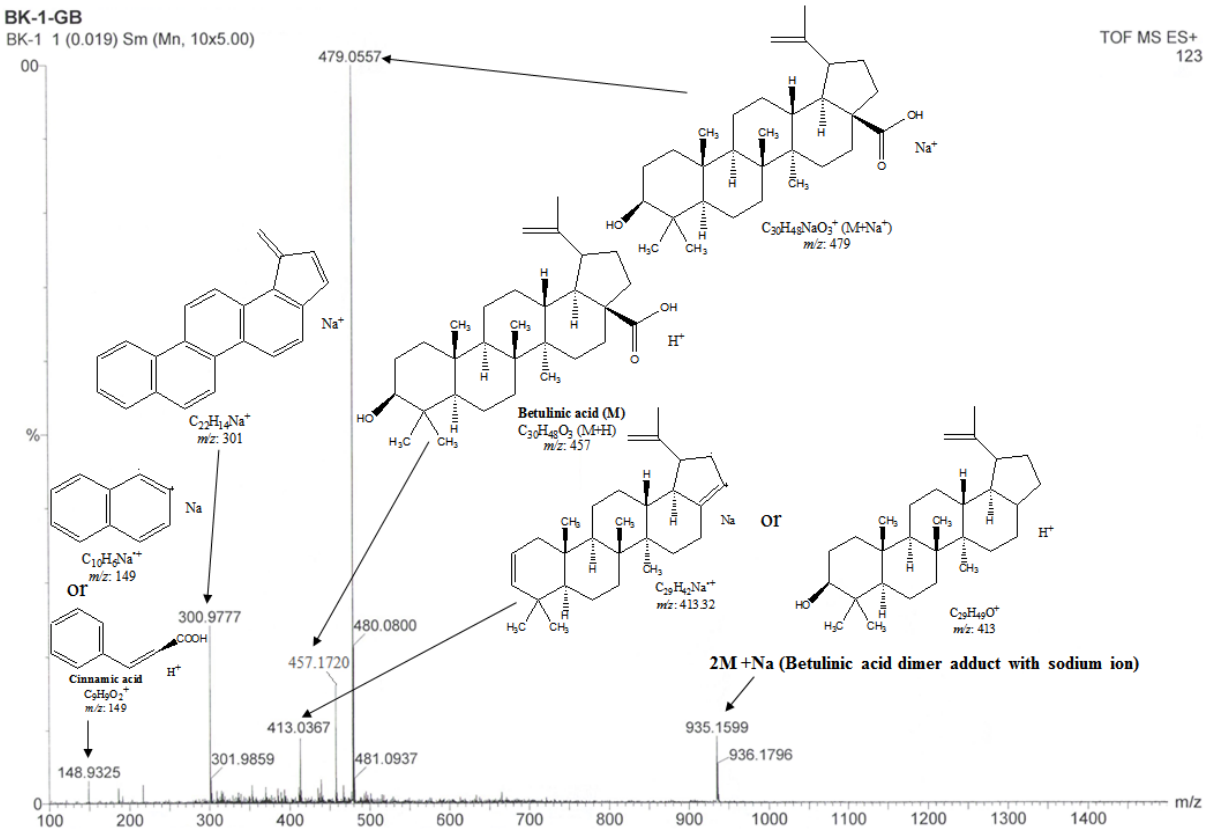
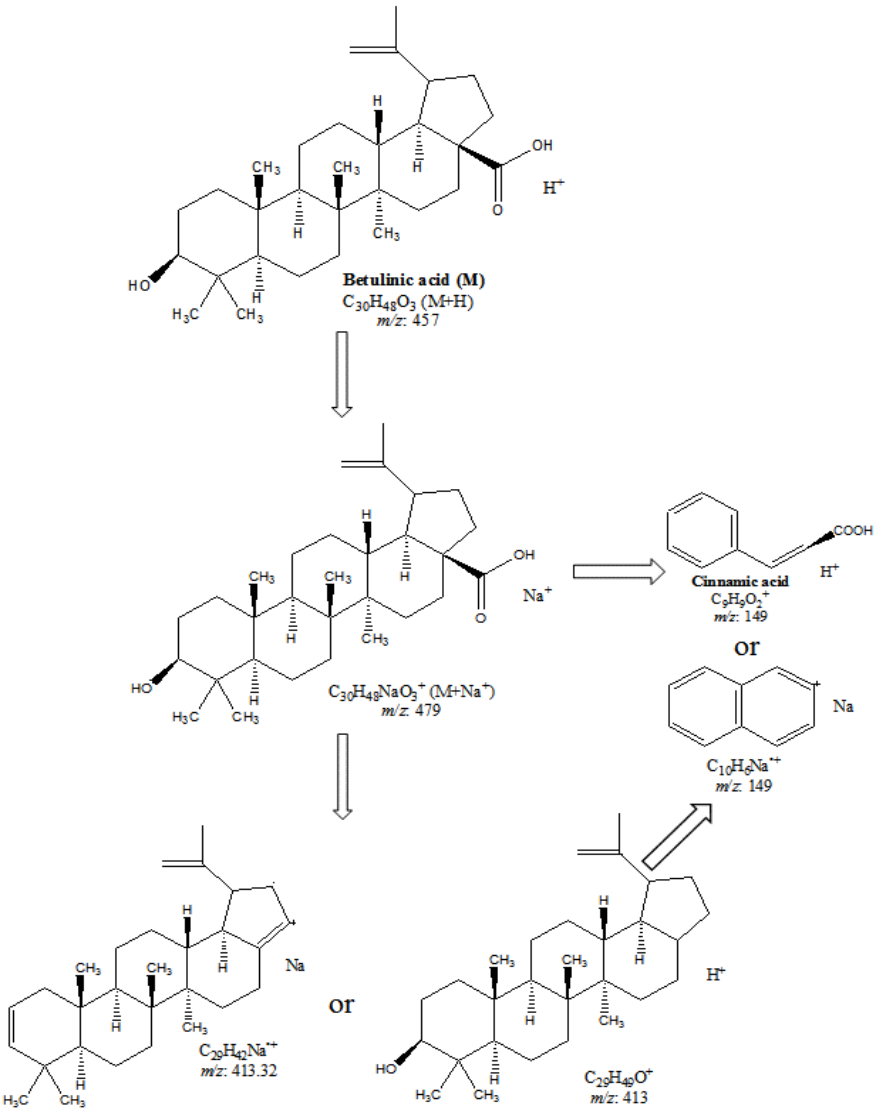


Figure - 4.5: Mass fragmentations pattern of the proposed isolated compound from the methanol extract of *Nyctanthes arbor-tristis* leaves.



4.6.2. Cell viability assay

The Cytotoxicity of all crude extracts (500 µg/mL), and sub-fractions of ethyl acetate extract (1–100 µg/mL) from leaves of *Nyctanthes arbor-tristis*, isolated compound (betulinic acid) and all positive controls (boswellic acid, ascorbic acid, dexamethasone, and doxorubicin) was tested in RAW 264.7 macrophages using MTT assay. The data revealed that at the chosen concentrations of the test items the cell viability was greater than 80% (data not shown). RAW 264.7 cells release various inflammatory mediators including COXs, 5-LOX, nitrite and cytokine, TNFα in response to stimuli like LPS. In addition, LPS (0.1 µg/mL) alone had no impact on cell viability.

4.6.3. Effects of crude extracts, ethyl acetate sub-fractions and isolated compound on Ant-inflammatory, Anticancer and Antioxidant activity

To assess the bioactivity of different crude extracts of *Nyctanthes arbor-tristis* leaves, we measured the test item-mediated percentage inhibition of COX-1, COX-2, 5-LOX enzyme activity, and percent free radical scavenging activity using DPPH, inhibition of the production of nitrite and TNFα in LPS-induced macrophage RAW 264.7 cells, and inhibition of HepG2 cell proliferation (cell-based assays) at a single concentration of 500 µg/mL. The results are shown in **Table 4.2**. Ethyl acetate extract showed noticeable percentage inhibition of COX-1 (82.33%), COX-2 (80.10%), 5-LOX (75.89%), Nitrite (78.64%), TNFα (77.55%), HepG2 cell proliferation (89.37%) and percentage free radical DPPH scavenging effect (69.68%) at 500 µg/ mL. Methanol, and n-butanol extracts of *Nyctanthes arbor-tristis* leaves showed lower percentage inhibition (<50%) of anti-inflammatory, anticancer and lesser DPPH scavenging effect. The aqueous extract did not show any activity (**Table 4.2**). Boswellic acid (50 µg/mL) was used as a positive control for inhibition of COX-1, COX-2, and 5-LOX; dexamethasone (15 µg/mL) was used as a positive control for NO and TNFα; doxorubicin (10 µg/mL) was used as a positive control for anticancer activity, and ascorbic acid (50 µg/mL) as a positive control for free radical scavenging activity (**Table 4.2**). Positive controls data were well matched with the literature values. The bioactivity data indicated that ethyl acetate extract from *Nyctanthes arbor-tristis* leaves had the highest anti-inflammatory, anticancer and antioxidant activity at nontoxic tested concentration. Based on these results, one part of the ethyl acetate extract (EtOAc fraction) was selected and proceeded for the further separation using column chromatography and eluted in a gradient manner with chloroform - ethyl acetate – methanol (20:60:20), and collected fourteen sub fractions (labelled as sub fractions (sub of EtOAc: 1 to 14). These sub fractions were tested for the evaluation of their anti-inflammatory activity in vitro assay. The sub-fractions 6-9 of ethyl acetate extract were showed almost similar and better anti-inflammatory activities as compared to other sub-fractions (**Table 4.3**). Based on the various anti-inflammatory effects, the sub fractions (6-9) were pooled and further purified in a similar manner using a silica gel column chromatography with stepwise gradient elution with chloroform – methanol (60:40 *v/v*) at the final step and purified by crystallization in methanol, yielding a compound, which was white color powder.

4.6.4. Structure identification of isolated compound as Betulinic acid

The bioactive compound was identified by detailed analysis of melting point, FT-IR, ^{1}H NMR, ^{13}C NMR and LC-MS data and all these data were compared with the literature data. The melting point of isolated compound was 317^{o}C, which was well matched with reported m.p. of betulinic acid [14]. The isolated compound showed a single peak with Rt-9.5 min (**Figure 4.6 A**) in LC-MS analysis, indicated the compound was pure (> 99%). The full scan mass spectra of the isolated compound are shown in **Figure 4.6 B**. The molecular ion $[M+1]^{+}$ peak was obtained at 457.7 m/z which confirms molecular weight of the isolated compound at 456.7 [1]. FT-IR spectroscopy was carried out to ascertain functional groups. The FT-IR spectrum of the compound showed dominant IR absorption bands in the high wave region at 3467 cm^{-1}, and 2942 cm^{-1} attributed to -OH, and -CH_2 asymmetric and symmetric stretching vibrations respectively. In finger print region, the FT-IR spectrum represented dominant bands at 1672, 1457, 1376, 1239, 1035, 885, 722 cm^{-1} and many other bands of medium to weak intensity. The observed band at 1672 cm^{-1} can be assigned to C=O stretching of –COOH functional group. Other bands in the spectral range were assigned to bending vibrations of –OH, - CH_2 and CH_3 groups as well as to skeletal bending bonds. The band at 1239 cm^{-1} was due to C-O stretching among others. The intense band as 885 cm^{-1} in IR spectrum was due to vibration of the CH_2 in alkene group. Theoretical wave numbers responsible for the functional groups were compared with observed wave numbers and closely matched with the structure of lupane triterpenoid i.e., betulinic acid [15,16]. The ^{1}H NMR and ^{13}C NMR experiments of the isolated compound were carried out, and the results obtained mentioned in **Table 4.4**. The ^{1}H NMR spectrum showed six tertiary methyl singlets at δ 0.62, 0.66, 0.69, 0.74, 0.93, 0.97 and one more secondary hydroxyl group showed broad triplet at δ 3.23 and two olefinic protons at δ 4.54 and 4.67 representing the exocyclic double bond. The ^{13}C NMR spectrum confirmed the presence of signals in vinyl 150.9 and 109.6 ppm (double bond), carbonyl acid in 180.03 ppm and secondary alcohol in 79.69 ppm. The appearance of carbonyl group at δ 178.4 in ^{13}C NMR spectrum suggested the presence of the acid group in its structure (**Table 4.4**) [1,17]. Based on the above spectral data, the structure of the isolated compound was assigned as being betulinic acid (**Figure 4.6 C**), which was further corroborated by the physical and spectral data reported from the literature [14,18].

4.6.5. Anti-inflammatory and Antioxidant Activity of Isolated Betulinic acid

The pure isolated compound, betulinic acid showed significant percentage inhibition on COX-1 (99.43%), COX-2 (92.63%), 5-LOX (84.43%), NO (97.43%), TNFα (93.67%),

HepG2 cell cytotoxicity (99%) and percentage free radical scavenging effect (79.18%) at 40 µg/mL. The percentage inhibition of pure isolated compound also demonstrated dose-dependent anti-inflammatory and antioxidant activity with different concentrations (1–40 µg/mL), which are shown in **Figure 4.7 A & 4.7 B**. Betulinic acid's IC_{50} values against COX-1 (10.34 µg/mL), COX-2 (12.92 µg/mL), 5-LOX (15.53 µg/mL), NO (15.21 µg/mL), and TNFα (16.65 µg/mL). There was a significant enhancement of the inhibitory effects of ethyl acetate fraction on various inflammatory markers with the progress of purification with a decrease in IC_{50} from 160.56 -180.44 to 16.65 µg/mL (**Table 4.2**). The bioactivity-guided fractionation of the ethyl acetate extract indicated that the isolated betulinic acid was responsible for the anti-inflammatory activity. Also, betulinic acid had also shown a strong percentage of free radical scavenging activity (antioxidant) in DPPH assay with an IC_{50} of 18.03 µg/mL (**Figure 4.7 C**).

4.6.6. Anticancer activity of isolated Betulinic acid on various cancer cell lines

The anticancer activity of crude extracts (500 µg/mL) from *Nyctanthes arbor-tritis* leaves was evaluated in preliminary screening assay against hepatocellular carcinoma cell line-HepG2. The percentage inhibition of the cell proliferation data is shown in **Table 4.2**. The methanol and ethyl acetate leaves extract of *Nyctanthes arbor-tritis* were shown 39.27% and 89.37% inhibition, respectively against HepG2 and the IC_{50} value of crude ethyl acetate extract was 145.48 µg/mL. To isolate and identify the most potent bioactive compound, which may be responsible for the anticancer activity, ethyl acetate fraction was extracted successively with the various solvent systems using column chromatography and the cytotoxicity of each sub-fraction of ethyl acetate extract was assessed in HepG2. The sub fractions were also shown anti-proliferative activity against HepG2 (data not shown). The antiproliferative activity of isolated pure compound betulinic acid was further investigated with the MTT bioassay on various human cancer cell lines (liver-HepG2, lung-A549, Leukaemia-HL-60, breast-MCF-7, colon-HCT-116, prostrate-PC-3, and cervix-HeLa). These cancer cells were treated with betulinic acid (for 24 h incubation) at different test sample concentrations (1– 40 µM), which showed dose-dependent cytotoxic effects (**Figure 4.8**). It exhibited excellent anti-proliferative activity against HepG2 (IC_{50} = 6.53 ± 0.26 µM), A549 (IC_{50} = 9.34 ± 0.30 µM), HL-60 (IC_{50} = 14.92 ± 0.27 µM), MCF-7 (IC_{50} = 16.90 ± 0.16 µM), HCT-116 (IC_{50} = 17.07 ± 0.12 µM), PC-3 (IC_{50} = 13.27 ± 0.17 µM), and HeLa (IC_{50} = 12.55 ± 1.02 µM) (Table 4.4).

4.6.7. Table Legends

Table- 4.2 : Effects of the crude ethyl acetate extract and other three different solvent extracts (500 µg/mL) of *Nyctanthes arbor-tristis* (NAT) leaves on the anti-inflammatory activity (COX-1, COX-2; 5-LOX, Nitrite, TNFα), anticancer activity (cell proliferation in HepG2) and antioxidant activity (free radical scavenging activity-DPPH). Results are summarized with the mean values of n = 3 ± S.D. NR- no reaction under experimental condition. PC (Positive Control).

Name of Fraction (Leaves of *Nyctanthes arbor-tritis*-NAT)	% Inhibition of COX-1, COX-2, 5-LOX, Nitrite, TNFα, Cell proliferation and % Free radical scavenging activity (DPPH)						
	COX-1	COX-2	5-LOX	Nitrite	TNFα	Cell proliferation (HepG2)	Antioxidant-DPPH
Methanol extract of NAT	31.64 ± 4.21	33.11± 6.37	36.78 ± 3.22	35.64 ± 4.21	40.10 ± 3.46	39.27 ± 3.80	35.90 ± 2.43
Ethyl acetate extract of NAT	82.33 ± 2.11	80.10 ± 1.26	75.89 ± 4.53	78.64 ± 5.66	77.55 ± 4.36	89.37 ± 5.49	69.68 ± 4.15
n-Butanol extract of NAT	21.22 ± 1.11	22.00 ± 1.78	24.64 ± 3.58	26.25 ± 5.20	21.64 ± 2.44	28.22 ± 2.58	30.64 ± 3.11
Aqueous extract of NAT	11.22 ± 0.32	8.00 ± 0.75	NR	NR	NR	5.17 ± 0.27	7.13 ± 0.31
IC_{50} of Ethyl acetate extract (µg/mL)	160.56 ± 8.81	165.33 ± 6.31	180.44 ± 7.20	169.37 ± 6.12	171.30 ± 5.05	145.48 ± 4.18	190.75 ± 7.46
IC_{50} of isolated Betulinic acid (µg/mL)	10.34 ± 0.72	12.92 ± 0.54	15.53 ± 0.88	15.21 ± 0.61	16.65 ± 0.87	6.53 ± 0.37	18.03 ± 0.19
PC-Boswellic Acid (50 µg/mL)	97.36 ± 2.57	94.32 ± 3.70	92.64 ± 4.18	–	–	–	–
PC-Dexamethasone (15 µg/mL)	–	–	–	97.99 ± 3.52	98.34 ± 3.22	–	–
PC-Doxorubicin (10 µg/mL)	–	–	–	–	–	99.87 ± 2.30	–
PC-Ascorbic acid (50 µg/mL)	–	–	–	–	–	–	98.66 ± 3.15

Table- 4.3 : Effects of the different sub-fractions (50 µg/mL of each fraction) of ethyl acetate (EtOAc) extract of *Nyctanthes arbor-tristis* (NAT) leaves on the anti-inflammatory activity (COX-1, COX-2, 5-LOX, Nitrite, TNFα). Results are summarized with the mean values of n = 3 ± S.D. NR- no reaction under experimental condition, ND- not determine, PC, positive control.

Name of Sub-fraction of Ethyl acetate (EtOAc) extract of NAT	% Inhibition of COX-1, COX-2, 5-LOX, Nitrite, and TNFα				
	COX-1	COX-2	5-LOX	Nitrite	TNFα
SubF1	25.32 ± 1.22	20.21 ± 0.98	23.44 ± 1.76	18.32 ± 1.36	15.32 ± 1.02
SubF2	30.03 ± 2.22	28.18 ± 1.07	25.89 ± 1.77	23.38 ± 1.67	29.03 ± 1.37
SubF3	28.00 ± 1.41	20.10 ± 1.08	24.58 ± 1.45	23.04 ± 1.08	19.37 ± 1.11
SubF4	36.73 ± 2.07	38.26 ± 1.09	15.77 ± 4.56	37.16 ± 1.56	29.17 ± 1.43
SubF5	46.23 ± 2.15	50.61 ± 1.27	53.38 ± 2.35	50.36 ± 2.55	54.95 ± 2.16
SubF6	80.12 ± 3.40	78.20 ± 3.01	79.34 ± 4.01	86.22 ± 2.71	88.11 ± 2.25
SubF7	92.10 ± 3.05	89.99 ± 3.51	87.56 ± 3.24	90.27 ± 3.44	92.20 ± 2.10
SubF8	84.33 ± 4.31	80.30 ± 3.30	80.12 ± 3.16	87.11 ± 2.31	89.37 ± 2.56
SubF9	78.44 ± 2.66	75.33 ± 2.07	73.42 ± 3.63	79.64 ±1.46	78.22 ± 1.21
SubF10	48.21 ± 2.30	40.11 ± 1.48	45.89 ± 2.16	39.61 ± 2.17	38.25 ± 1.44
SubF11	25.16 ± 1.42	30.16 ± 1.23	22.57 ± 1.32	20.46 ± 1.41	23.50 ± 1.36
SubF12	10.83 ± 1.02	7.19 ± 1.06	5.67 ± 1.03	3.35 ± 0.53	6.25 ± 0.24
SubF13	NR	NR	NR	ND	ND
SubF14	NR	NR	NR	ND	ND
Boswellic Acid (50 µg/mL)	95.86 ± 2.59	94.64 ± 1.37	93.20 ± 3.21	–	–
Dexamethasone (15 µg/mL)	–	–	–	96.55 ± 2.54	97.72 ± 2.14

Table- 4.4 : Comparison of the observed melting point, mass spectra, FTIR, 1H-NMR and 13C-NMR data with reported literature values of betulinic acid isolated from *Nyctanthes arbor-tristis* leaves.

Parameter	Betulinic acid (isolated compound from *Nyctanthes arbor-tristis* leaves)		References
	Observed Values	Reported Values	
Melting point (m.p.)	317^{o}C	316-319^{o}C	Cichewicz and Kouzi, 2004
Mass spectrometry	m/z-457.71 $[M+1]^{+}$ (Molecular Wt - 456.73)	m/z-456.7	Cichewicz and Kouzi, 2004; Falamaş et al., 2011
FTIR(cm-1)	CDCl3: 3467 cm-1(OH str), 1672 (C=O), 1457 (OH bend), 1376 (CH2=CH-CH3), 1239 (C=O, str), 722 (=CH, bending)	CDCl3: 3465 cm-1, 1670 cm-1, 1450 cm-1, 1373 cm-1, 1235 cm-1, 716 cm-1	Cichewicz and Kouzi, 2004; Anwar et al., 2008; Parvin et al., 2009; Kovac-Besovic et al., 2009
^{1}H-NMR (δ in ppm)	$CDCl_3$; δ 4.67 (1H, s), δ 4.542 (1H, s,m); δ 3.233 (1H, d); δ 0.926 (3H, s, Me-26)	δ 4.75, δ 4.62, δ 3.20, δ 3.00, δ 0.99	Cichewicz and Kouzi, 2004; Anwar et al., 2008; Parvin et al., 2009; Kovac-Besovic et al., 2009
^{13}C-NMR (δ in ppm)	$CDCl_3$; δ 178.4, δ 150.9, δ 109.6, δ 78.20, δ 76.70, δ 76.82, δ 57.2, δ 49.14, δ 42.52	δ 178.8, δ 151.3, δ 109.9, δ 78.4, δ 77.0, δ 76.81, δ 56.01, δ 49.8, δ 42.9	Cichewicz and Kouzi, 2004; Anwar et al., 2008; Parvin et al., 2009; Kovac-Besovic et al., 2009

Table- 4.5 : Cytotoxicity of betulinic acid isolated from *Nyctanthes arbor-tristis* (NAT) leaves on various cancer cell lines. Results summarized are the mean values of n = 3 ± S.D. Pc, positive control.

IC_{50} values of betulinic acid on various cancer cell lines							
Cancer Type	Liver	Lung	Leukaemia	Breast	Colon	Prostate	Cervix
Cell line	HepG2	A549	HL-60	MCF-7	HCT-116	PC-3	HeLa
IC_{50} of Betulinic acid (µM)	6.53 ± 0.26	9.34 ± 0.30	14.92 ± 0.27	16.90 ± 1.02	17.07 ± 0.12	13.27 ± 0.17	12.55 ± 0.42
IC_{50} of Doxorubicin (µM)	3.56 ± 0.13	11.62 ± 0.27	2.57 ± 0.16	6.52 ± 0.76	15.33 ± 0.72	10.23 ± 0.84	9.32 ± 0.73

4.6.8. Figure Legends

Figure - 4.6:

Liquid chromatogram of purified betulinic acid isolated from *Nyctanthes arbor-tristis* leaves (A) and its mass spectra of the protonated molecular ion [M+H]+ of an isolated and purified betulinic acid under the positive mode (B). m/z of betulinic acid is 456.7 and it's mass of protonated molecular ion [M+H]+ is 457.71. (C). chemical structure of betulinic acid.

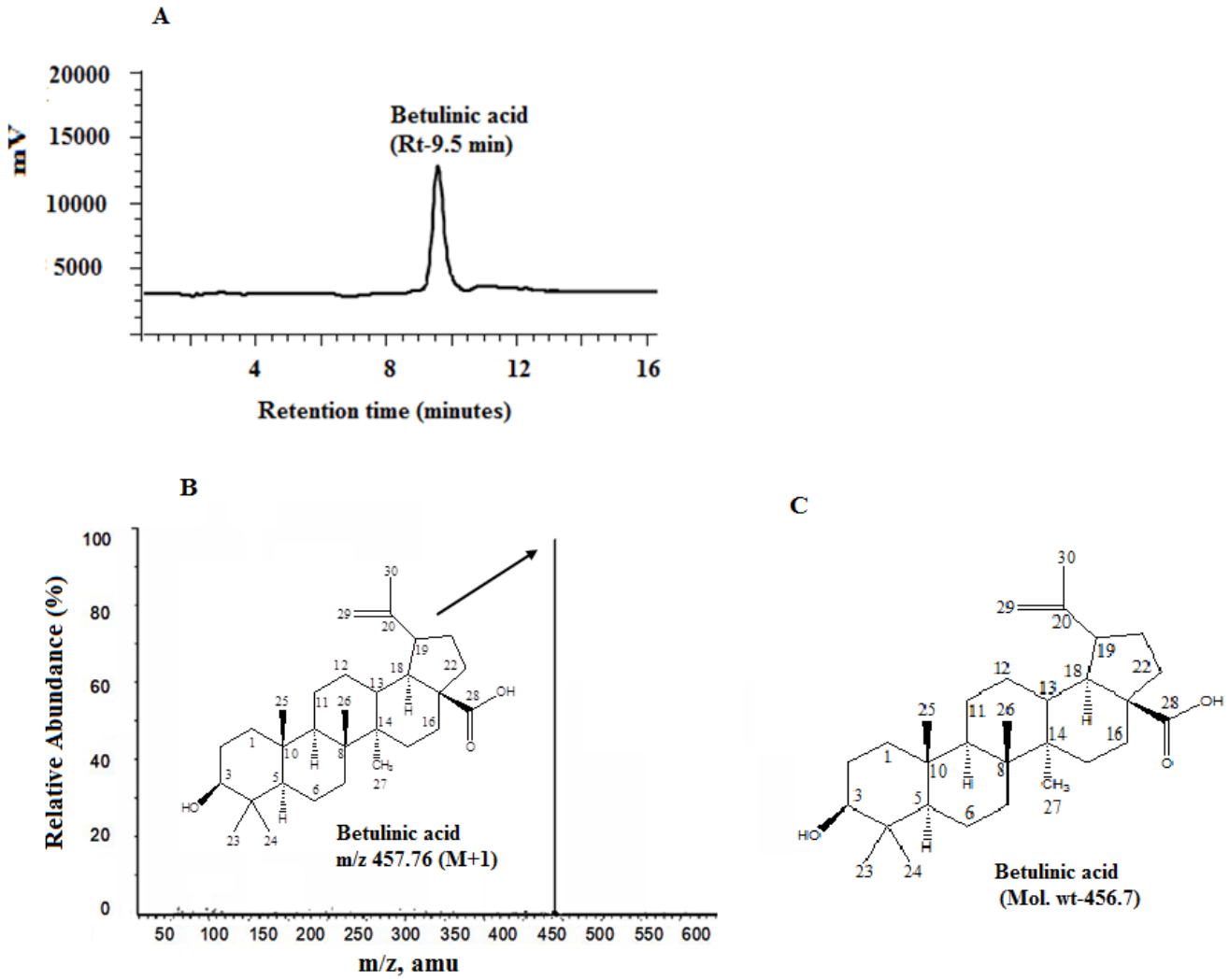


Figure - 4.7:

The inhibitory effect of purified betulinic acid isolated from *Nyctanthes arbor-tristis* on COX-1, COX-2, 5-LOX enzyme activity (A), nitrite and TNF-α production in RAW264.7 cells (B) and percent free radical scavenging activity in DPPH assay (C). COX-1, COX-2, 5-LOX and DPPH are the cell free assays. RAW264.7 cells were stimulated with LPS (1 µg/mL) for 24 hours, or were left untreated (control). The production of nitrite, and TNF-α level was measured using ELISA. Data presented is the mean ± S.D. of three independent experiments. Error bars denote S.D. IC50 value was determined using Graph pad prism (version 5) software.

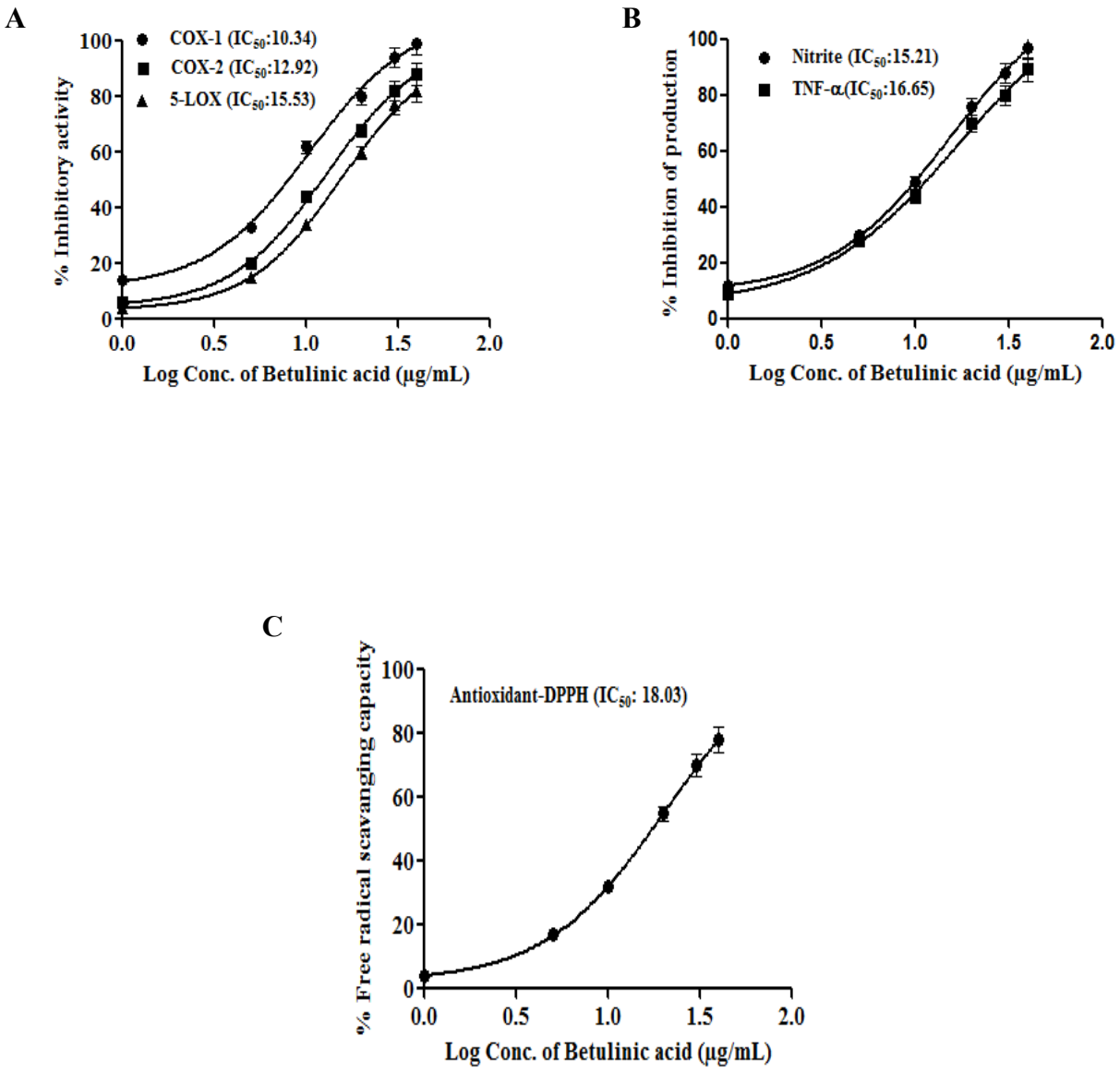
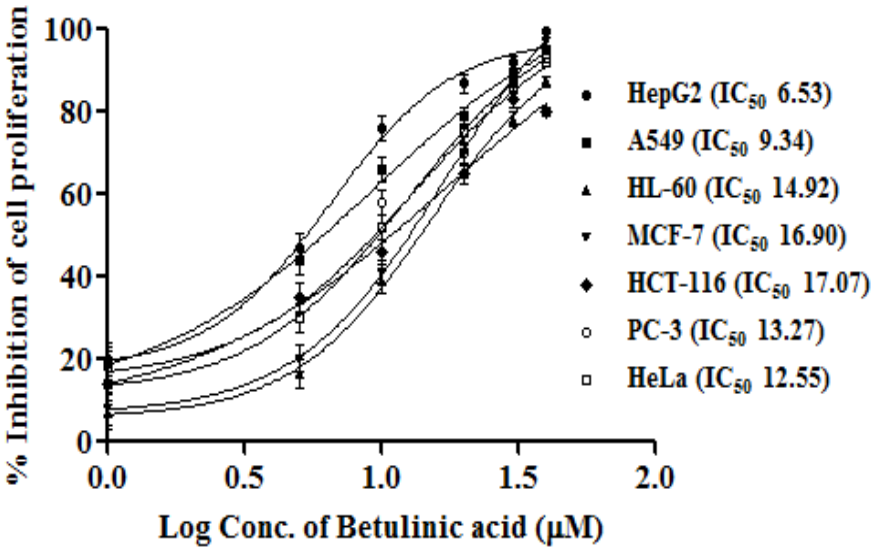


Figure - 4.8:

The anti-proliferative effect of purified betulinic acid isolated from *Nyctanthes arbor-tristis* on was performed with the MTT bioassay using various human cancer cell lines such as liver-HepG2, lung-A549, Leukaemia-HL-60, breast-MCF-7, colon-HCT-116, prostrate-PC-3 and cervix-HeLa. Cells were pretreated with various doses of betulinic acid. Percentage inhibition of cell proliferation was determined. Data presented is the mean ± S.D. of three independent experiments. Error bars denote S.D. IC50 value was determined using Graph pad prism (version 5) software.



4.7. Discussion

Nyctanthes arbour-tristis plant is widely used in traditional system of medicine for a varied ailment [19]. Literature data revealed that the phytochemical constituents such as alkaloids, phytosterols, phenolics, tannins, flavonoids, glycosides and saponins which are present in the crude extract of plant and these might be responsible for the anti-inflammatory and other pharmacological activities of *Nyctanthes arbor–tristis* [20-24]. There are very few reports, which have been documented on the anti-inflammatory and anti-cancer effects of leaves extracts *Nyctanthes arbor-tritis* [25,26]. There is no detail phytochemical and bioactivity analysis, which claimed that betulinic acid is present as one of the major bioactive phytoconstituent in the leaves of *Nyctanthes arbor-tritis*. Therefore, authors designed this research work to isolate and characterize the most bioactive compound(s) via bioactivity guided isolation and fractionation from the leaves extracts of *Nyctanthes arbor-tritis*. All crude extracts of *Nyctanthes arbor-tritis* leaves were tested (500 µg/mL) for their anti-inflammatory, anti-proliferative and antioxidant activity in various cell-free and cell-based assays. It was found that ethyl acetate fraction of *Nyctanthes arbor-tritis* showed better anti-inflammatory, anti-proliferative and antioxidant activity as compared to other three extracts (methanol, n-butanol and aqueous). On the other hand, these three crude extracts had very little or nonsignificant inhibition observed on COX-1, COX-2, and 5-LOX enzyme activity, on the production of NO, and TNF-α in mouse macrophage RAW264 cells, on the proliferation of cancer cell (HepG2) and percent free radical scavenging-DPPH assay (Table 4.2). Based on the results obtained from in vitro screening experiments, bioassay-guided fractionation of ethyl acetate was carried-out to isolate the most bioactive fraction. There was total fourteen sub fractions obtained from ethyl acetate extract with the gradient solvent elution (chloroform, ethyl acetate, and methanol) and each sub fraction was tested for their anti-inflammatory activity using cell-free assays (COX-1, COX-2, and 5-LOX) and cell-based assays (the production of NO, and TNF-α observed in RAW264 cells) (Table 3). Sub fractions 6-9 showed similar and best anti-inflammatory activity as compared to other sub fractions. The most bioactive fraction was obtained with the eluent chloroform: methanol (60:40), as eluent. This fraction was further purified by column chromatography, and the fraction was crystalized with methanol, yielding the newly isolated compound. The melting point, purity, and structure elucidation of the isolated compound were carried out using the melting point determination, FTIR, NMR and liquid chromatography-mass spectrometry. All spectroscopic data suggest that the isolated compound was being as betulinic acid (**Figure 4.6**

C). Betulinic acid, 3β-hydroxy-lup-20(29)-en-28-oic acid, a C-28 carboxylic acid derivative of the ubiquitous triterpene betulin, is a member of the class of the lupane-type pentacyclic triterpenes. It is a natural product with a range of biological and pharmacological activities such as: anti-inflammatory, antimicrobial, antileshminiasis, antimalarial, anti-HIV, and potent antitumor activity [27]. Its anticancer property is linked to its ability to induce apoptotic cell death in cancer cells by triggering the mitochondrial pathway of apoptosis [28].

The betulinic acid was isolated from the EtoAc fraction, which was found to possess strong anti-inflammatory, antiproliferative and antioxidant effects *in vitro*. Till date; no report is available on its presence in the leaves of *Nyctanthes arbor-tristis*. This is for the first time; authors report the presence of the newly identified lupane-type triterpenoid, betulinic acid in the leaves of *Nyctanthes arbor-tristis*, which showed potent anti-inflammatory, antiproliferative, and antioxidant activity. In the cell-free assay, betulinic acid inhibited purified ovine COX-1, COX-2 and 5-LOX enzymes at the range of IC_{50} values from 10.34 – 16.65 µg/ mL., yielding a ratio for COX-1 IC_{50}/ COX-2 IC_{50} of 0.8. This value was close to that for marketed NSAID, ibuprofen (1.1) (Botting, 2006). These results illustrate the direct effect of betulinic acid on arachidonic acid metabolism. To test whether betulinic acid could affect specific inflammatory markers involved in chronic disease, a murine RAW264.7 cells were chosen and stimulated with 1 µg/mL LPS and in the presence of betulinic acid. It showed to have a pronounced inhibitory effect on NO and TNF-α production by lipopolysaccharide (LPS)-activated RAW 264.7 macrophages [29]. A variety of triterpenoids are known to modulate inflammation and cancer cell proliferation by affecting NF-kB levels primarily through their antioxidant activities [30,31]. In agreement with this idea, betulinic acid significantly reduced the oxidative stress. The antioxidant capacity of betulinic acid has been confirmed by *in vitro* DPPH assay, which had shown the significant percent free radical scavenging capacity (IC_{50} value 18.36 µg/ mL) (**Table 4.2**). Control of oxidative stress may also account for the observed gene expression and protein down-regulation by betulinic acid *via* modulation of NF-kB and IκB-α [32]. Oxidative stress can trigger an inflammatory cascade in which NF-kB plays a significant role. NF-kB is an important central transcription factor complex that regulates the expression of many genes involved in immune and inflammatory responses in chronic human disease [33,34]. In unstimulated cells (Baldwin, 1996), NF-kB is constitutively localized in the cytosol as a heterodimer by physical association with an inhibitory protein called IκB-α [35]. Following activation, the NF-kB heterodimer is rapidly translocated to the nucleus, where it activates the transcription of target

genes, including the genes encoding the pro-inflammatory cytokines; adhesion molecules; chemokines; and inducible enzymes such as COX-1, COX-2, 5-LOX and iNOS [36,37]. Our data indicate that betulic acid may act *via* inhibition of NF-kB pathway in LPS-stimulated macrophages. Betulinic acid has recently been reported to be a promising agent for the treatment of multiple forms of cancer including those of the melanoma, liver, lung, colorectum, breast, prostate, cervix, ovary, kidney, leukemia and brain [38,39]. The current anti-proliferative assay results showed the significant inhibitory effects observed by betulinic acid in seven different human cancer cell lines and showed the IC_{50} values of it was a range from 6.53 – 17.07 μM (Table 4.5). Betulinic acid has been reported to suppress inflammation and modulate the immune response by interfering with NF-κB activation triggered by inflammation [29]. At the concentrations tested (1 – 40 μg/mL), it was shown potent antiproliferative effects on various cancer cell lines, which could be through down-regulating the expression of COX, 5-LOX, NF-κB, TNF-α, and i-NOS. Activation of NF-kB during inflammation regulates the expression of almost 500 different genes, which include enzymes such as the iNOS which are involved in inflammation and cellular injury [40]. Activated macrophages transcriptionally express iNOS, which catalyzes the oxidative deamination of L-arginine to produce NO, and is responsible for the prolonged and profound production of NO [41]. The high output of NO by iNOS can provoke deleterious consequences such as septic shock and other inflammatory diseases [42]. NO production by iNOS is regulated mainly at the transcriptional level, and the expression of the iNOS gene in macrophages is under the control of several transcription factors, which include NF-kB [29]. TNF-α is another NF-kB-inducible product that has an early and crucial role in the cascade of pro-inflammatory cytokine production and subsequent inflammatory processes [43]. The betulinic acid produced a significant reduction in the levels of TNF-α in Raw264.7 cells. The data on inflammatory markers along with the data relating to dual inhibition of AA metabolism suggest that betulinic acts *via* a variety of mechanisms to inhibit inflammation, and cell proliferation making it a promising therapeutic agent for different inflammatory disorders.

4.8. References

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Chapter- 5

In vivo study of Anti-Cancer Activity from Methanol Leaf Extract (MENA) of *Nyctanthes arbor- tristis* Linn. on EAC Cell bearing Swiss Albino Mice

5.1. Reagents and Chemicals

Propylene glycol, 5-Fluorouracil, 0.4% Trypan blue, N/10 HCl, Distilled water (D/W), Sodium sulphate, sodium chloride, mercuric chloride, glacial acetic acid, gentian violet, DNPH (2,4-dinitrophenylhydrazine) reagent, total nitrite reagent, NaOH solution, Biuret reagent, picric acid reagent, Buffer reagent, creatinine standard. All the reagents are purchased in LR grade.

5.2. Animals

The anti-cancer in vivo study was carried out on the male Swiss Albino mice weighing 20±2gm. after obtaining the permission from Animal Ethics Committee of Jadavpur University (permission letter Ref. No. AEC/PHARM/1502/12/2015). Animal experiment was carried out as per the guide line of CPCEA (Govt. of India) and UGC, India. The animals were kept under standard environmental condition, fed with standard laboratory diet and water *ad libitum*.

5.3. Acute toxicity study

The acute toxicity study of methanol leaf extract of *Nyctanthes arbor-tristis* (MENA) was determined as per the guide line of OECD No. 420. Swiss albino mice (male) of 25-30 g body weight were divided into six groups having six(n=6) mice in each group depending upon the dose of MENA. The dose groups were 200,400,800,1000,1600,2000 mg/kg b.w. of MENA. The MENA was administered orally as per the dose schedule. The mice were attentively observed for behavioral changes if any up to four hours after administration of the MENA as per dose schedule. Then the mice were kept for 14 days under strict and careful observation for mortality if any or behavioral change [1,2]. There was no mortality up to 2000 mg/kg b.w. So the test drug MENA is considered to be safe up to 2000 mg/kg b.w.

5.4. Study of Anti-tumor activity

5.4.1. Transplantation of Tumor

Ehrlich Ascites Carcinoma (EAC) cells, cells were obtained from Chittaranjan National Cancer Research Centre (CNCRI), Kolkata, India. The EAC cells were sub cultured by intraperitoneal transplantation (i.p.) at the dose of $2x10^7$ cells/mouse every ten days interval. Cells of nine days old were used for investigation of antitumor activity of MENA.

5.4.2. Experimental Protocol

The mice were divided into five groups, each group containing 6 mice.

Group I: Normal control (Propylene glycol 5ml/kg b.w.)

Group II: Group II: EAC control ($2x10^7$cells/mouse)

Group III: Group III: Test Drug (EAC + MENA 200 mg/kg b.w.)

Group IV: Group IV: Test Drug (EAC + MENA 400 mg/kg b.w.)

Group V: Group V: Standard Drug (EAC + 5- Fluorouracil 20mg/kg b.w.)

In the day '0', at the dose of 0.1ml ice cold normal saline suspension containing $2x10^7$ EAC cells were transplanted i.p. to all the mice of groups (II), (III), (IV) and (V). MENA was suspended with Propylene glycol and two doses (200mg/kg and 400mg/kg) were injected i.p. to the mice of groups (III) and (IV) just 24 hours interval of EAC cells transplantation. The standard anticancer drug 5-(FU) was injected i.p. to the mice of group (V) at the dose of 20mg/kg b.w. [3]. Group I mice were injected i.p. propylene glycol at the dose of 5ml/kg b.w. for nine days. Thus treatment was continued by MENA and 5-FU for nine consecutive days and development of tumors of mice in various groups were observed. 24 hours of last dose administration and 18 hours fasting, 50% mice of each group were sacrificed by cervical dislocation in the day 10 for determination of various hematological parameters, tumor cell count, percentage inhibition of tumor cell count, tumor volume, percentage inhibition of tumor volume, viable and non-viable cell count. Rest mice of each group were kept providing standard diet and water *ad libitum* for determination of percentage increase in life span (%ILS). The antitumor activity of MENA was evaluated in EAC mice as per the following parameters: [4]

5.4.3. Tumor growth

The growth was measured by tumor cell count, percentage inhibition of tumor cell count, tumor volume, percentage inhibition of tumor volume, viable and non-viable cell count.

5.4.4. Tumor cell count

The ascitic fluid was drawn from the tumor by WBC pipette and diluted 100 times. A drop of EAC cell suspension was placed on cleaned and dry Neubauer's counting chamber and the numbers of cells in 64 small squares was counted under the compound microscope in 10x10 =100 magnification.

5.4.5. Viable and Non-viable tumor cell count

These cells were collected from ascitic fluid and stained with 0.4% trypan blue in normal saline. The viable cells were not stained with trypan blue and non-viable cells were stained with trypan blue. These viable and non-viable cells were counted according to the following formula [5].

$$\text{Cell count} = \frac{\text{No.of cells X Dilution}}{\text{Area X Thickness of liquid film}}$$

5.4.6. Tumor volume measurement

The ascetic fluid was drawn by hypodermic syringe and needle from peritoneal cavity of various group of mice and measured in graduated centrifuged tube.

5.4.7. Percentage increase in Life span

The percentage increase in life span (%ILS) after administration of MENA in EAC cells bearing mice was calculated according to the mortality of mice [6].

$$\text{ILS (\%)} = \left(\frac{\text{mean survival time of treated group}}{\text{mean survival time of control group}} - 1 \right) \text{x}100$$

$$\text{Mean Survival Time*} = \frac{\text{first death+ last death}}{2}$$

*Time is denoted in days.

5.5. Study of Hematological Parameters

On the tenth day, blood was collected separately from the heart of the sacrificed mice from each group and white blood corpuscles (WBC), red blood corpuscles (RBC) count and Hb level was determined by standard method. These parameters of treated groups of mice was compared with the EAC group (II) of mice for statistical analysis [7-9].

5.5.1. Estimation of Hemoglobin (Hb)

Estimation of Hemoglobin was done by Sahli's Hemoglobinometer Method [7].

Reagents:

(1) N/10 HCl (2) Distilled Water

Apparatus:

(1) Sahli's Apparatus (2) Hemoglobin Pipette (0.02ml /0.02µl) (3) Sahli's graduated Hemoglobin Tube (4) Thin glass rod stirrer for Hemoglobin tube (5) Sahli's comparator box with brown glass standard.

Method:

N/10 HCl was taken in the Hemoglobin tube up to the mark 20. Capillary blood of each group of mice was drawn up to 20 µl mark exactly of the hemoglobin pipette by capillary action. This blood hemoglobin pipette was added to the N/10 HCl and stirred with glass rod. It was placed proper position of Hemoglobinometer and kept for 10 minutes for complete conversion of Hemoglobin to acid hematin. Then drop by drop distilled water was added and mixed by stirring to match the standard reference color of the Hemoglobinometer glass. This procedure was continued until the color was matched. After color matching reading of Hemoglobin level was taken in g/dl (g/100ml) of blood.

5.5.2. Determination of Total Erythrocyte (Red Blood Cell /RBC) Count

Apparatus:

Improved Neubauer's counting chamber, RBC pipette, RBC diluting fluid (Hayem's Fluid); compound microscope, cover-slips, pricking apparatus [7].

Composition of RBC diluting fluid (Hayem's Fluid):

Sodium Sulphate (Na_2SO_4) - 2.5gm

Sodium Chloride (NaCl) - 0.5gm

Mercuric Chloride ($HgCl_2$) - 0.25gm

Method:

Improved Neubauer's counting chamber, cover-slips, RBC pipette and the lenses of the microscope were cleaned and dried. Sufficient amount of diluting fluid was taken in watch glass. The fingertip was disinfected by rectified spirit and pricked. Fiest drop of blood was wiped off and then blood was sucked by RBC pipette upto the mark 0.5 with attention to avoid air bubbles. Then Hayem's Fluid was sucked upto the mark 101 holding the pipette nearly horizontal position and gently rolled between the palms to mix the blood with fluid. A clean cover slip was placed in the Neubauer's counting chamber so as to cover the ruled area on both sides. The RBC squares of Neubauer's chamber were focused under the low power o[8]bjective (x10) of the microscope. Few drops of fluid from RBC pipette were discarded. A small drop fluid was allowed to form at the tip of the pipette and this drop was gently brought in contact with the edge of cover slip. The fluid was drawn into the chamber by capillary action and both sides of the Neubauer's were charged. The RBC squares were focused under high power objective (x40) and RBCs in 4 medium size corner squares and one medium size central squares (1/20 mm side).

Calculation of Dilution Factor:

0.5 parts of the blood was mixed with 99.5 parts of the diluting fluid in the bulb of the pipette to form 100 (0.5 + 99.5) parts of the solution. So the dilution factor was calculated as:

$$\frac{\text{Final volume achieved (100 parts)}}{\text{Original volume taken (0.5 parts)}} = 200$$

Calculation of volume of fluid examined:

Area of 5 medium sized RBC squares =5x1/5mm x 1/5mm = $1/5^{th}$ sq.mm.

Depth of the chamber = 1/10 mm

Hence, volume of fluid in 5 RBC squares = 1/5 sq.mm. x 1/10 mm

$$=1/50 \text{ mm}^3$$

Calculation of total RBC count:

Suppose N= total number of RBCs counted in 5 medium sized squares in 1/50 mm^3 of the diluted blood.

So the Number in 1 mm^3 of undiluted blood = N x 50 x Dilution factor (200) = N x 10,000

5.5.3. Determination of Total Leucocyte (White Blood Cell /WBC) Count

Apparatus:

Improved Neubauer's counting chamber, WBC pipette, WBC diluting fluid (Turk's Fluid); compound microscope, cover-slips, pricking apparatus [8].

Composition of WBC diluting Fluid (Turk's Fluid):

Glacial acetic acid – 3 ml

Gentian violet – (1%) -1 ml

Distilled water to – 100ml

Method:

WBC diluting fluid was taken in a watch glass. The fingertip was disinfected by rectified spirit and sucked the second drop of blood with a WBC pipette up to 11 mark after finger pricking. The blood was diluted in the ratio 1:20 with Turk's Fluid. The WBC pipette was rotated for 3 to 4 minutes. First few drops of WBC fluid in the stem of the pipette were discarded. A small drop fluid was allowed to form at the tip of the pipette and this drop was gently brought in contact with the edge of cover slip which was already placed on Neubauer's chamber. It was allowed for time to settle the cells. The low power objective (x10) was focused. WBC cells were checked and identified in four corners of WBC squares of Neubauer's chamber. WBC cells were observed and counted as per rule.

Calculation of dilution factor:

$$\frac{\text{Final volume achieved (10 parts)}}{\text{Original volume taken 0.5parts)}} = 20$$

Calculation of volume of fluid examined:

Area of 4 WBC squares = 4 x 1mm x 1mm

= 4 sq.mm.

Depth of the Chamber = 0.1 mm

Volume of fluid in the 4 WBC squares

= 4 sq.mm x 0.1 mm

Calculation of Total Leucocyte Count:

Let 'N' be the total number of WBCs in 4 WBC squares (in 0.4 mm^3 of diluted blood)

There the Total number of WBCs in mm^3 of undiluted blood

$$= \frac{\text{N x Dilution factor (20)}}{0.4}$$

= N x 50

5.6. Biochemical constituents Test

The level of Serum glutamate pyruvate transaminase (SGPT), Serum glutamate oxaloacetate transaminase (SGOT), Total bilirubin (TBIL), Total protein, serum creatinine etc. [10-14] are measured by standard method from the cardiac punctured blood serum of sacrificed mice. The elevated level of the above mentioned parameters consider as the risk factor of cancer [15,16]. The transfer of amine group from aspartic acid, alanine and keto group from ketoglutaric acid by catalytic reaction of transaminases and specific electron transport system (ETS). These biochemical reactions maintain the normal function of liver. But the elevated level of SGPT, SGOT, TBIL, Total protein, signifies the abnormal liver function or hepatic disease, cancer etc. Elevated level of Serum creatinine signifies the kidney damage. This test is performed to detect whether the MENA damage the mice kidney or not.

5.6.1. Determination of SGPT

Serum Glutamate pyruvate transaminase (SGPT) was determined by **Reitman & Frankel's method [10].**

SGPT is present in various tissues including liver of human & animals. Increased level signifies the obstructive jaundice, hepatitis, cirrhosis, liver cancer and other liver ailments.

Reactions:

L-alanine + α-ketoglutarate $\xrightarrow[\text{pH 7.4}]{\text{SGPT}}$ Pyruvate + L-glutamate

Pyruvate + 2,4-DNPH $\xrightarrow[\text{medium}]{\text{Alkaline}}$ 2,4-dinitrophenyl hydrazine (Brown colored complex)

Method:

Two dry and cleaned test tubes were taken and labeled as Blank and Test. 0 .50ml substrate reagent (L1) was taken in each test tube and incubate for 3minutes at 37^{o}C. 0.10ml serum sample of each group of mice was added in the Test marking test tube. It was mixed well and incubated for 30 minutes. Each 0.50 ml DPNH reagent (L2) was added to the Blank & Test tubes. It was mixed well and kept for 20 minutes at room temperature. 0.10 ml distilled water was added into the blank marking test tube. The two mixtures were made alkaline by NaOH reagent (L3). The contents of the test tubes were mixed well and kept for 10 minutes at room temperature. Then absorbance of Test sample was measured at 505nm against Blank.

5.6.2. Determination of SGOT

Serum Glutamate Oxaloacetate Transaminase (SGOT) was determined by **Reitman & Frankel's method** [10].

Reactions:

L-aspartate + α-ketogluterate $\xrightarrow[\text{pH 7.4}]{\text{SGOT}}$ Oxaloacetate + L-glutamate

Oxaloacetate + 2,4-DNPH $\xrightarrow[\text{medium}]{\text{Alkanine}}$ 2,4-dinitrophenyl hydrazine (Brown colored complex**)**

Method:

Two dry and cleaned test tubes were taken and labeled as Blank and Test. 0 .50ml substrate reagent (L1) was taken in each test tube and incubate for 3minutes at 37^{o}C. 0.10 ml serum sample of each group of mice was added in the Test marking test tube. It was mixed well and incubated for 30 minutes. Each 0.50 ml DPNH reagent (L2) was added to the Blank & Test tubes. It was mixed well and kept for 20 minutes at room temperature. 0.10 ml distilled water was added into the blank marking test tube. The two mixtures were made alkaline by NaOH reagent (L3). The contents of the test tubes were mixed well and kept for 10 minutes at room temperature. Then absorbance of Test sample was measured at 505nm against Blank.

5.6.3. Estimation of total Bilirubin:

Total bilirubin level (TBL) was estimated by **Mod. Jendrassik & Graffs method** [11].

Reactions :

Serum Bilirubin + Diazotised Sulphanilic acid ⟶ Azobilirubin compound (Color)

Method:

Two dry and clean test tubes were marked as Blank and Test. Total Bilirubin reagent (L1) 1.0 ml each was added into the 'Blank' and 'Test' tubes. Total Nitrite reagent (L2) 0.05 ml was added into the Test marking test tube. Then 0.1ml Sample from each group of mice was added to both test tubes. The contents were mixed well and incubated for 10 minutes at room temperature. Then absorbance of the test (AbsT) sample was measured at 546 nm wave length against respective blank.

Calculation:

Total Bilirubin in mg/dl = AbsT x13

5.6.4. Determination of Total Protein

Total Protein was determined by **Biuret Method**. Protein was bound with cupric ions of biuret reagent and produced blue violet colored complex [12].

Reaction: Serum protein + Cu^{++} ⟶ Blue violet colored complex

Method:

Three clean and dry test tubes were taken and marked as Blank (B), Standard (S), and Test (T). Biuret reagent (L1) 1.0 ml was taken in each test tube. 0.02ml distilled water was added into the B mark test tube only. 0.02 ml protein standard (S) was added into marked standard test tube (S). 0.02 ml serum protein sample of each group of mice was added into the marked test (T) tube. The contents were mixed well and incubated at 37^{o}C for 10 minutes. Then absorbance was measured at 550 nm of Standard (AbsS), Test (AbsT) against Blank (Abs B).

Calculation:

Total Protein in mg/dl = $\frac{\text{Abs T}}{\text{Abs S}}$ x 8

[Standard protein concentration is 8 g/ dl]

5.6.5. Determination of creatinine

Serum creatinine was determined by **Alkaline Picrate method** [13,14].

Creatinine phosphate is used by skeletal muscle and after metabolism creatinine is produced. Elevated level signifies various diseases like renal dysfunction, congestive heart failure, prostate cancer etc.

Reactions:

Creatinine + Alkaline picrate $\longrightarrow$ Orange colored complex

Reagents: (L1) Picric acid reagent

(L2) Buffer reagent

(S) Creatinine Standard (2mg/dl)

Method:

Two clean and dry test tubes were taken and labeled as Test (T) and Standard (S). Picric acid (L1) 0.5ml and Buffer reagent (L2) 0.5 ml was taken in each test tube. 0.1 ml creatinine standard(S) was added into the (S) marked test tube. Serum creatinine of each group of mice 0.1 ml was added into the (T) marked test tube. The solutions were well mixed and initial absorbance A_1 was measured for Standard (A_1S) and Test (A_1T) after 30 seconds at 520 nm wave length. Another reading A_2 was taken just after 60 seconds for both Standard (A_2S) and Test (A_2T).

For ΔStandard $\Delta AS = A_2 - A_1S$

For Test $\Delta AT = A_2T - A_1T$

Calculation: Creatinine in mg/dl = $\frac{\Delta AT}{\Delta AS}$ x 2.0

5.7. Statistical Analysis

All data are expressed as mean ± SEM. (n = 6 mice per group). The data were analyzed by one-way ANOVA between the treated groups and the EAC control followed by Dunnett's Multiple Comparison test.

5.8. Results

Table- 5.1 : Anticancer activity of MENA against EAC bearing mice (Tumor cell count, percentage inhibition Tumor cell count, tumor volume, percentage inhibition of and percentage inhibition tumor volume)

Group	Compound	Tumor cell count ($2x10^7$)	%TCI	Tumor volume (mL)	%TVI
I	Normal	----	----	---	-----
II	EAC + Control	7.617± 0.078	0.00	6.89± 0.16	0.00
III	EAC + MENA(200mg/kg)	1.161± 0.095	84.75	1.66± 0.283	75.52
IV	EAC + MENA(400mg/kg)	0.7338 ± 0.0164	90.36	0.2495± 0.034	96.37
V	EAC + 5-FU	0.5992± 0.006	92.30	0.15±0.030	97.88

Each value represents the mean ± SEM (n = 6 mice per group). Experimental groups were compared with EAC control group ($P < 0.01$).

Table- 5.2 : Anticancer activity of MENA against EAC bearing mice (Total Count of viable, non-viable and % inhibition of viable, non-viable)

Group	Compound	Viable cell count ($2x10^7$)	Non-viable cell count ($2x10^7$)	% of viable cells	% of non-viable Cells
I	Normal	----	----	----	----
II	EAC + Control	6.903±0.049	0.711±0.097	90.62	9.33
III	EAC + MENA(200mg/kg)	0.794±0.064*	0.3665± 0.1345**	68.38	31.52
IV	EAC + MENA(400mg/kg)	0.3790±0.044*	0.354± 0.048**	51.64	48.24
V	EAC + 5-FU	0.1835±0.022*	0.415± 0.023***	30.62	69.28

Each value represents the mean ± SEM (n = 6 mice per group)

*Experimental groups were compared with EAC control group ($P < 0.01$)

**Experimental groups were compared with EAC control group ($P < 0.05$)

***Experimental groups were compared with EAC control group ($P > 0.05$)

Table- 5.3 : Percentage increase in life span (%ILS)

Group	Compound	MST (in days)	% ILS
I	Normal	----	----
II	EAC + Control	19.35	0.00
III	EAC + MENA(200mg/kg)	29.44	52.14
IV	EAC + MENA(400mg/kg)	35.22	82.01
V	EAC + 5-FU	43.33	123.92

Table- 5.4 : Hematological parameters- WBC, RBC and Hemoglobin content

Group	Compound	WBC count ($x10^9$/L)	RBC count (10^{12}/L)	Hemoglobin (g/dL)
I	Normal	5.415± 0.189	10.19±0.285	14.29±0.168
II	EAC + Control	17.88± 0.378	4.045± 0.222	5.117±0.2065
III	EAC + MENA(200mg/kg)	8.818± 0.4164	6.795± 0.2452	8.434±0.1687
IV	EAC + MENA(400mg/kg)	7.082 ±0.04688	8.564 ± 0.2140	12.05±0.2149
V	EAC + 5-FU	6.423±0.1601	9.020 ± 0.095	13.44±0.120

Each value represents the mean ± SEM (n = 6 mice per group).

Experimental groups were compared with EAC control group ($P < 0.01$).

Figure - 5.1: Percentage Inhibition of Total Cell Count

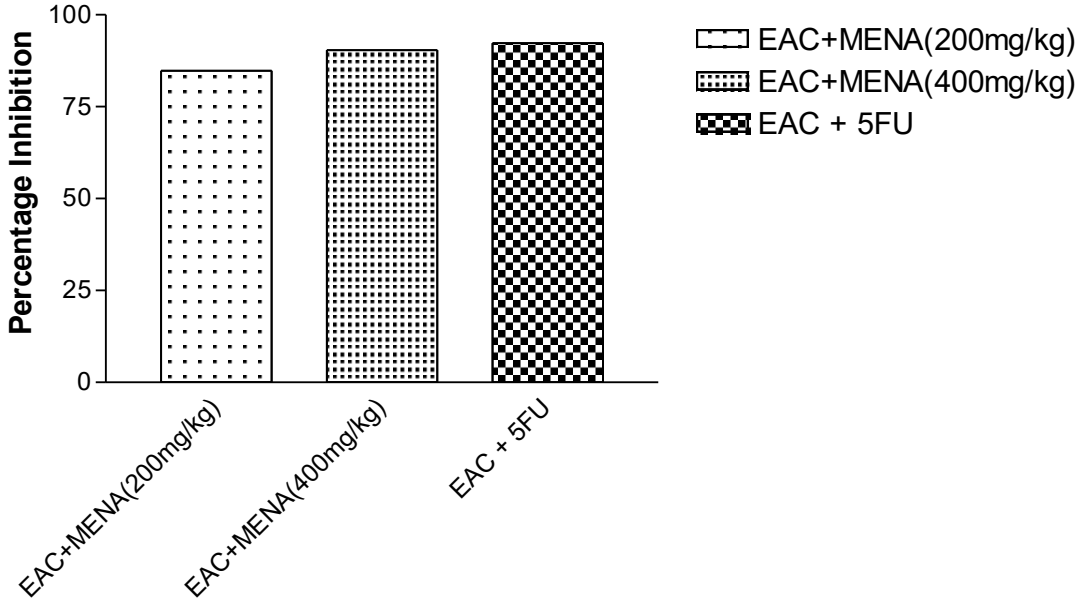


Figure - 5.2: Percentage Inhibition of Tumor Volume

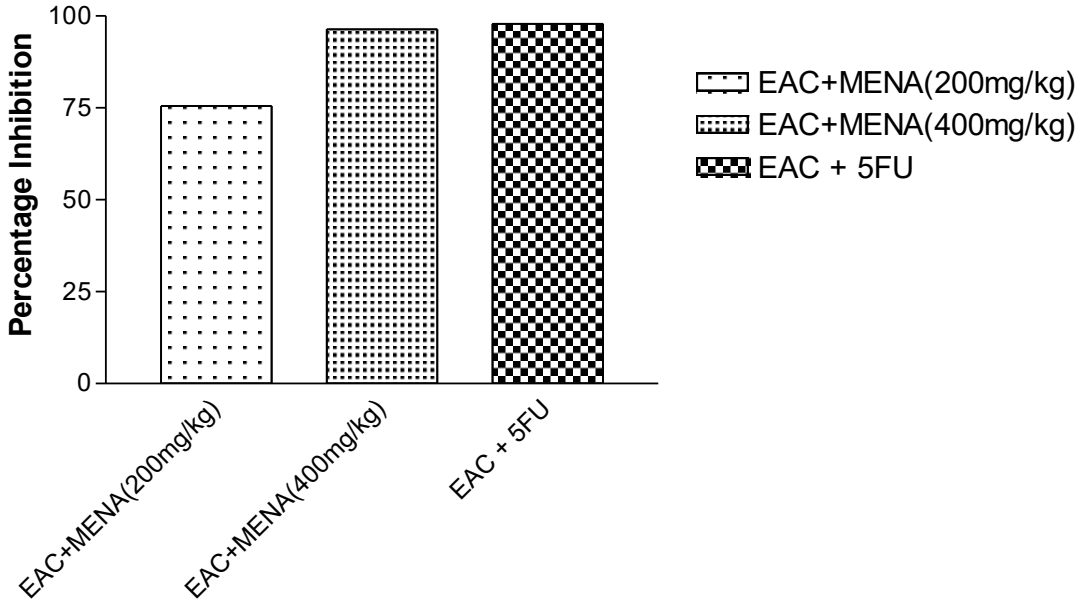


Figure - 5.3: Percentage Inhibition of nonviable cell count

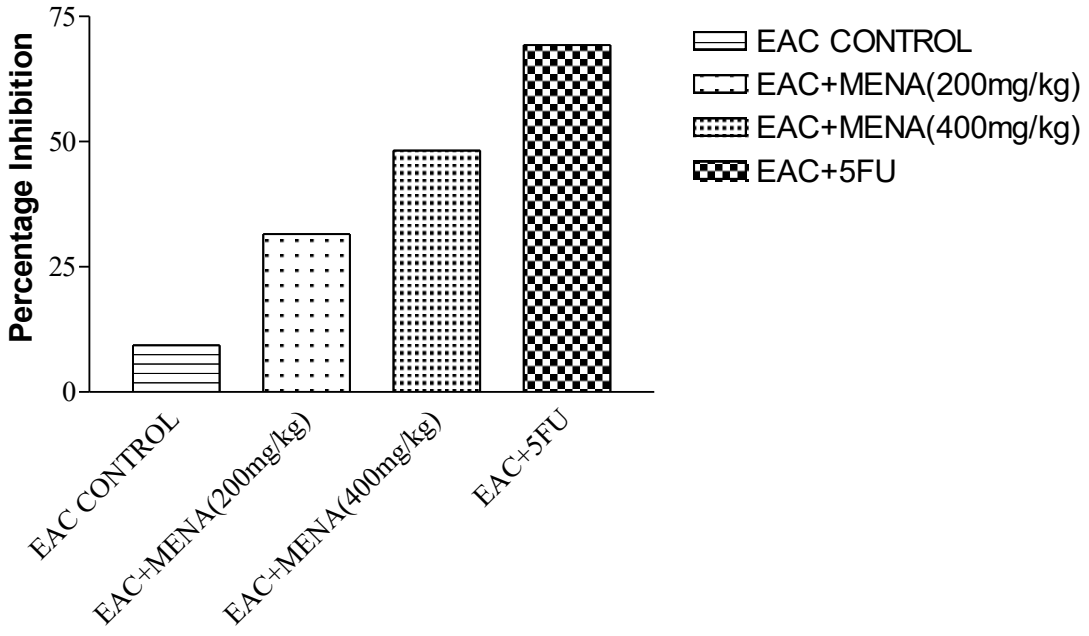


Figure - 5.4: Percentage Inhibition of viable cell count

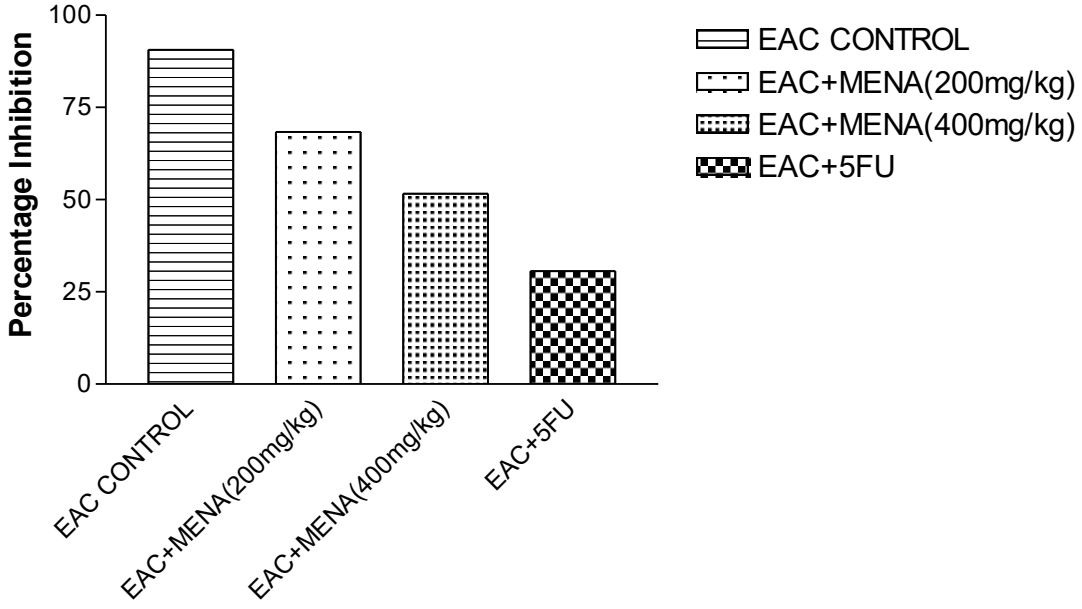


Figure - 5.5: Percentage increase in life span (%ILS)

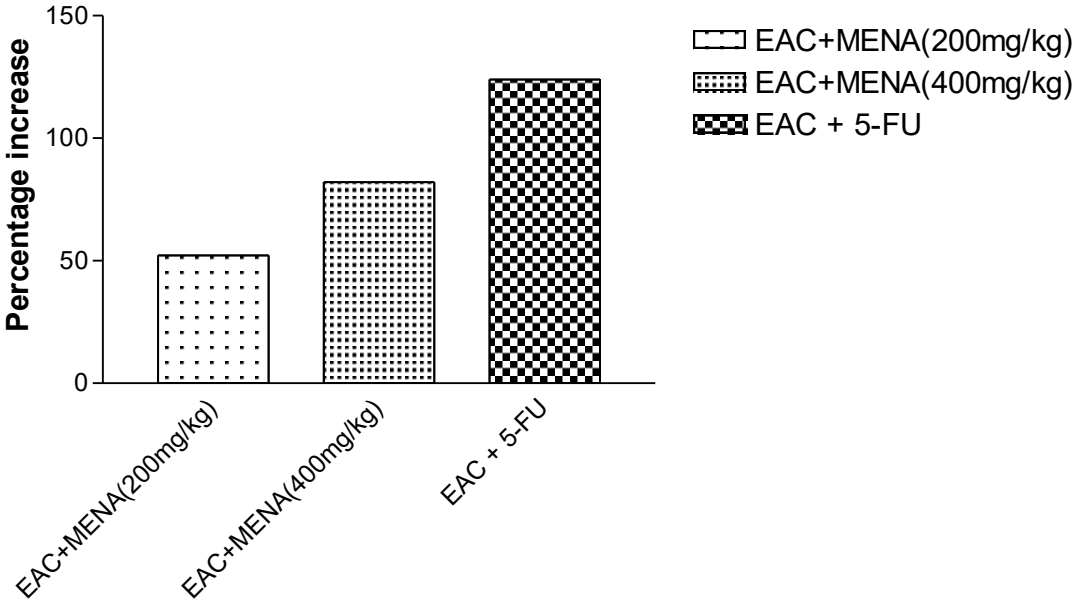


Figure - 5.6: Haemoglobin

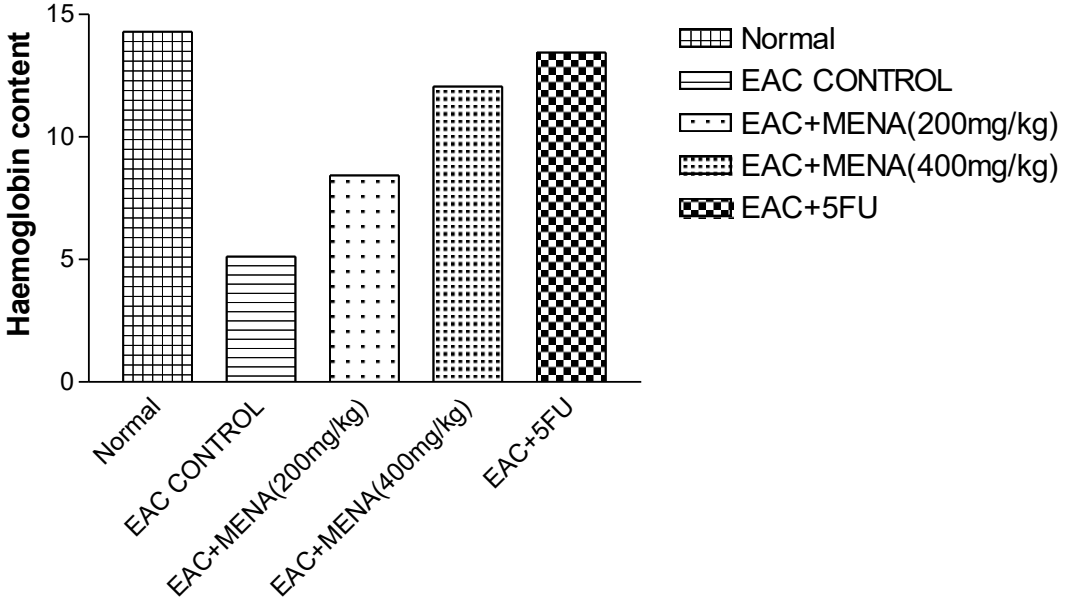


Figure - 5.7: Red Blood Cell

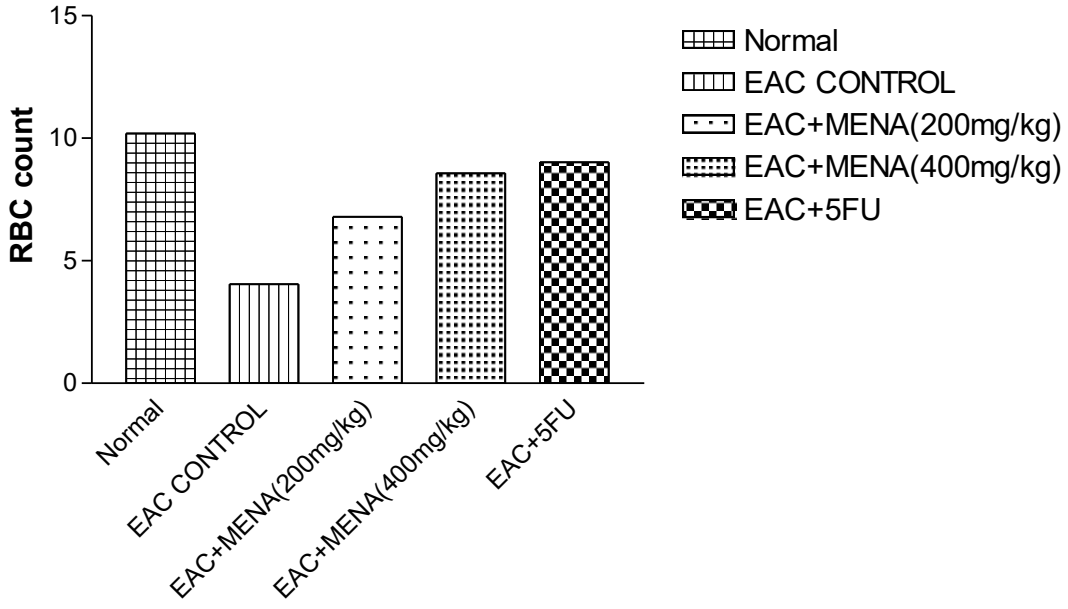


Figure - 5.8: White Blood Cell

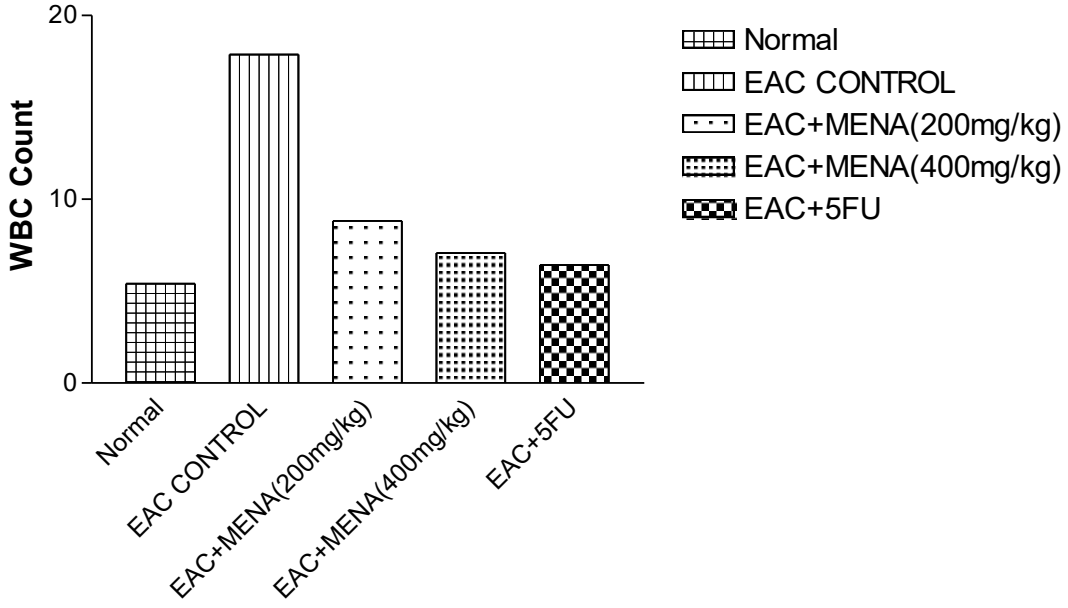


Table- 5.5 : Measurement of Biochemical constituents' level relating to antioxidant activity of MENA:

Group	Compound/ Drug Treatment	SGPT (U/L)	SGOT (U/L)	Creatinine Mg/dl	Total Protein mg/dl	Total bilirubin (mg/100ml)
I	Normal	34.66 ± 1.36*	254.3 ±2.96*	0.707 ±0.003*	5.82 ± 0.32*	0.35 ± 0.01*
II	EAC + Control	207.5±4.19	860.7 ±13.14*	0.608 ± 0.008	9.39 ± 0.76	3.25 ± 0.14
III	EAC + NA(200mg/kg b.w.)	44.6±1.43*	419.9 ± 7.36*	0.877 ± 0.011*	6.00 ± 0.29*	1.68 ± 0.12*
IV	EAC + NA(400mg/kg b.w.)	35.85±0.71*	243.8 ±4.9*	0.908 ± 0.003*	6.04 ± 0.7*	0.72 ± 0.03*
V	EAC + 5-FU (20 mg/kg b.w.)	44.26±0.46*	260.0 ± 0.88*	0.950 ± 0.012*	5.4 ± 0.18*	0.38 ± 0.02*

Each value represents the mean ± SEM (n = 6 mice per group).

*Experimental groups were compared with EAC control group ($P < 0.01$).

Figure - 5.9: SGPT

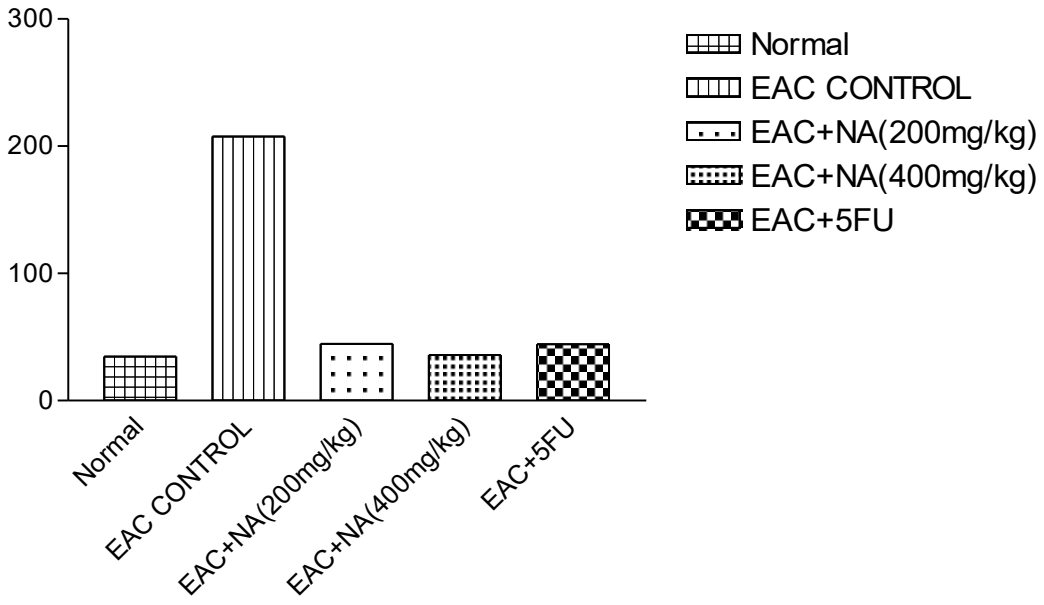


Figure - 5.10: SGOT

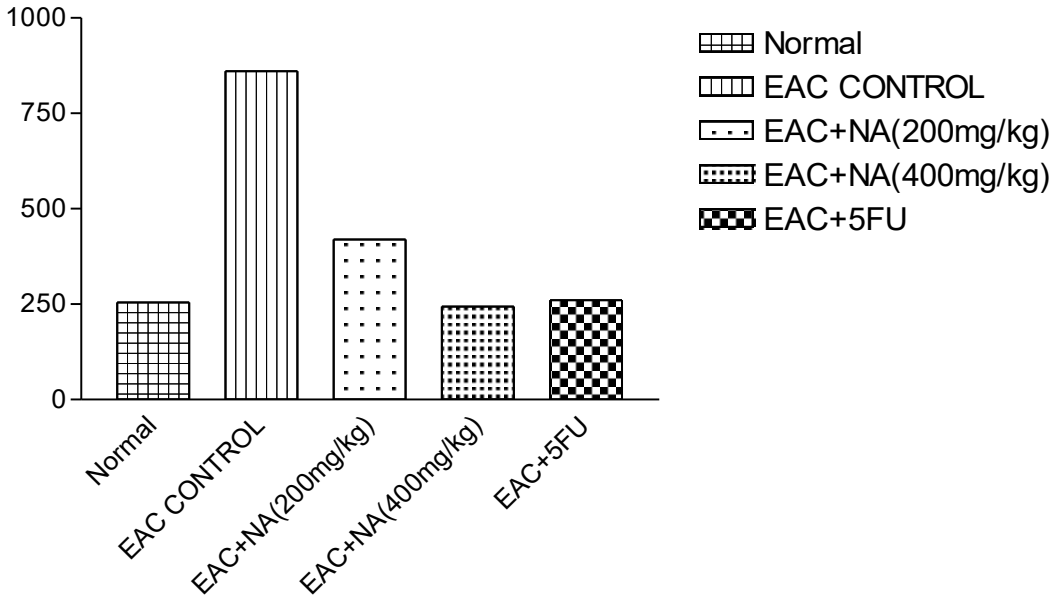


Figure - 5.11: CREATININE

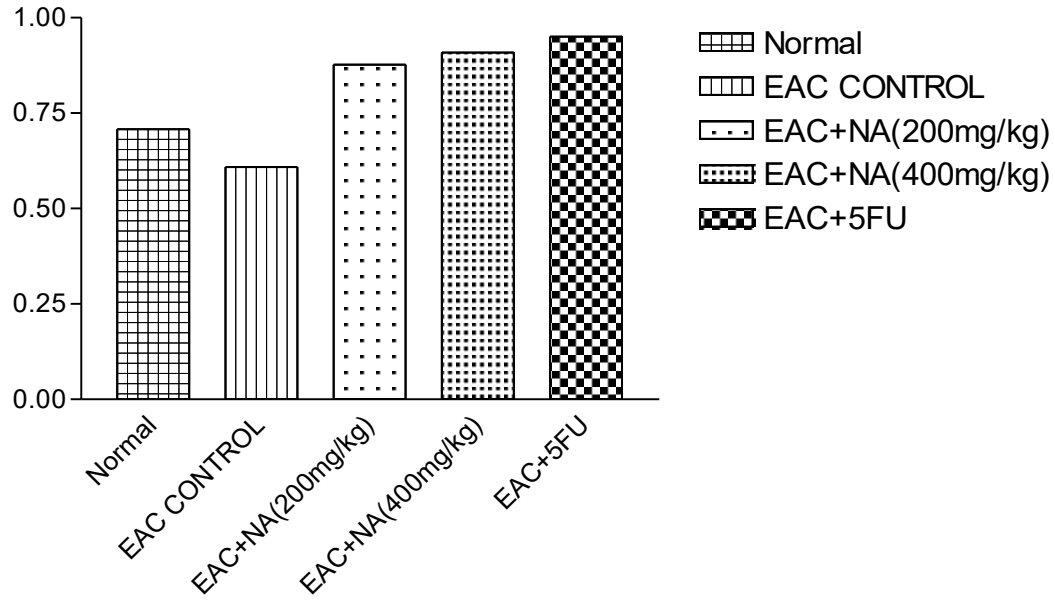


Figure - 5.12: TOTAL PROTEIN

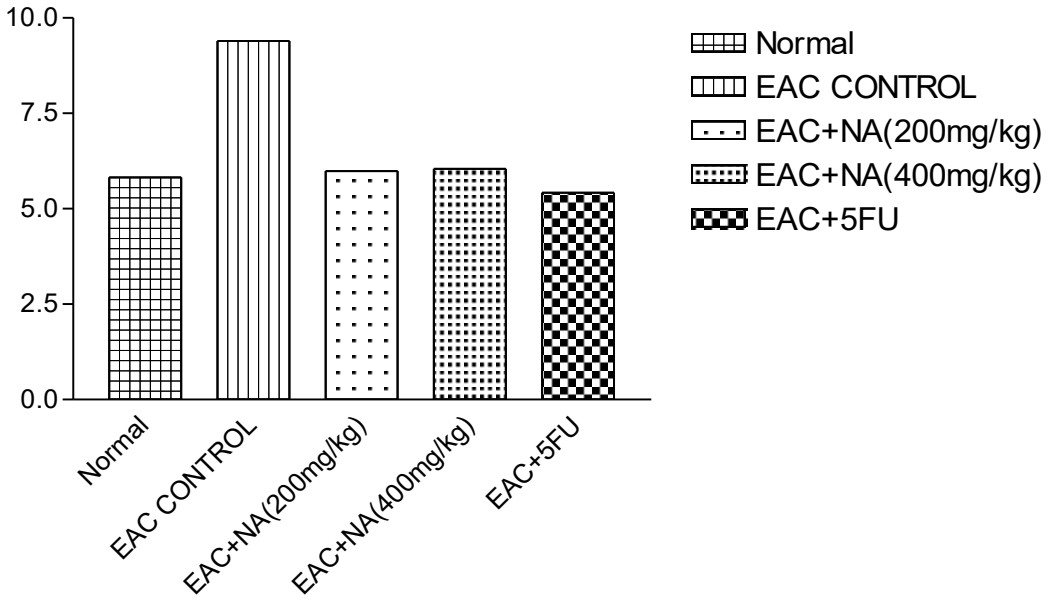
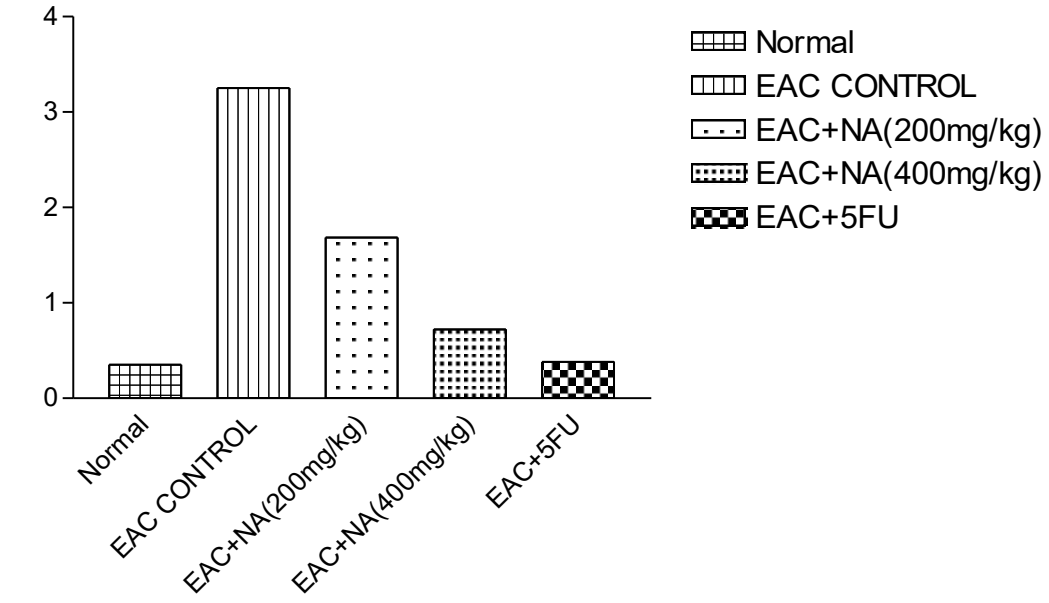


Figure - 5.13: TOTAL BILIRUBIN



5.9. Discussion

The anticancer property of MENA was evaluated by their ability to inhibit cancer cell growth in ascetic fluid of Swiss Albino mice. Various parameters like percentage inhibition of tumor volume (%TVI), percentage inhibition of total cell count (%TCI), percentage inhibition of viable and nonviable cell count, percentage increase in life span (%ILS) and hematological parameters were taken to be considered to establish the potency of the anticancer property of MENA.

Administrations of MENA at the doses of 200 mg/ kg b.w. and 400 mg /kg b.w. significantly reduced the total cell count and tumor volume when compared to EAC control group. Percentage inhibitions of total cell count (%TCI), percentage inhibition of tumor volume (%TVI) were observed as 84.75% and 75.52% respectively at the dose of 200 mg/kg b.w. of MENA. Similarly, percentage inhibitions of total cell count (%TCI), percentage inhibition of tumor volume (%TVI) were observed as 90.36% and 96.37% respectively at the dose of 400 mg/kg b.w. of MENA. (**Table 5.1, Figure 5.1 & 5.2**). Administration of MENA at the doses of 200 mg /kg b.w. and 400 mg /kg b.w. significantly ($P < 0.01$) decreased the viable cell count (**Table 5.2, Figure 5.4**). Non-viable cell count was significantly ($P < 0.05$) increased in MENA treated animals when compared with EAC control animals (**Table 5.2, Figure 5.3**).

One of the most reliable criteria for judging the efficiency of any anticancer drug is the prolongation of the life span of animals. Life span was also increased in MENA treated animals (Group III and IV) when compared with EAC control animals (Group II) (**Table 5.3, Figure 5.5**).

Treatment with MENA at the doses of 200 mg/kg b.w. and 400mg/kg b.w.in EAC bearing mice significantly ($P < 0.01$) increased both the level of RBC and hemoglobin (Hb) while significantly ($P < 0.01$) reduced level of WBC when compared with EAC control group (**Table 5.4, Figure 5.6, 5.7, 5.8**).

The biochemical constituents- SGPT, SGOT, Total bilirubin, creatinine, Total protein etc. were present in nomal level in animals or mice blood. But these biochemical parameters were elevated or reduced in certain disease like cancer of the animals. Liver is the exocrine gland. So enzyme released from this gland through duct. During infective condition the hepatic enymes were releasd and came into circulation due to hepatocellular injury. So SGPT and SGOT were the parameters for investigation of liver damage [17].

The elevated levels of SGPT, SGOT, Total bilirubin (TBL) and Total protein were observed in the blood of EAC control group/Gr. II but the level of these parameters were decreased and almost normalized in the blood of MENA (Group III & IV) and 5-FU/Gr.V treated groups of mice. Another biochemical parameter, creatinine showed elevated level in MENA and 5–FU treated group compare to normal control group/Gr. I of mice (**Table 5.5; Figure 5.9, 5.10, 5.12, 5.13**). Generally cancer chemotherapy produced elevated level of creatinine upon administration to the animal body. Both the standard cancer chemotherapy 5-Fluoruracil (5-FU) and methanol leaf extract of *Nyctanthes arbor-tritis* (MENA) showed the elevated creatinine level than the normal control group (**Table 5.1; Figure 5.12**).

5.10. References

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Chapter- 6

Investigation of in vivo antioxidant activity of the leaf extract of *Nyctanthes arbor-tristis* Linn. on EAC cell-bearing Swiss Albino mice

6.1. Chemicals and reagents

0.4M Tris buffer, Ethylene diamine tetra acetic acid (EDTA), tricarboxylic acid (TCA), 0.01M 5,5'-dithio-(2-nitrobenzoic acid)/DTNB, Sodium pyrophosphate, phenazine methosulphate, nitroblue tetrazoline (NBT), Nicotinamide adenine dinucleotide phosphate (NADH), Hydrogen peroxide $(H_2O)_2$, N/15 Phosphate buffer, N/150 Phosphate buffer.

6.2. Materials and Methods

The antioxidant activity was evaluated from methanol leaf extract of *Nyctanthes arbor-tristis* Linn on Ehrlich Ascites Carcinoma (EAC) cells bearing Swiss Albino mice and its correlation with histopathological parameters of liver. The MENA was administered intraperitoneally (i.p.) in the doses of 200 and 400mg/kg body weight (b.w) per day for nine consecutive days just after 24 hours inoculation of EAC cells in the dose of $2x10^7$cells/mouse. 5-Flurouracil (5-FU) was administered (i.p.) as standard anticancer drug in the dose of 20 mg/kg b.w. After administration of last dose, the mice were fasting for 18 hours and sacrificed. The present study deals with biochemical parameters like Serum Glutamate Pyruvic Transaminase (SGPT), Serum Glutamate Oxaloacetate Transaminase (SGOT), Creatinine, Catalase, Total bilirubin, Total protein from the blood of the mice heart [1-5]. Enzyme assay like Superoxide dismutase (SOD), lipid peroxidation (LPO), Glutathione (GSH), Catalase (CAT) were performed taking homogenized content of liver [6-9]. These parameters and findings were helpful to assess the antioxidant and anticancer activity of the mice.

6.3. Evaluation of *In Vivo* antioxidant activity

Reactive oxygen species (ROS) are produced due to aerobic cellular biochemical reactions. ROS are cytotoxic and high level causes mutation, aberration of chromosome, cell death, carcinogenesis etc. [10] Three types of primary antioxidant enzymes are present in mammalian cells. They are Superoxide dismutase (SODs), Catalase (CAT-found in peroxisome, cytoplasm), Substrate specific peroxidase, Glutathione peroxidase (GPx-found in mitochondria, nucleus). The types SODs are Manganese containing SOD (MnSOD-found in mitochondria), copper and zinc containing SOD (CuZnSOD- found in cytoplasm &

nucleus), extracellular SOD (ECSODo). These SODs are converted superoxide radicals to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). This H_2O_2 is converted into water and oxygen (O_2) in presence of catalase. But the peroxidases are converted the H_2O_2 to water. Thus harmful superoxide and hydrogen peroxide converted harmless water and oxygen by the catalytic reaction of three types of primary antioxidant enzymes.

Sequences of biochemical reactions are as follows:

$$\text{Superoxide Radicals} \xrightarrow{\text{SODs}} H_2O_2 + O_2$$

$$H_2O_2 \xrightarrow{\text{Catalase}} O_2 + H_2O$$

$$H_2O_2 \xrightarrow{\text{Peroxidase/ GPx}} H_2O$$

Livers of sacrificed mice in various above mentioned five groups were collected separately, rinsed with ice cold normal saline (O.9% w/v of NaCl in water) and soaked by filter paper. Then these livers of various groups were homogenized one by one for the estimation of Lipid peroxidation (MDA), Glutathione (GSH), Superoxide dismutase (SOD), Catalase (CAT) according to the standard method.

6.3.1. Estimation of Lipid Peroxidation

Lipid Peroxidation was assayed by Fraga *et al* (1981) method. Liver tissue was homogenized and 10% W/V of homogenized was prepared with 0.15M Tris-HCl (pH 7.4). The uniform mixture of 0.5ml tissue homogenate, 1 ml 10% Tricarboylic acid (TCA), 0.5 ml normal saline was centrifuged at 3000 rpm for 20 minutes maintaining the temperature 4^o C. 1 ml supernatant was taken and 0.25 ml of Thiobarburic acid (TBA) was added and mixed. This content was boiled at 95^o C for 1 hour, cooled optical density was measured at 532 nm.

6.3.2. Estimation of Superoxide dismutase (SOD)

The SOD was estimated by Kakkar *et al*.(1984) method. A mixture consist of 1.2 ml Sodium Pyrophosphate buffer, (pH 8.3, 0.025mol/L), 0.1 ml phenazine methosulphate/PMS (186mM/L), 0.3ml Nitrobenzene Tetrazoline/ NBT(300mM/L), 0.2 ml NADH (780mM/L) was mixed with 0.1 ml tissue homogenate and volume was adjusted to 3 ml by water. It was incubated at 30^oC for 90 seconds. 1 ml glacial acetic acid was added and stirred vigorously and stirring was continued for few minutes after addition of 4 ml *n*-butanol. The intensity of

color of chromogen formed in the butanol layer. The optical density was measured at 560nm against *n*-butanol.

6.3.3. Estimation of Reduced Glutathione (GSH)

GSH was assayed according to Ellman *et al*. (1958) method. 0.2 ml of tissue homogenate was mixed with 1.8ml of Ethylene Diamine Tetra Acetic Acid (EDTA) solution. Then 3.0ml of precipitating reagent consist of 1.57 g of meta phosphoric acid, 30 g Sodium Chloride, 0.2g EDTA in 1 L distilled water was added to the homogenate mixture. It was mixed well and kept for 5 minutes. Then it was centrifuged at 15000 rpm for 20 minutes maintaining the temp. 4^{o}C. The supernatant 2.0 ml was taken and mixed with 4.0ml of 0.3M di-sodium hydrogen phosphate solution , 1.0ml of DTNB (5,5-dithio-bis -2-nitrobenzoic acid) reagent. Then optical density was measured at 412 nm.

6.3.4. Estimation of Catalase (CAT)

CAT assay was followed by Mahely and Chance (1954) method. Liver tissue was homogenized with M/150 phosphate buffer (pH 7). 2.5 ml tissue homogenate was centrifuged at 5000 rpm at 4^{o}C. A reaction mixture was prepared mixing with 0.01M phosphate buffer (pH 7),enzyme extract, 2mM hydrogen peroxide(H_2O_2) . The optical density was read at 230 nm. The specific activity of catalase is expressed as mole of H_2O_2 consumed /min/mg of protein.

6.4. Result

Table- 6.1 : Measurement of Enzyme assay for determination of MENA

Group	Compound/ Drug Treatment	CAT Catalase (U/mg protein)	SOD (U/mg protein)	Lipid peroxidation (nmol MDA/mg protein)	GSH (mg/g wet tissue)
I	Normal	26.62 ± 0.25*	16.81± 0.42*	2.294 ± 0.15*	3.186 ± 0.23*
II	EAC + Control	2.98 ±0.46	3.77±0.34	7.92 ± 0.67	1.105 ± 0.08
III	EAC + NA(200mg/kg b.w.)	13.15 ±0.40*	9.08±0.47*	4.77 ± 0.45*	1.79 ± 0.11**
IV	EAC + NA(400mg/kg b.w.)	25.51 ±0.83*	14.42±0.51*	3.0 ± 0.40*	2.72 ± 0.15*
V	EAC + 5-FU 20mg/kg b.w.)	23.20 ± 0.93*	16.20±0.70*	2.725 ± 0.22*	2.794 ± 0.11*

Each value represents the mean ± SEM (n = 6 mice per group).

*Experimental groups were compared with EAC control group ($P < 0.01$).

**Experimental groups were compared with EAC control group ($P < 0.05$)

Figure - 6.1: CATALASE

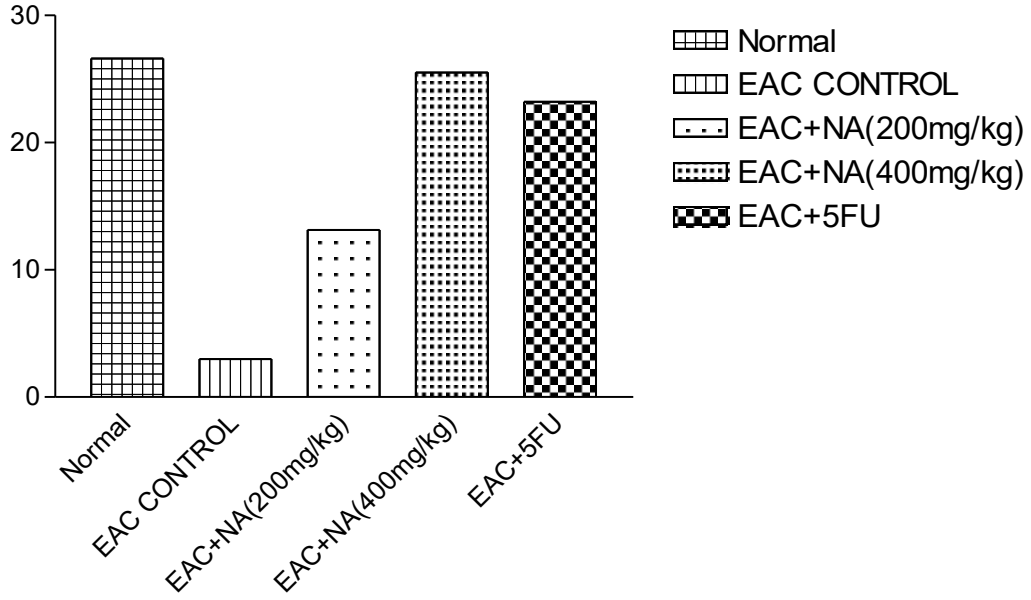


Figure - 6.2: SOD

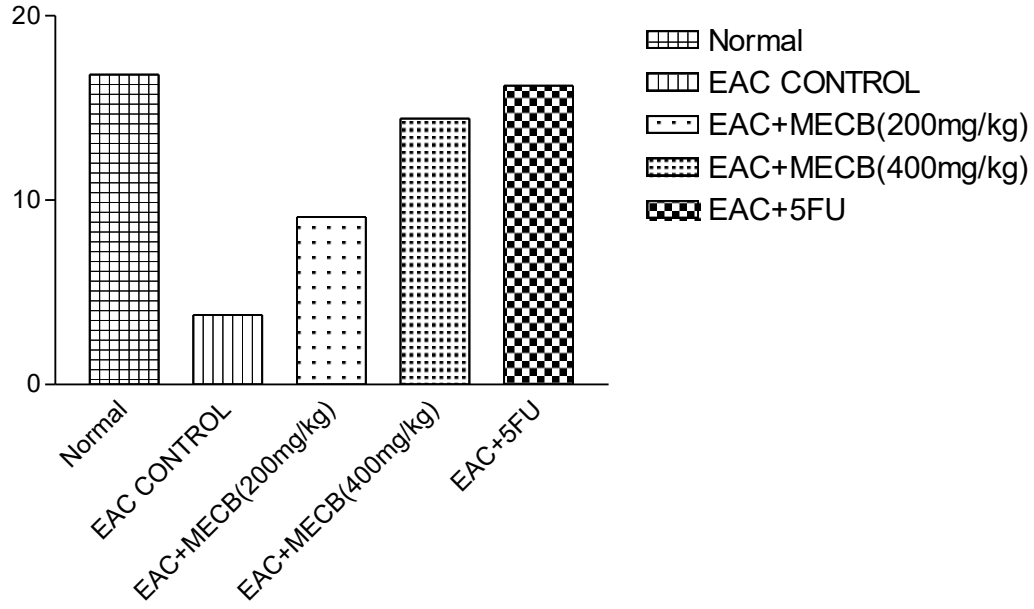


Figure - 6.3: LIPID PEROXIDATION

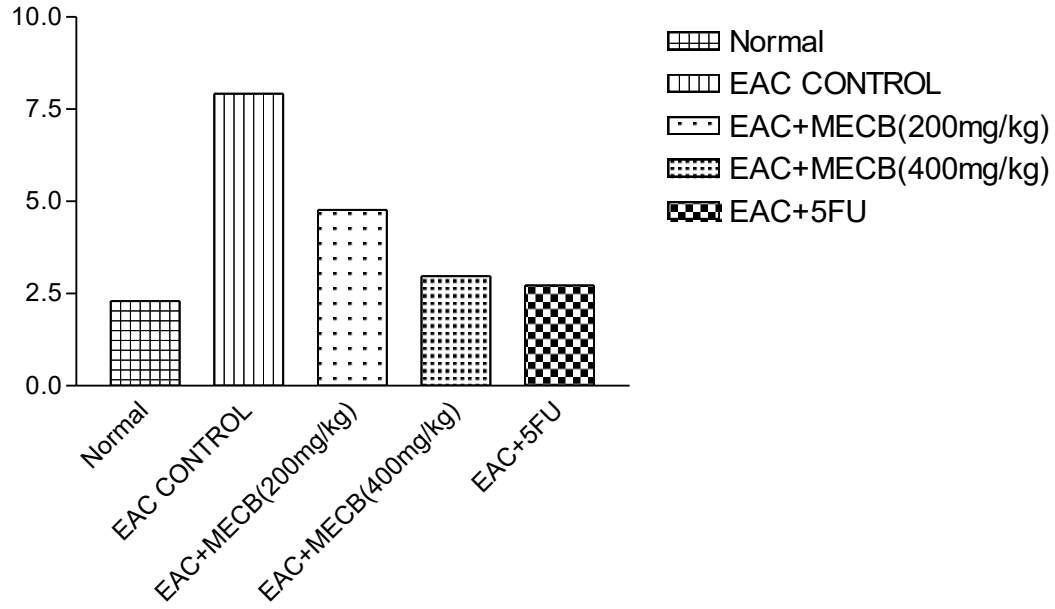
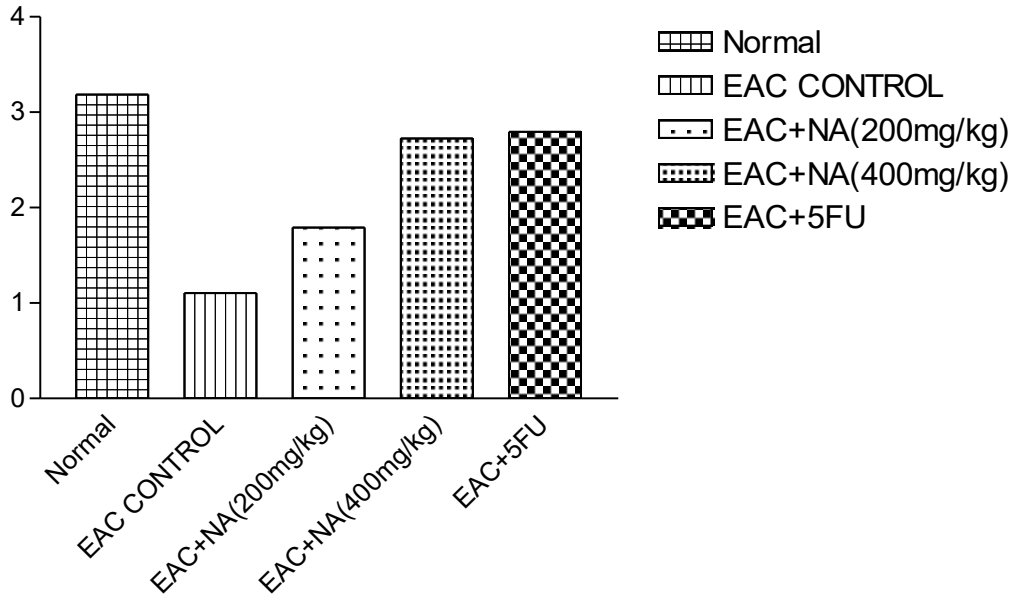


Figure - 6.4: GSH



6.5. Discussion

Certain histopahological changes occur due to i.p. inoculation of EAC cells. Carcinogenic abnormal cell proliferation may be inhibited by administration of chemotherapy may be natural, synthetic or semisynthetic origin. For this reason liver is the selected organ or largest digestive gland for investigation of oxidative stress and pathological findings. Due to production of free radicals in excess oxidative stress may occur which may produce LPO and produce degeneration of the hepatocytes. Malondialdehyde (MDA) is synthesized from LPO. This is mainly produced from cancerous animals than normal animals. After i.p. inoculation of EAC cells to the Swiss Albino Mice the LPO level of liver was highly increased and this parameter was observed through Lipid peroxidation assay using homogenized content of EAC control(Group II) mice. This LPO level was reduced of the MENA (200mg/kg &400 mg/kg b.w.) and standard drug (5-FU) treated groups through Lipid peroxidation assay using homogenized content of liver (**Table 6.1, Figure 6.3**). Glutathione (GSH) has very important role for inhibition of abnormal and uncontrolled mitosis as an antioxidant. The GSH assay using homogenized liver content showed reduced level of GSH in EAC control group(Group II) and elevated level of GSH in MENA and 5-FU/Gr. V treated mice in the said dose (**Table 6.1, Figure 6.4**). Similarly two important enzymes namely Superoxide dismutase (SOD) and Catalase (CAT) are present in all aerobic oxidation cells which have antioxidant activity. The SOD and catalase assays using homogenized livers contents showed reduced level of SOD and CAT in EAC control group(Gr.II) and elevated level of SOD and CAT in MENA and 5-FU(Gr.V) treated mice (**Table 6.1; Figure 6.2 & 6.1**)

6.6. References

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Chapter- 7

Study of histopathological architecture and determination of Histology Activity Index (HAI) from liver of MENA treated and EAC cells bearing Swiss Albino mice

7.1. Reagents and chemicals

10% buffered formalin, dehydrated alcohol, Paraffin, hematoxylin, eosin, clean and dry glass slides livers of five groups of mice.

7.2. Methods

The dissected livers of the sacrificed mice which belong to the Normal Control Group (Gr-I), EAC Control Group (Gr-II), Methanol Extract of *Nyctanthes arbor-tristis*(MENA) Treated Group (Gr-III 200 mg/kg b.w. and Gr-IV 400 mg/kg b.w.) and 5-FU Treated Group (Gr - V 20 mg/kg. b.w.) were initially kept in 10% buffered formalin. This formalin treated livers then dehydrated in alcohol and embedded in paraffin. The paraffin blocks were sectioned at 5 μm intervals and stained with hematoxylin and eosin for histological examination.

7.3. Result and Discussions

Histological findings such as steatosis and lymphocyte accumulation were observed in the liver of EAC bearing mice by histological examination. The liver section of EAC treated control group was compared with the tested formulations in different treated groups (200 mg/kg b.w., 400 mg/kg b.w. of MENA, and 20 mg/kg b.w. of 5-FU). The histological observations of the test groups suggested that normal architecture of liver nucleus, parenchyma, and hepatic cells were regenerated, which was damaged in EAC disease (tumor) control group. All three tested formulations (with 200, & 400 mg/ kg body weight of leaf extract of MENA and 20 mg/kg of 5-Fluorouracil) showed significant improvement of the damaged central vein (CV), decreased degenerated hepatocytes and deformed necrosis of tissues, which were damaged in tumor /cancer group (EAC control) [1-7].

Photomicrograph of the liver section of the normal mice was composed of a number of lobules and hepatic tissue showing a thin walled central vein (CV) from which cords of hepatic cells radiate. Branches of the hepatic portal vein (PV), hepatic artery (A) and bile duct (BD) forming the portal triads are also seen (H&E, x100)

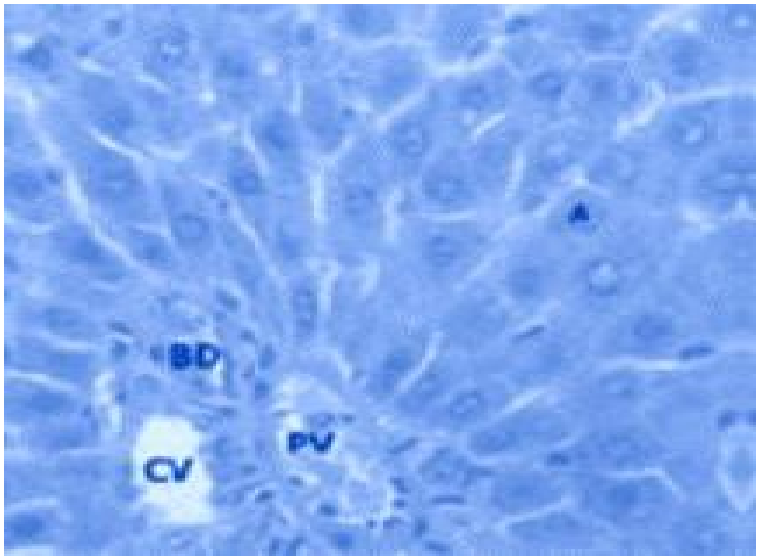

Figure - 7.1: Normal

Photomicrograph of mice liver in disease (tumor) control group showing thick walled central vein (CV) plates of highly differentiated (hypertrophy), large neoplastic hepatocytes (N) (prominent nucleoli and finely granular cytoplasm) without discernible hepatic architecture (H&E, x100).

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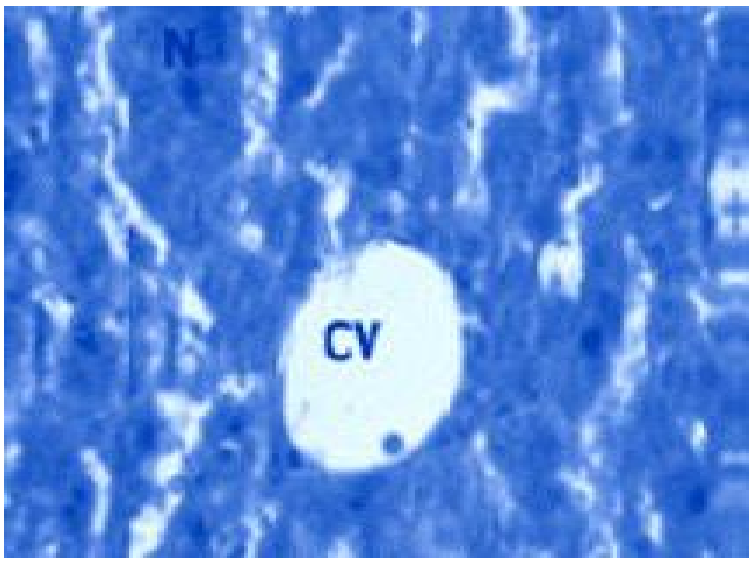

Figure - 7.2: EAC Control

Photomicrographs of mice liver in EAC treated with 200 & 400 mg/ kg body weight of methanol extract of *Nyctanthes arbor-tristis* (MENA), showing gradual improvement the structural damages of the liver tissue, showing less damage of central vein (CV), less degenerated hepatocytes and deformed necrosis of tissue (N) (H&E, x100).

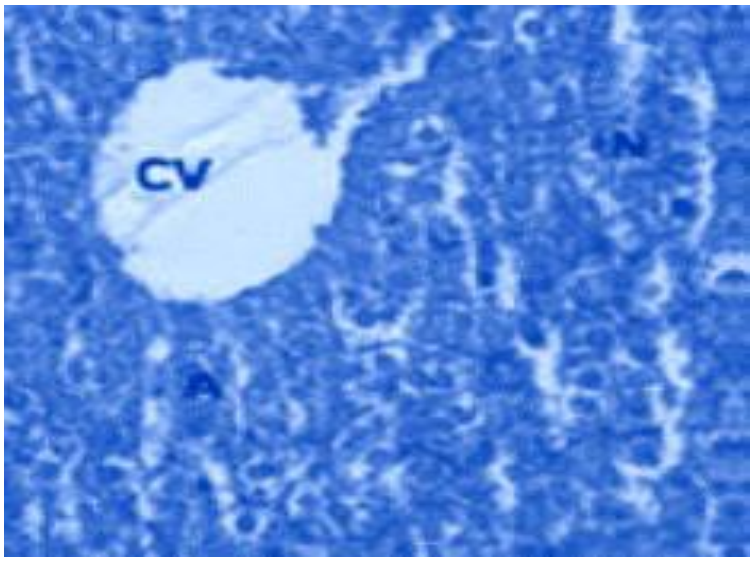

Figure - 7.3: 200 mg/kg, b.w.

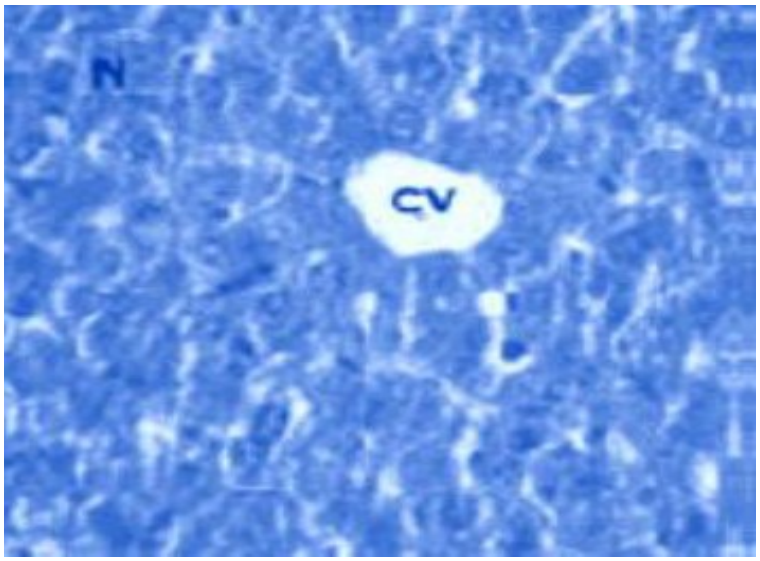

Figure - 7.4: 400 mg/kg, b.w.

Photomicrograph of mice liver in EAC treated with 5-Fluorouracil (20 mg/kg b.w., i.p.) showing significant improvement and more or less normal structure of the liver tissue, showing thin wall central vein(CV), hepatic portal vein (PV) and large neoplastic hepatocytes (N) are regenerated look like to normal liver section (H&E, x100).

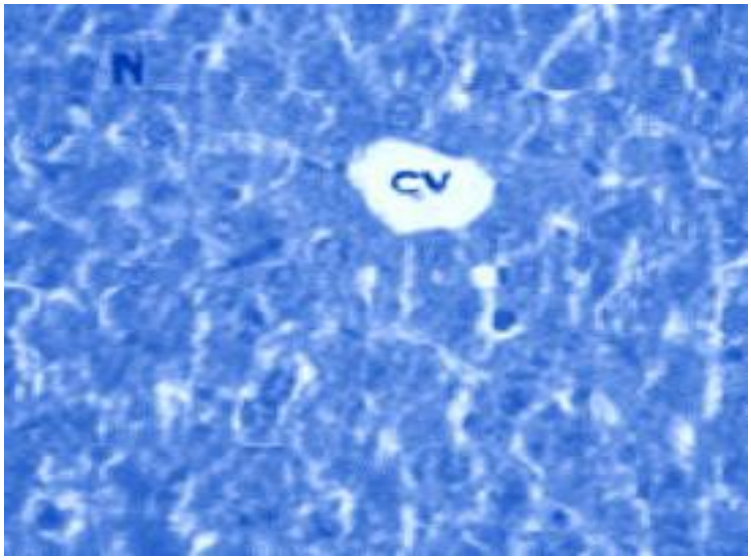

Figure - 7.5: 5-FU 20mg/kg b.w.

Table- 7.1 : Histology activity index (HAI) of MENA

Histology Activity Index (HAI) using the Knodell score- ***Nyctanthes arbor-tristis*** *L.leaf extract.*						
Organ	**Findings**	**Gr'A' Normal**	**Gr 'B' EAC control**	**Gr 'C' 200mg/kg MENA treated**	**Gr 'D' 400mg/kg MENA treated**	**Gr 'E' 20mg/kg 5-FU treated**
Liver	*Sinusoidal dilatation around central vein*	0	2	1	0	0
	Eosinophilic hepatocytes	0	1	0	1	0
	MNC infiltration	0	3	2	1	1
	Degeneration (binucleated hepatocytes) near central vein	0	2	1	1	0
	Inflammatory cells foci	0	2	1	0	0
	MEAN	0.00	2.00	1.00	0.60	0.20
	SEM	0.00	0.32	0.32	0.24	0.20

These scores are a very good way to show differences in histologic response between control versus receiving different doses of test items, and these have been used successfully for better interpretation of the experimental results.

7.4. Histopathological findings

Figure 7.1 showed a normal hepatic architecture with no specific histopathological lesions. Liver sections of mice bearing Ehrlich Ascites Carcinoma (EAC) showed various histopathological alternations including, vacuolization of hepatocellular cytoplasm, sporadic multiple cell necrosis of individual hepatocytes with deeply pyknotic nuclei (arrow), inflammatory foci, congestion of central vein(C) associated with brown pigment deposition(arrow) as shown in **Figure 7.2.**

Liver sections in EAC - bearing mice treated with EEP and different doses of MENA appeared in normal histological arrangement pattern with very few mononuclear cells infiltrating the hepatic sinusoids figure, sporadic cell necrosis of individual hepatocyte with mild vacuolization of hepatocellular as shown in **Figure 7.3 and 7.4**.

Liver sections in EAC - bearing mice treated with EEP plus 20 mg/kg of 5-FU (positive control, reference anticancer drug), appeared in normal histological arrangement pattern with very few mononuclear cells infiltrating the hepatic sinusoids and sporadic cell necrosis of hepatocytes with mild vacuolization of hepatocellular as shown in **Figure 7.5**.

7.5. Discussion

Carcinogenesis is associated with cirrhosis. Cirrhosis correlates with primary liver disease. Liver is easily affected by various types of diseases, cancer being one of them. EAC easily affects the liver of mice. From the experiment, the liver section of EAC disease control group was compared with normal; 5-Fluorouracil and MENA treated groups. The liver of normal control group of mice (**Figure 7.1**) appeared to be formed of the classical hepatic lobules. Each lobule showed radially arranged hepatocytes forming cords around the central veins. Hepatocytes appeared polygonal in shape with rounded central vesicular nuclei. Blood sinusoids were seen separating the cords of the liver cells and lined by flattened endothelial cells and Von Kupffer cells. Minimal amount of thin fine collagen fibers was seen around the central veins and portal areas (**Figure 7.1**). In **Figure 7.2**, mice liver sections showed hypertrophy of hepatic cells, vacuolated and necrosis in the cytoplasm with deeply stained pyknotic nuclei. A widening of the portal tract and mononuclear cellular infiltration around the congested portal vein were seen. The cellular architecture of the hepatic lobules seemed to have degenerated after inoculation of EAC cells in mice. The cells were positively stained with H & E and the cytoplasmic material was less vacuolated.

The best way to use histologic data such as Histology Activity Index (HAI) has been already reported using Knodell score [8]. The original Knodell score was calculated as the sum or scores of periportal necrosis, intralobular injury, portal inflammation and fibrosis.

7.6. Reference

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Chapter- 8

Conclusion

Epidemiological studies have consistently shown that the phytochemicals, obtained from different medicinal plants have significant protective effects against different diseases. Traditional medicines, which use plant extracts, have a prominent role in treating major diseases throughout the world in which India, no doubt have become a hub of the herbal medicines. Several medicinal plants are found in this sub-continent which possess anticancer activity as they have a significant amount of phytochemicals having anti-oxidant property which is helpful for the treatment of cancer. Cancer is a major threat to the world which is a major cause of death. A prospective study by ICMR has predicted that 17.3 lakh people of India will be affected to cancer of which 8.8 will succumb to death by 2020.

This research work entitled **'Bioactivity guided isolation/ fractionation of anti-cancer principle from *Nyctanthes arbor-tristis* Linn'** has been carried out to investigate secondary plant derived metabolite which has significant anti-cancer activity in human cancer cell lines.

Several studies and spectral analysis pave the way to conclude that the isolated compound is Betulinic acid. This is the first report on isolation and identification of lupane type triterpenoid compound from leaves of *Nyctanthes arbor tristis* Linn. using bioactivity guided fractionation. The betulinic acid *in vitro* study shows significant and potential anti-inflammatory activity through the COX-1, COX-2 and 5-LOX enzyme inhibition assay and also have inhibition activity of nitric oxide (NO), tumor necrosis factor-α (TNF-α) production in lipopolysaccharide (LPS) stimulated RAW 264.7. Betulinic acid shows significant antioxidant activity in DPPH assay as well as anticancer activity on human cancer cell lines like HEPG2, MCF-7, A549, HL-60, PC-3, HCT-116 and HeLa.

Simultaneously, *in vivo* study of methanol leaf extract of *Nyctanthes arbor tristis* Linn (MENA) also showed significant antioxidant activity through the increased level of enzyme CAT, SOD, GSH and reduced level of lipid peroxidation (LPO) in MENA treated group compare to EAC control group. Pathological parameters SGPT, SGOT, Total protein, Creatinine, Total bilirubin is also normalized due to intra-peritoneal administration of MENA which has also helped the judgment of antioxidant and anticancer activity.

The MENA significantly reduces tumor volume; viable cells count; normalized hematological parameters (RBC count, WBC count, and Hemoglobin level) and increased

life span (ILS) as compared with EAC control group. These observations suggested that *Nyctanthes arbor tristis* leaf extract has antitumor and anticancer activity.

The histopathological findings of MENA test groups shows normal architecture of liver tissue, less thick wall central vein (CV), deformed necrosis of tissue (N), and nucleus, parenchyma, hepatic cells are regenerated. Those were damaged in EAC cells inoculated mice. Histology Activity Index (HAI) or Knodell scores also showed better result of the MENA treated group. These parameters and observations indicate that methanol leaf extract of *Nyctanthes arbor tristis* Linn (MENA) has significant anticancer property.

The present *in vitro* and *in vivo* studies reveal that methanol leaf extract of *Nyctanthes arbor-tristis* Linn (MENA) and its isolated compound Betulinic Acid from ethyl acetate fraction have significant anti-inflammatory, antioxidant, antitumor and anticancer activity.

As the scope and objective of the study revolves around the anti-cancer activity of the derived compound, 'Bitulinic Acid', still there is a scope to investigate the other compounds present in *Nyctanthes arbor tristis* Linn. Further molecular level of study can be performed to find out the other pharmacological activities including anti-HIV property of the compound.

Appendix – I : Identification of the Plant Specimen

Phone No: +91(033) 2668 3235
GOVERNMENT OF INDIA
MINISTRY OF ENVIRONMENT & FORESTS
BOTANICAL SURVEY OF INDIA
OFFICE OF THE SCIENTIST -' D'
CENTRAL NATIONAL HERBARIUM
P O - BOTANIC GARDEN
HOWRAH- 711 103

Fax: +91(033) 2668 6226
भारत सरकार
पर्यावरण एवं वन मंत्रालय
भारतीय वनस्पति सर्वेक्षण
वैज्ञानिक 'डी' का कार्यालय
केन्द्रीय राष्ट्रीय पादपालय
पत्रालय-बोटेनिक गार्डन
हावड़ा -711103

No.: CNH/31/2013/Tech.II/1065 Dated:19-05-2013

To
Mr. Birendra Nath Kaan
Department of Pharmaceutical Technology
Jadavpur University
Kolkata -700032

Sub.: Identification of four plant specimens – reg.

Dear Mr. Kaan,

Please refer to your letter dated 19th April 2013 along with four plant specimens for identification. The specimens have been identified by the concerned expert as:

Sl. No.	Specimen No.	Scientific Name	Family
1.	CB -01	**Commelina longifolia** Lam.	Commelinaceae
2.	CV -02	**Clerodendrum infortunatum** L.	Lamiaceae
3.	GP -03	**Glycosmis pentaphylla** (Retz.) DC.	Rutaceae
4.	NA -04	**Nyctanthes arbor-tristis** L.	Oleaceae

You are hereby requested to remit a sum **of ₹ 200/-** (Rupees two hundred only) by cash or Demand Draft drawn in favour of **'Accounts Officer, P.A.O. (BSI & ZSI), Kolkata'** towards identification charges.

Your specimens are returned herewith.

With best wishes,

Yours sincerely

20-6-2013

(V.P. Prasad)
Scientist –'C'

वैज्ञानिक "सी" Scientist 'C'
केन्द्रीय राष्ट्रीय पदपालय
Central National Herbarium
भारतीय वनस्पति सर्वेक्षण
Botanical Survey of India
हावड़ा/Howrah-711103

Appendix – II : Approval of Animal Ethics Committee

Dr. Biswajit Mukherjee
M.Pharm., Ph.D., F.I.C., F.I.C.S.
Professor in Pharmaceutics
Coordinator, QIP Nodal Cell (Pharmacy)
Joint Coordinator,
Centre for Advance Research in Pharmaceutical Sciences,
Jadavpur University, Kolkata
Former DAAD Fellow (Germany) and Ex-guest Scientist,
German Cancer Research Center (DKFZ)
Heidelberg, Germany
Indo-Hungarian Education Exchange Fellow,
Budapest, Hungary
Former Fellow Scientist, School of Pharmacy,
University of London, London, U.K.
Ex. Biotechnology Overseas Associate,
Department of Biotechnology
(Government of India) and Worked in DKFZ
Heidelberg, Germany

Department of Pharmaceutical Technology
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E-mail : biswajit55@yahoo.com

To,
Prof. T. K. Maity
Dept. of Pharm. Tech.
Jadavpur University
Kolkata-32, West Bengal

Project title: Bioactivity guided isolation/fractionation of anticancer principle from selective indian medicinal plants

Ref No.: AEC/PHARM/1502/12/2015
Date: 30.07.2015

Dear Sir/Madam,

As per the meeting of Animal Ethics Committee (AEC), Jadavpur University, held on 23.07.2015 , I am pleased to inform you that the AEC, Jadavpur University, has approved your project for fresh /renewal applied to the respective funding agencies. However, you are requested to abide by the following guidelines-

1) All the animal experiments should be conducted strictly following the guidelines of CPCSEA (Govt. of India) and the UGC, New Delhi.
2) Animals are to be purchased from registered breeders only and record should be maintained and required to be submitted to the convenor, AEC, Jadavpur University.
3) Details of animals housed and sacrifice of animals being carried out should be given to the AEC time to time.

Furthermore, you are requested to submit the project no. to the AEC once the project is sanctioned. Moreover, you are requested to note further that the expenditure regarding maintenance of the animals has to be made from your project only. **Special attention should be provided to prohibit any unlawful cruelty to the animals.**

Thanking you
Yours sincerely,

30/7/15
(Dr. Biswajit Mukherjee)

Dr. Biswajit Mukherjee
Convener
Animal Etheics Committee
JADAVPUR UNIVERSITY
Kolkata-700 032

Appendix – III : List of Publications

NATURAL PRODUCT RESEARCH, 2018
https://doi.org/10.1080/14786419.2018.1470171

Taylor & Francis
Taylor & Francis Group

SHORT COMMUNICATION

Check for updates

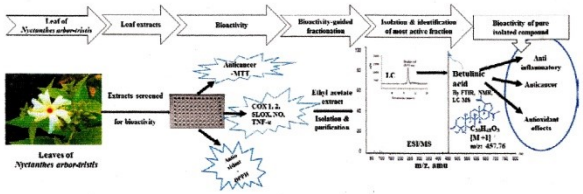
Betulinic Acid, the first lupane-type triterpenoid isolated via bioactivity-guided fractionation, and identified by spectroscopic analysis from leaves of *Nyctanthes arbor-tristis*: its potential biological activities *in vitro* assays

Birendra Nath Karan[a,b], Tapan Kumar Maity[b], Bikash Chandra Pal[b], Tanushree Singha[b] and Snehasis Jana[c]

[a]Pharmaceutical Research Division, Institute of Pharmacy, Kalyani, India; [b]Department of Pharmaceutical Research, Jadavpur University, Kolkata, India; [c]Pharmaceutical Research and Development, Trivedi Science Research Laboratory Pvt. Ltd, Bhopal, India

ABSTRACT

Betulinic acid was first time isolated via bioactivity-guided fractionation from ethyl acetate extract of *Nyctanthes arbor-tristis* leaves. Its structure was established by FTIR, ^{1}H and ^{13}C- nuclear magnetic resonance and high-resolution mass spectrometry. It had shown excellent inhibition of anti-inflammatory properties with IC_{50} of 10.34 µg/mL (COX-1), 12.92 µg/mL (COX-2), 15.53 µg/mL (5-LOX), 15.21 µg/mL (Nitrite), 16.65 µg/mL (TNF-α), and also exhibited potent antioxidant activity with IC_{50} of 18.03 µg/mL. The anticancer activity of betulinic acid was evaluated against different human cancer cell lines. It showed significant cytotoxicity against various cancer cell lines with an IC_{50} of 6.53 (HepG2), 9.34 (A549), 14.92 (HL-60), 16.90 (MCF-7), 17.07 (HCT-116), 13.27 (PC-3), and 12.55 µM (HeLa). This is the first report on isolation and identification of the unreported lupane-type triterpenoid, betulinic acid from leaves of *Nyctanthes arbor-tristis*, which showed potent anti-inflammatory, antiproliferative, and antioxidant activity *in vitro* assays.



ARTICLE HISTORY
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KEYWORDS
Nyctanthes arbor-tristis; bioactivity-guided fractionation; anti-inflammatory; anti-cancer; FTIR; NMR; LC-MS

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Research Journal of Pharmaceutical, Biological and Chemical Sciences

Evaluation of Anti-Cancer Activity of Methanol Extract of *Nyctanthes arbor- tristis* Linn.

Birendra Nath Karan[1, 2], Tanushree Singha[2], Tanmoy Guria[2], Puspita Roy [2], TK Maity[2]* and Bikash Chandra Pal[3].

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ABSTRACT

Nyctanthes arbor tristis Linn Family-Oleaceae, is a well-known plant in India. The Methanol Extract (ME) of its leaves was prepared and injected into intraperitoneal route (200 and 400mg/Kg body weight) of Swiss Albino mice to observe anti-cancer activity against Ehrlich Ascites Carcinoma (EAC) cells (10^7cells/mouse). 5-Fluorouracil (5-FU) 20mg/kg body weight (b.w.) was administered into intraperitoneal (i.p.) route as standard Anti-cancer drug. After strictly maintaining the standard experimental protocol; EAC cell count (cancer cell), total tumor volume, percentage inhibition of total cell count, percentage inhibition of tumor volume, percentage inhibition of viable and non- viable cells, percentage increase in life span(%ILS) and Hematological parameter (WBC, RBC, Hb) were evaluated. These evaluated parameters were comparing with EAC control group. A significant decrease of EAC cell count, viable cell count, percentage inhibition of total cell count, percentage inhibition of tumor volume, percentage increase of life span (%ILS), percentage increase of non-viable cell count, increase of RBC count and Hemoglobin level were observed. The above evaluations of present study suggest that Methanol Extact of *Nytanthes arbor- tristis* leaves has an Anti-cancer activity against Ehrlich Ascites Carcinoma cells.

Keywords: *Nyctanthes arbor –tristis* Linn.(NA),Ehrlich Ascites Carcinoma, Methanol Extract of NA (MENA)

Corresponding author

ORIGINAL ARTICLE

Evaluation of anticancer activity of methanol extract of *Commelina benghalensis* Linn. against Ehrlich ascites carcinoma in albino mice

Birendra Nath Karan[1,2], Tanushree Singha[2], Tapan Kumar Maity[2], Bikash Chandra Pal[3], Tanmoy Guria[2], Puspita Roy[2], Avik Maji[2], Sanjib Das[2]

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Abstract

Aim: Evaluation of anticancer activity of methanol extract of *Commelina benghalensis* L. (MECB) by the experimental parameters such as tumor volume, tumor cell count, viable and non-viable cell count, mean survival time, increase lifespan, red blood cells (RBC) and white blood cells count and hemoglobin level (Hb), and histological architecture of liver section of three treatment group of mice compared to Ehrlich Ascites Carcinoma (EAC) control group of mice. **Materials and Methods:** The anticancer activity of methanol extract of the aerial part of CB, family Commelinaceae has been evaluated against EAC cells (EAC 10^7 cells/mouse) in Swiss Albino mice at the dose of 200 and 400 mg/kg body weight. The MECB was administered intraperitoneal (i.p.) route for 9 consecutive days to tumor-bearing mice. 5-Flurouracil (5-FU) 20 mg/kg body weight was also administered to i.p. for 9 days as a standard anticancer drug. **Result:** The MECB decreased the EAC cell count, viable cell count, and percentage inhibition of total cell count. At the same time, there was an increase in the percentage of lifespan (% ILS), non-viable cell count, RBC count, and Hb level. These parameters were compared with the EAC bearing mice in a dose-dependent manner. The histological architecture of liver section in EAC bearing mice was observed steatosis, lymphocyte accumulation and normal architecture of nucleus, parenchyma, and regeneration of hepatic cells in three treated group of mice. **Conclusion:** This study reveals that the MECB has significant anticancer activity against EAC cell in mice.

Key words: *Commelina benghalensis* L, Ehrlich ascites carcinoma, histology activity index, methanol extract of *Commelina benghalensis*, tumor volume

INTRODUCTION

Phytochemical compounds and extract obtained from medicinal plants have an important role in the treatment of various diseases.[1-3] According to the WHO, 2004 approximately 12.5% population was affected by cancer, and the mortality rate was high. Large numbers of drugs are available from natural sources, either by structural modifications or by semi-synthetic chemical compounds for the treatment of cancer. Modern anticancer drug has cytotoxicity. Hence, compounds obtained from plants have major importance in the field of oncological therapy due to their low toxicity and comparatively low cost[4] than the modern anticancer drug. Herbal compounds vinca alkaloids - vincristine, vinblastine, vinorelbine; taxanes-paclitaxel, docetaxel; camptothecin derivatives-topotecan, irinotecan; epipodophyllotoxins - etoposide, etoposide phosphate, teniposide, etc. are used for cancer treatment.[5]

Commelina benghalensis (CB) is a succulent, creeping perineal plant, height of the creeping stem approximately

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|UGC Approved Journal |

SYNTHESIS OF 2,4,6-TRISUBSTITUTED PYRIMIDINE ANALOGUES VIA CHALCONE DERIVATIVES AND THEIR ANTICANCER EVALUATION

Puspita Roy, Tanmoy Guria, Birendra Nath Karan, Subbiah Ramasamy, Tapan Kumar Maity*

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ABSTRACT

*A number of 2,4,6-trisubstituted pyrimidine derivatives **5a-f** were synthesized from different chalcone moieties **3a-f**. Compounds **3a–f** were prepared from reaction between various substituted acetophenones **1a–c** and various aromatic aldehydes **2a-d** in presence of catalytic amount of sodium hydroxide. All these compounds were identified by FT-IR, ^{1}HNMR spectral studies. The anticancer evaluation of all synthesized compounds has been explored in vitro and in vivo against Ehrlich's ascites carcinoma cell line. Compound **5f** was found to be most active among all the prepared compounds in comparison with 5-Fluorouracil as standard.*

KEY WORDS

Anticancer activity, Chalcone, Ehrlich's ascites carcinoma, Pyrimidine.

INTRODUCTION:

The burden of cancer is increasing across the World and thus it is the leading cause of deaths in economically developed countries and second leading cause of deaths in developing countries [1]. Each year, tens of millions of people are diagnosed with cancer around the world, and more than half of the patients eventually die from it [2]. Cancer is considered to be one of the most intractable diseases because of the innate characteristics of cancer cells to proliferate uncontrollably, avoid apoptosis, invade and metastasize [3].

Pyrimidine is familiar as a versatile heterocyclic compound, which has been subjected to a various structural modification in order to synthesize some derivatives with different activity [4]. They are present throughout nature in various forms. Hundreds of pyrimidine-containing compounds have been found in biological system which control normal physiology [5, 6]. Pyrimidine ring is present in several pharmacologically active compounds, showing a wide range of biological activities, such as diuretic [7], anesthetic [8], anthelmintic [9], analgesic and anti-inflammatory [10], antibacterial [11], antifungal [12] etc. The presence of a pyrimidine base in thymine, cytosine and uracil, which are the essential binding blocks of nucleic acids, DNA and RNA is one possible reason for their activity [13].

In this article, we have reported the synthesis of 2, 4, 6-trisubstituted pyrimidine derivatives as well as their anti-cancer activity against Ehrlich's ascites carcinoma (EAC) cell line. The anticancer activity was screened by determining various parameters like *in vitro* cytotoxicity, percentage change in body weight, percentage tumor weight and tumor cell inhibition, haematological parameters.

MATERIALS AND METHODS:

Chemistry

Substituted acetophenone (0.01mol) **(1a-c)** and Substituted benzaldehyde (0.01mol) **(2a-d)** were

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Available Online at www.ijpba.info
International Journal of Pharmaceutical & Biological Archives 2018; 9(4):15-22

RESEARCH ARTICLE

Synthesis, Characterization, and Biological Evaluation of Some Novel Phthalimide Derivatives

Tanushree Singha[1], Tapan Kumar Maity[1]*, Saumen Karan[1], Birendra Nath Karan[1], Subbiah Ramasamy[1], Nimai Chand Ganguly[2]

[1]Department of Pharmaceutical Technology, Division of Pharmaceutical Chemistry, Jadavpur University, Kolkata, West Bengal, India,[2]Department of Chemistry, Kalyani University, Kalyani, West Bengal, India

Received: 20 August 2018; Revised: 11 September 2018; Accepted: 15 September 2018

ABSTRACT

Objective: Different Phthalimide derivatives (4,5,6,7-tetrachloro-2-[1,2,4]triazol-4-yl]-isoindole-1,3-dione derivatives) were synthesized and biological activities of them were evaluated. **Materials and Methods:** In the present study, four new phthalimide derivatives were synthesized. The structures of final compounds were characterized on the basis of spectral data. Then, biological evaluation of all the synthesized compounds means *in vivo* anticancer activity was evaluated on the Ehrlich ascites carcinoma (EAC) bearing Swiss albino mice model, and *in vitro* antioxidant activity was assessed using 1,1-diphenyl-2-picryl hydrazine (DPPH) radical scavenging assay. **Results:** The titled compounds (2A-2D) were found to reduce tumor volume, viable cell count and increase non-viable cell count, and percentage increase in life span. All compounds showed significant activity in quenching DPPH free radical. **Conclusion:** All compounds showed significant ($P<0.01$) anticancer activity compared to EAC control group. All synthesized compound exhibited considerable antioxidant activity against free radicals, and they showed significant IC_{50} values and can, thus, ensure protection against oxidative stress.

Keywords: 1,1-diphenyl-2-picryl hydrazine, anticancer, antioxidant, ehrlich ascites carcinoma cell, phthalimide

INTRODUCTION

Cancer is a frightful disease and major global challenge[1] because it is the second most common cause of death worldwide after cardiovascular diseases. Cancer is a non-communicable disease, but it spreads like a communicable disease in spite of its non-contagious character. Extensive research has been carried out to combat this silent killer. Various synthetic drugs have been developed from different chemical entities. Various techniques such as surgery, immunotherapy, radiotherapy, and chemotherapy are used to treat cancer. Maximum number of disease treatment is only possible with nitrogen containing chemical entity. Among them, phthalimide (IUPAC name - Isoindole-1, 3-Dione) is bicyclic non-aromatic nitrogen containing heterocyclic compound which possesses a structural feature –CO-N(R)-CO- and an imide ring.[2]

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Phthalimide has received attention due to their diverse biological activities such as androgen receptor antagonists,[3] anticonvulsant,[4] antimicrobial,[5] hypoglycemic,[6] anti-inflammatory,[7] antitumor,[8,9] anxiolytics,[10] anti HIV-1 activities,[11] antitubercular,[12] antivirus,[13] histone deacetylase inhibitory,[14] liver X receptor antagonist,[15] leukotriene D4 receptor antagonist,[16] and antioxidant.[17]

Antioxidants are the reducing agents which used to stabilize some free radicals. Free radicals are atoms or groups of atoms with an odd number of electrons and can be formed when oxygen interacts with certain molecules in the mitochondrial respiratory chain reaction, atmospheric pollutants, and from drugs. These free radicals play a vital role in damaging the various cellular macromolecules and cause various pathological conditions such as diabetes mellitus, atherosclerosis, myocardial infraction, arthritis, anemia, asthma, inflammation, neurodegenerative diseases, and carcinogenesis.[18,19] In human body, this oxidative damage can be prevented by enzymatic and non-enzymatic antioxidant. However, this

Appendix – III : List of Conferences and Seminars

IAPST/ABS/P- 219

Comparative Chemometric Modeling of p53-HDM2 Inhibitors as Antiproliferative Agents

Chanchal Mondal, Nilanjan Adhikari, Amit Kumar Halder, Tarun Jha*
Natural Science Laboratory, Division of Medicinal and Pharmaceutical Chemistry, Department of Pharmaceutical Technology, P.O. Box-17020, Jadavpur University, Kolkata-700032, India
*E-mail: tjupharm@yahoo.com

p53, a tumor suppressor protein, regulates the cell cycle and inhibit the tumors growth. It is inactivated by mutation or binding itself with human double minute 2 (HDM2) protein. Small molecule inhibitors of p53-HDM2 is a promising target for treating several p53 protein related cancers. 2D-QSAR and 3D-QSAR pharmacophore mapping were performed on 155 compounds having p53-HDM2 inhibitory activity to understand structural and physicochemical requirements for higher biological activity. 87 diverse compounds were selected as the training set for 2D-QSAR model building, 29 compounds were selected as the test set and remaining 39 compounds were selected as the external test set for 2D-QSAR model validation. 3D-QSAR pharmacophore models were generated on 27 diverse compounds and validations of these models were done on remaining 115 compounds. The best 2D-QSAR model showed q^2 and R^2_{pred} value of 0.785 and 0.618 respectively and external R^2_{pred} value of 0.674 respectively. The correlation of 10 3D-pharmacophore hypotheses were ranging from 0.989 to 0.940 and R^2_{pred} values of all these pharmacophore hypotheses were greater than 0.5. Among the pharmacophore hypothesis, only hypothesis 3 showed external R^2_{pred} value greater than 0.500 on same 39 compounds used as the external test set compounds used in 2D-QSAR study. These pharmacophore hypotheses fulfill all the validation criterias. This work may help to find better p53-HDM2 inhibitors.

IAPST/ABS/P- 220

Synthesis and Evaluation of *in vivo* Antiproliferative Activity some 1, 2, 4-Triazole Derivatives

Tanushree Singha[1], Jagadish Singh[2], Tanmoy Guria[1], Satarupa Acharjee[3], S Ramasamy[1], Rohit Viswakarma[1], Puspita Roy[1], Benu Pani Sahu[1], Birendra Nath Karan[1] and Tapan Kumar Maity[1]*
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4-Amino- 5-mercapto- 3-(substituted)-1, 2, 4-triazole derivative were synthesized according to the literature methods. The final compounds were characterized by using FT-IR, proton NMR, C, H, N analysis and evaluation of their antiproliferative activity. Here Ehrlich Ascites Carcinoma (EAC) cells used as tumor cells to perform the above said activity.

Compound at a dose level of 25 mg/kg, body weight intraperitoneally was administered in Swiss albino mice. Here, 5-Fluoro Uracil was used as a standard drug given at 20 mg/kg body weight. All The compounds showed moderate activity to inhibit cancer cell growth except compound **4d** which is found to be most potent one. The result of the present investigation may encourage us to develop and improve similar other related compounds and test them against various anticancer models.

2nd Pharm. Tech. IAPST International Conference on

New Insights into Diseases and Recent Therapeutic Approaches

17th – 19th January, 2014

Certificate

This Certificate is awarded to Ms./ Mr./ Dr./ ~~Prof.~~ Birendra nath Karan

for participation as Delegate /~~Resource Person~~ /~~Chairing a Session~~ /~~Presenting a Paper (Oral/Poster)~~.

Prof. Malay Chatterjee
Chairman

Prof. Biswajit Mukherjee
Organizing Secretary

Prof. Subhasish Maity
Joint Organizing Secretary

Dr. Ambika C. Banerjee
Chairperson, Scientific Committee

Prof. Banasri Hazra
Chairperson, Scientific Committee

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Indian Association of Pharmaceutical Scientists and Technologists, Kolkata

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NPR 2011

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Jadavpur University

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Appendix – IV : List of Abbreviations

NAT	Nyctanthes arbor tristis
MENA	Mathanol extract of Nyctanthes arbor tristis
FTIR	Fourier transforms infrared
NMR	Nuclear magnetic resonance
^{1}HNMR	Proton Nuclear magnetic resonance
^{13}CNMR	Carbon- 13 Nuclear magnetic resonance
MS	Mass spectrometry
LC – MS	Liquid mass spectroscopy
ESI	Electrospray ionization
LC-Q-T	Liquid chromatography quadrupole time of fight
UPLC	Ultra performance liquid chromatography
AJS-ESI	Agilent jet stream electrospray ionization
COX – 1	Cyclooxygenase – 1
COX – 2	Cyclooxygenase – 2
5- LOX	5 – Lipoxygenase
ELISA	Enzyme linked immunosorbent assay
TNF-α	Tumor necrosis factor α
IC_{50}	Inhibitory concentration- 50
LD_{50}	Lethal Dose -50
PC	Positive control
DPPH	1, 1-Diphenyl-2-picrylhydrazyl
MTT	(3- (4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide)
PBS	Phosphate buffer saline
DMSO	Dimethyl sulphoxide
HIFBS	Heat inactivated fetal bovine serum

TMPD	N, N, N, N'- tetramethyl phenylenediamine
NCCS	National center for cell sciences
ATCC	American type culture collection
PS	Penicillin/Streptomycin
EtoAcF	Ethyl acetate Fraction
n-BuF	n- butanol Fraction
AqF	Aqueous Fraction
EAC	Ehrlich ascites carcinoma
5 – FU	5- Fluorouracil
WBC	White blood corpuscles
RBC	Red blood corpuscles
Hb	Hemoglobin
ILS	Increase of life span
MSD	Median survival time
TCI	Inhibition of total cell count
TVI	Inhibition of tumor volume
SOD	Superoxide dismutase
GSH	Glutathione
CAT	Catalase
CV	Central vein
S	Sinusoids
HAI	Histology activity index
N	Necrosis
CPCEA	Committee for the purpose of control and supervision of experiments on animals
OECD	Organization for economic co-operation and development
ANOVA	Analysis of variation
SEM	Standard error of the mean

HDAC	Histone deacetylase inhibitors
SOS	Osteosarcoma
BRs	Brassinosteroids
CHO	Chinese Hamster ovary
LDH	Lactate dehydrogenase