In-vitro and *in-vivo* assessments of therapeutic potentials of Curcuma longa (turmeric) rhizomes along with its nano-composite synthesis and polymer encapsulation for enhanced wound dressing applications

A THESIS

Submitted by

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Jadavpur University

Under the guidance of

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DEDICATION

This service is whole heartedly dedicated to God and well-being of its creations

Acknowledgements

I am glad to write this note of thanks after the end of my thesis work. It was a period of intense learning for me, not only in the scientific arena, but also on a personal level. I would like to reflect on the people who have supported and helped me so much throughout this period.

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Last, but not the least, I wish to express my profound gratitude and my deep feelings for my family who have been the constant source of my energy, inspiration and determination for going ahead with my academic pursuit.

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text. All information in this document has been obtained and presented in accordance with academic rules and ethical conduct.

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This is to certify that the thesis entitled "***In-vitro* and *in-vivo* assessments of therapeutic potentials of Curcuma longa (turmeric) rhizomes along with its nano-composite synthesis and polymer encapsulation for enhanced wound dressing applications**" that is being submitted by Sri Tathagata Adhikary, in partial fulfillment for the award of degree of Master in Biomedical Engineering of Jadavpur University is a record of bona fide work carried out by him under my guidance and supervision. The project, in our opinion, is worthy for its acceptance.

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Certificate of Approval

The forgoing thesis is hereby approved as a creditable study of an engineering subject carried out and presented in a manner satisfactory to warrant its acceptance as a prerequisite to the degree for which it has been submitted. It is understood that by this approval the undersigned do not necessarily endorse or approve any statement made, opinion expressed or conclusion drawn therein but approve the thesis only for the purpose for which it is submitted.

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List of figures

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Preface

Curcuma longa (turmeric) has been traditionally harnessed in different fields including both daily life and science. Although the fact that turmeric is a potent medicinal pant is well established, scientific data is needed to support the fact and evaluate its extent of potency. This work highlights the positive qualities that turmeric possesses and aims for the betterment of them.

Walking through this thesis, chapter one deals with the understanding of basic principles used in this work. All the assays and their mechanisms are discussed here. The statements given are well established and behaves as a tool to begin the work.

In chapter two, the knowledge behind this presentation in noted in a nutshell. It contains the results of different researches which are being linked or used in some way to accomplish the work.

Chapter three and four claims the motivation behind the work, its objectives and a plan along with the requirements and procedure of the experiments performed.

All the findings, their probable interpretations and conclusion after the end of the work is presented in chapter five and six.

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Chapter One

INTRODUCTION

1] INTRODUCTION

From ancient times, turmeric showed its use as a spice and medicine. Hence its potential in different fields has been investigated in recent years. Although the goodness of turmeric is claimed traditionally, providing proper scientific data will not only support the statements making it more evident but will also search for possible ways to enhance its potential.

Turmeric (Curcuma longa) is a rhizomatous herbaceous perennial plant of the ginger family, Zingiberaceae.[1] It requires temperatures between 20 and 30 °C (68–86 °F) and a substantial amount of annual rainfall to survive, making it popular in Southeast Asia and Indian subcontinent.

Scientific classification

Kingdom:	Plantae
Clade:	Angiosperms
Clade:	Monocots
Clade:	Commelinids
Order:	Zingiberales
Family:	Zingiberaceae
Genus:	*Curcuma*
Species:	*C. longa*

Figure1.1: Turmeric (Curcuma longa) with it botanical view

1.1 Phytochemistry

Turmeric powder comprises of approximately 6–13% water, 60–70% carbohydrates, 5–10% fat, 6–8% protein, 3–7% dietary minerals, 2–7% dietary fiber, 3–7% essential oils, and 1–6% curcuminoids.[2]

1.1.1 Phenols:

Phenols or phenolics, being a class of chemical compounds in organic chemistry consist of a hydroxyl group (—OH) which is bonded directly to an aromatic hydrocarbon group. When compared to alcohols, they have higher acidities because the aromatic ring is tightly coupled with the oxygen whereas a relatively loose bond exists between the oxygen and hydrogen.

Phenols have found its use as drugs. For example, a drug Crofelemer (USAN, trade name Fulyzaq), still under development, is used for the treatment of diarrhea associated with anti-HIV drugs. Additionally, derivatives have been made of phenolic compound, combretastatin A-4, an anticancer molecule, including nitrogen or halogens atoms to increase the efficacy of the treatment.[3]

1.1.2 Flavonoids:

Flavonoidis derived from the Latin word *flavus* meaning yellow indicating their color in nature. They are identified as a class of plant and fungus secondary metabolites. [13]

Chemically, C6-C3-C6 is the general structure flavonoids (also called bioflavonoids) which consists of a 15-carbon skeleton, having two phenyl rings and heterocyclic ring.

Flavonoids such as the catechins are polyphenolic compounds most commonly found in the human diet and everywhere in plants.[4] They have comparatively low toxicity when compared to other bioactive components of plant, for instance alkaloids. Flavonoids, being an important plant pigment, found its role in coloration of flowers. It produces yellow or red/blue pigmentation in petals which attract pollinator animals. In higher plants, flavonoids also play a part in UV filtration and symbiotic nitrogen fixation along with floral pigmentation. They

also act as chemical messengers, cell cycle inhibitors and physiological regulators. [18]

In *in vitro* studies. flavonoids witnessed a wide range of biological and pharmacological activities. Examples include anti-allergic,[5]antioxidant,[6]anti-inflammatory,[5][6] anti-cancer,[6][7] anti-microbial (antifungal,[8][9] antibacterial, [10][11] and antiviral[12][13]) and anti-diarrheal activities.[14] In future in vivo or clinical research has to performed to establish any beneficial or detrimental effect of flavonoids on human health. Research at the Linus Pauling Institute and the European Food Safety Authority showed poor absorption of flavonoids in the human body which is less than 5%.Moreover most of the absorbed flavonoids is metabolized and excreted very quickly.[15][16][17]This proves the fact that flavonoids have paltry antioxidant activity in a biological system. The increase in antioxidant capacity of blood observed after consumption foods rich in flavonoids is due to uric acid produced as a result of flavonoid depolymerization and excretion.[18]

1.1.3 Curcumin:

Phytochemical components of turmeric include diarylheptanoids which consists of numerous curcuminoids, such as curcumin, demethoxycurcumin, and bisdemethoxycurcumin[19]. Approximately 3.14% of turmeric powder constitutes curcumin. Major reported pharmaceutical activities of turmeric are antioxidant; antibacterial, anti-inflammatory, anti-tumor and anti-cancer activities and these are due to the presence of curcumin in it, hence claiming it to be the most bioactive component in turmeric. Curcumin is considered as safe by the United States Food and Drug Administration (FDA) because of its low toxicity at relatively high levels of ingestion. Curcumin is a phenolic constituent and is hydrophobic in nature [20]. So it can be extracted efficiently by many organic solvents. It is a tautomeric compound existing in enolic and keto form in organic solvents and water respectively.[21] The aromatic ring systems, which are phenols, are connected by two α,β-unsaturated carbonyl groups. The diketones form stable enols and are readily deprotonated to form enolates; the α,β-unsaturated carbonyl group is a good Michael acceptor and undergoes nucleophilic addition.

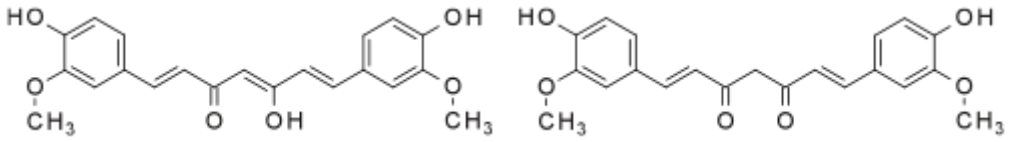

Figure 1.2: Structure of curcumin in enol and keto form respectively

1.2 Extraction techniques

In pharmaceutics, extraction involves the separation of medicinally active components of plant or animal tissues from other inactive or inert components by using selective solvents in standard extraction procedures. The general techniques of extraction include maceration, infusion, percolation, digestion, decoction, hot continuous extraction (Soxhlet), aqueous-alcoholic extraction by fermentation, counter-current extraction, microwave-assisted extraction, ultrasound extraction (sonication), supercritical fluid extraction, and phytonic extraction (with hydrofluorocarbon solvents). For aromatic plants, hydro-distillation techniques (water distillation, steam distillation, water and steam distillation), hydrolytic maceration followed by distillation, expression and enfleurage (cold fat extraction) are applicable. Latest extraction methods for aromatic plants include headspace trapping, solid phase micro-extraction, protoplast extraction, micro-distillation, thermos micro-distillation and molecular distillation [24].

The portion of plants used as the starting material, solvent used for extraction and extraction procedure are some parameters influencing the extract quality [25]. The variations among different extraction methods affecting the quantity and secondary metabolite composition in an extract depends upon type and time of extraction, temperature, nature of solvent along with its pH, concentration and polarity, particle size of the plant tissues and solvent to sample ratio [25]. A good solvent in plant extraction processes should have certain qualities like low toxicity, ease of evaporation at relatively lower temperatures, preservative action and inability to cause the extract to complex or dissociate. The factors affecting the choice of solvent are quantity of phytochemicals to be extracted, rate of

extraction, diversity of different compounds extracted, diversity of inhibitory compounds extracted, ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process and potential health hazard of the extractants [26]. Since traces of residual solvent will be present in the end product, the solvent should be non-toxic so that it does not interfere with the bioassays [23][22].

Methods of extraction of medicinal plants used here are:

Maceration: In this process, coarsely powdered crude drug is placed in a container with a suitable solvent or mixture of solvents and allowed to stand at room temperature for a period of roughly 3 days with frequent agitation ensuring dissolution of the soluble matters in it. The mixture then is strained, the marc i.e. the damp solid material is pressed and is filtered or decanted after standing.

Digestion: It is a form of maceration in which gentle heat is used during the process of extraction. This increases the solvent efficiency of the menstruum but is preferred when moderately elevated temperature is not obnoxious [24].

1.3 Antioxidant activity

Oxidative stress is involved in pathogenesis of numerous chronic diseases. Free radicals and other reactive oxygen species (ROS) are plays an important role in causing diseases such as asthma, diabetes, inflammatory arthropathies, Parkinson's and Alzheimer's diseases, cancers and atherosclerosis. ROS are also responsible for the aging process in living beings[28],[29].

An antioxidant is defined as any substance that helps to delay or inhibit oxidative damage to a target molecule due its ability to trap free radicals[30]. Antioxidant compounds like polyphenols, phenolic acids and flavonoids scavenge free radicals such as peroxide, lipid peroxyl or hydroperoxide inhibiting the oxidative mechanisms responsible for degenerative diseases[31],[27].

DPPH assay is one of the technique to measure the antioxidant activity. This assay was developed by Blois in 1958using a stable free radical α, α-diphenyl-β-picrylhydrazyl (DPPH; $C_{18}H_{12}N_5O_6$,). The method is based on the measurement of the scavenging capacity of antioxidants towards it. The odd electron of nitrogen atom present in DPPH is reduced by an incoming hydrogen atom from

antioxidants to the corresponding hydrazine [32]. DPPH is a stable free radical. Its stability is by virtue of the delocalization of the spare electron over the molecule as a whole, which inhibits the molecules to dimerise, like most other free radicals. The delocalization also gives a deep violet color, with a maximum absorption at 520 nm in ethanol. Mixing DPPH solution with a substance that has the capability to donate a hydrogen atom gives rise to the reduced form and the loss of violet color is observed. If we represent the DPPH radical by Z* and the donor molecule by AH, the primary reaction will be:

$$Z^* + AH \longrightarrow ZH + A^*$$

Where ZH is the reduced form and A* is free radical produced in the first step [33].

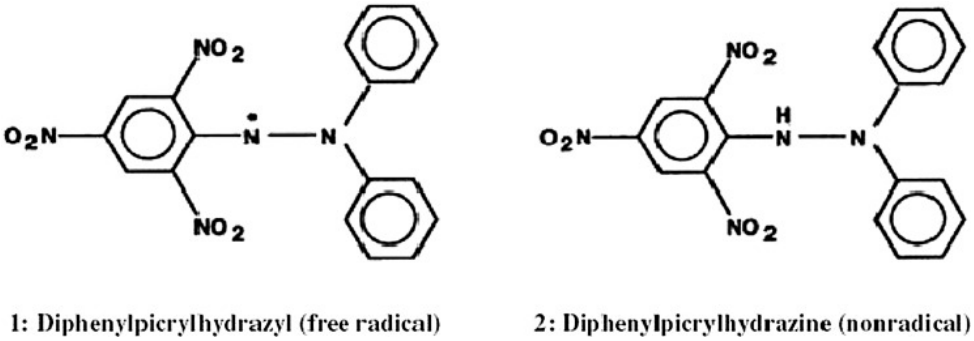

Figure 1.3: 1 and 2 depicts DPPH radical and its stable form respectively [33]

1.4 Bacterial strains

1.4.1 Escherichia coli:

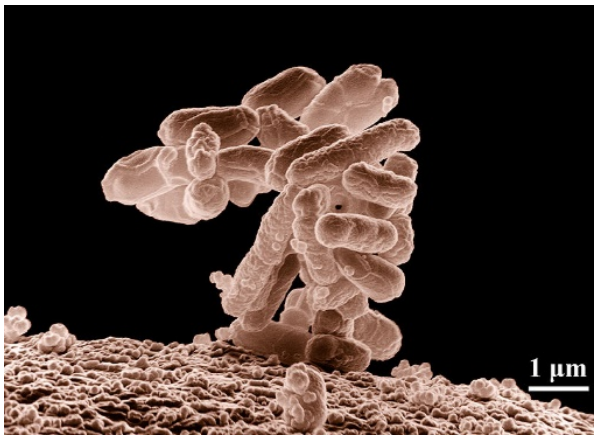

Figure 1.4: E.coli, its shape and size

E. coli is a Gram-negative bacteria. Its cell wall is framed with a thin peptidoglycan layer having an outer membrane which provides a barrier to certain antibiotics, for e.g., E.coli is resistant to penicillin.. It is mostly found in the lower intestine of warm-blooded organisms (endotherms) and is facultatively anaerobic, rod-shaped, coliform bacterium belonging to the genus Escherichia. Upon staining, E. coli takes up the color of safranin (i.e. the counterstain) and becomes pink in color. The outer membrane surrounding the cell wall [34].

Optimum growth temperature of E. coli is 37 °C (98.6 °F), although some strains used in the laboratory can proliferate at temperatures up to 49 °C (120 °F)[23].Regarding media, its growth can be achieved in a variety of defined laboratory media, such as lysogeny broth, or any medium containing glucose, ammonium phosphate, sodium chloride, magnesium sulfate, monobasic, dibasic, potassium phosphate and water. E.coli grows by both aerobic or anaerobic respiration by using variety of redox pairs which includes the oxidation of pyruvic acid, hydrogen, formic acid, and amino acids along with the reduction of substrates such as nitrate, oxygen, dimethyl sulfoxide, fumarate and trimethylamine N-oxide[35].

Scientific classification

Domain:	Bacteria
Phylum:	Proteobacteria
Class:	Gammaproteobacteria
Order:	Enterobacteriales
Family:	Enterobacteriaceae
Genus:	Escherichia
Species:	E. coli

1.4.2 Staphylococcus aureus:

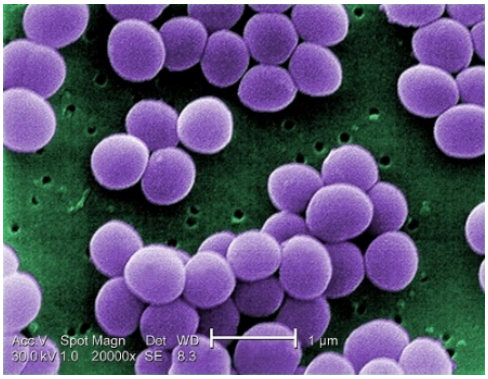

Figure 1.5: S. aureus, its shape and size

S. aureus is also known as "golden staph" and "oro staphira". It is a facultative anaerobic, gram-positive coccal (i.e. round) bacterium. It is non-motile and does not support spores formation.[14]It is often found in the nose, respiratory tract, and skin[36].

Scientific classification

Domain:	Bacteria
Kingdom:	Eubacteria
Phylum:	Firmicutes
Class:	Bacilli
Order:	Bacillales
Family:	Staphylococcaceae
Genus:	Staphylococcus
Species:	S. aureus

1.5 Antimicrobial activity

Antimicrobial susceptibility testing can be used for drug discovery, epidemiology and prediction of therapeutic outcome [37]. Among different procedures, diffusion methods which include agar disc diffusion method and agar well diffusion method are popular to check antimicrobial activity.

Agar disc-diffusion method:

Agar plates are inoculated with a standardized inoculum of the test microorganism. Then, filter paper discs (of size about 6 mm in diameter), containing the test compound at a desired concentration, are placed on the agar surface. The Petri dishes are incubated under sterile conditions at suitable environment to promote the growth of test microorganisms. The antimicrobial agent diffuses into the agar and helps to inhibit the germination and growth of the microorganism. The diameters of inhibition growth zones are measured which indicate the potency of the antimicrobial agent [37].

Agar well-diffusion method:

Similar to disc-diffusion method, the agar plates are inoculated by spreading a volume of the microbial inoculum over the entire agar surface. A hole with a diameter of about 6 mm is punched aseptically and a definite volume of the antimicrobial agent or extract solution at desired concentration is added into the well. Depending upon the test microorganism, agar plates are incubated at suitable conditions. The antimicrobial agent diffuses in the agar medium and inhibits the growth of inoculated microorganism [37].

1.6 Scanning electron microscope

A scanning electron microscope (SEM) is a type of electron microscope. It produces images of the sample under study by scanning its surface with a focused beam of electrons. The electrons interact with atoms present in the sample, producing various signals containing information about the topography and composition of the surface of sample being scanned. The most usual mode of SEM utilizes the principle of detection of secondary electrons emitted from atoms that are being excited by the electron beam. Among other factors, specimen topography influences the number of emitted secondary electrons. After scanning the samples and gathering the secondary electrons emitted using a detector, an image is formed presenting the topography of the surface. Very high-resolution images are displayed, exposing details less than 1 nm in size. The angle and velocity of the secondary electrons reflect the surface topography of the object. Samples for SEM need to withstand the vacuum conditions and high energy levels

of beam of electrons and should have a size so that it fit on the specimen stage. Samples are generally mounted rigidly to a specimen holder or stub using a conductive adhesive. Hence sample preparation is an essential step before performing SEM analysis [38].

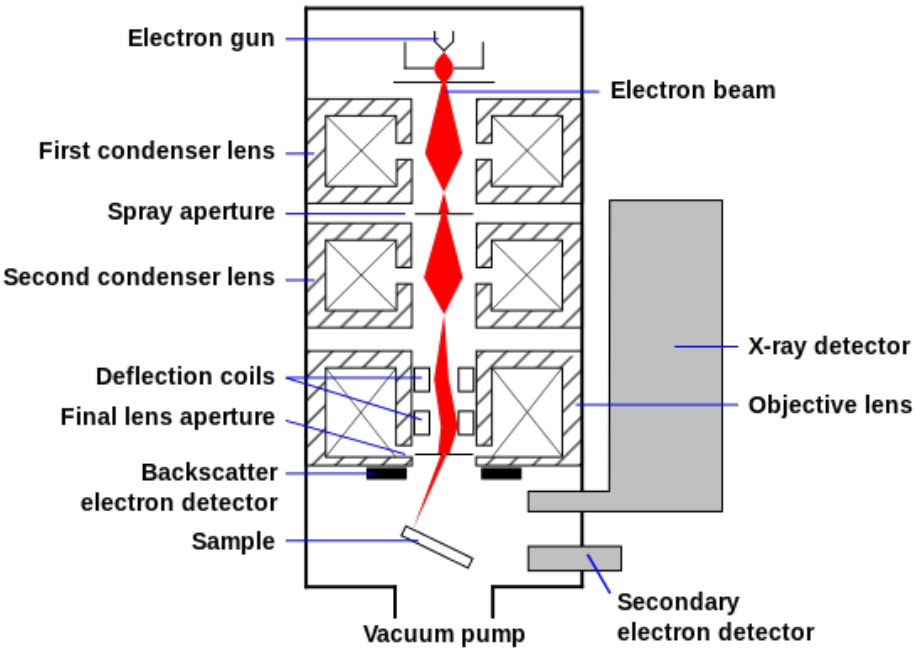

Figure 1.6: Schematic diagram of SEM

1.7 Fourier-transform infrared spectroscopy

Fourier-transform infrared spectroscopy (FTIR) is a method which is used to obtain an infrared spectrum of absorption or emission of a material. Materials for FTIR can be solid, liquid or gas. An FTIR spectrometer collects high spectral resolution data over a broad spectral range. Fourier transform is a mathematical process and this technique is applied to convert the raw data into the actual spectrum [39].

1.8Cell culture

1.8.1 Peripheral blood mononuclear cell (PBMC)

A PBMC is any peripheral blood cell with a round nucleus[40]. These cells include lymphocytes (T cells, B cells, NK cells) and monocytes, whereas erythrocytes and platelets have no nuclei, and granulocytes (neutrophils, basophils, and

eosinophils) have multi-lobed nuclei. In humans, lymphocytes contribute to the majority of the PBMC population, followed by monocytes, and amino percentage of dendritic cells[41]. By using ficoll (a hydrophilic polysaccharide) and gradient centrifugation, separate layers of blood can be observed from which PBMC in whole blood can be extracted[42].Top layer contains plasma, followed by a layer of PBMCs and a bottom fraction of polymorphonuclear cells (such as neutrophils and eosinophils) and erythrocytes.

1.8.2 MCF-7:

MCF-7 is a breast cancer cell line. It was isolated in 1970 from a 69 years old Caucasian woman[42].The cell line was established in 1973 by Herbert Soule and co-workers in Michigan Cancer Foundation-7 which coined its name. Tumor necrosis factor alpha (TNF alpha) inhibits the growth of MCF-7 breast cancer cells. Anti-estrogens treatment can regulate the secretion of insulin-like growth factor binding proteins. Omega-3 and 6 fatty acids such as EPA, DHA and AA has been accounted to inhibit the growth as well as proliferation of MCF-7 cell line[43].

1.8.3Cytotoxicity measurement using MTT assay:

Cytotoxicity determines the toxicity to cells. A variety of cell fates can occur when cells are treated with some cytotoxic compounds. Necrosis can occur in which cells lose their membrane integrity and die rapidly due to the occurrence of cell lysis. Also the cells can stop growing and splitting (causing a decrease in cell viability) dynamically, or the cells can undergo apoptosis by activating a genetic program of controlled cell death [44].

MTT assay can be used to measure cytotoxicity of different samples on cell cultures. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was the first homogeneous cell viability assay developed. Viable cells with active metabolism convert MTT into a formazan product (purple in color) having maximum absorbance value around 570 nm. Dead cells are incapable to convert MTT into formazan. Thus the formation of colored product (i.e. formazan) functions as a practicable and convenient marker of viable cells only eliminating dead cells. The exact cellular mechanism

of reduction into formazan is still under question, but probably involves reaction with NADH or similar kind of reducing molecules that can transfer electrons to MTT [45].

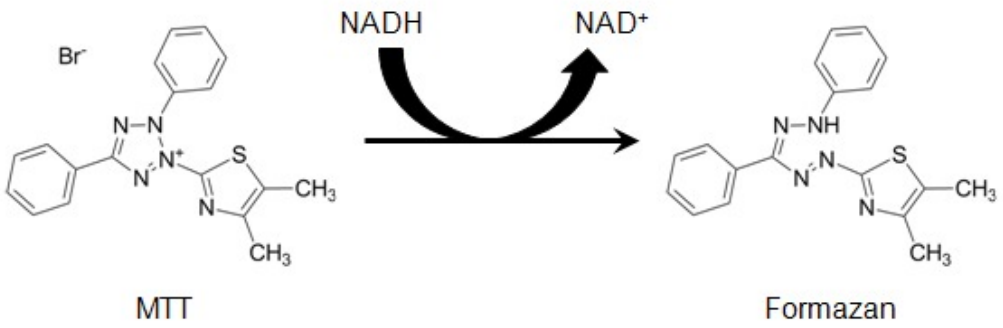

Figure1.7: Structures of MTT and its colored formazan product upon reduction

MTT tetrazolium when reduced to its formazan product gets accumulated as an insoluble precipitate inside cells besides being deposited in close proximity to the cell surface and also in the culture medium. The formazan is solubilized in order to record the absorbance values. A number of methods have been exploited for solubilizing the purple colored formazan product, stabilizing its color, avoiding evaporation and reducing interference due to phenol red and other constituents of culture medium [46]. Various techniques include using solvents like acidified isopropanol, dimethylformamide, DMSO, SDS and combinations of different detergents and organic solvents [47] [48].

1.9 Wound and its healing mechanism

A wound can be defined as the deprivation or breaking of cellular continuity (both anatomical and functional) of living tissues. Wound healing is a dynamic process. It aims to restore the structure of the tissue that suffers injury [49]. It is divided into 4 overlapping phases – inflammation, then coagulation followed by tissue formation and tissue remodelling [49]. The action begins right away after injury releasing platelet granules, growth factors and cytokines which plays a crucial role in recruiting inflammatory cells at the wound site. Inflammatory phase starts with an essential cellular influx for local debridementaiming to degrade any invading foreign particles and providing a provisional matrix to promote enhanced

proliferation of fibroblasts. This leads to the formation of granulation tissue marking the beginning of the phase of new tissue formation [50]. Vigorous fibroblast proliferation and its migration occur which helps to synthesize the new extracellular matrix covering the damaged portion of tissues [51]. Fibroblasts then starts synthesizing new matrix elements and principal components of the extracellular matrix (for e.g. proteoglycans, glycosaminoglycans and collagen) which gets deposited at the site of injury to substitute the provisional matrix that comprised initially of fibrin [51]. The most important structural constituent of granulation tissue is collagen. Collagen provides strength to the extracellular matrix. Collagen fiber is composed of an amino acid proline as its basic component and thus hydroxyproline can act as a biochemical marker for collagen content in tissues indicating the progress of wound healing process [49].

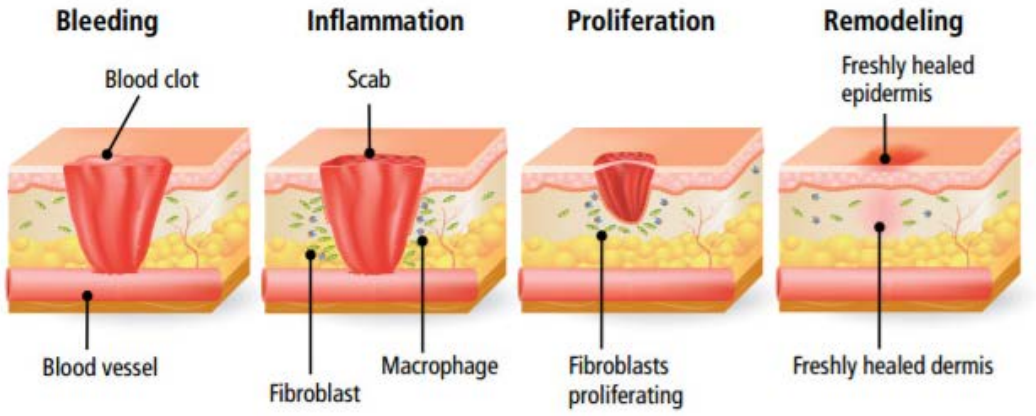

Fig 1.8: Stages in wound healing

Healthy wound healing process necessitates fibroblasts which is the basic connective tissue cells present in the body and macrophages which are produced in response to infection.

Generally, these types of wound models are employed to evaluate the wound healing activity:

1. Excision wound model
2. Incision wound
3. Burn wound model
4. Dead space wound model
5. Ear wound models
6. Superficial wound models [50]

1.10Histopathology

The term “histopathology” denotes the microscopic examination of tissues for studying the manifestations of diseases. Histology, also called microanatomy,[52] is the study of the anatomy of cells and tissues of plants and animals using microscopy. It generally utilize a light microscope or electron microscope for viewing the specimens that are being sectioned, stained, and finally mounted on a microscope slide.

The most frequently used stain in the field of histopathology is the combination of hematoxylin and eosin (often abbreviated H&E).

1.10.1 Hematoxylin:

Hematoxylin is extracted from the bark of a tree “hematoxylom campechianum”. It is not a dye does not have any coloring properties. To stain nucleus, it is compulsory to oxidize hematoxylin to hematin. This hematin is a weak anionic dye having a purple color. Anionic hematin will have no affinity towards nucleic acids of the nuclei. Therefore, a metallic salt or mordant is mixed with hematoxylin so that the dye gets positively charged by virtue of the metal action. The cationic dye-metal complex will bind to the nuclear chromatin which is anionic in nature. A mordant can be of different type such as ammonium or potassium alum ferric salt, chromalum and phosphotungstic acid. Hematoxylin lake is the term given to the combination of hematoxylin and mordant [52].

1.10.2 Eosin:

The nuclear staining is followed by counterstaining. This is achieved with an aqueous or alcoholic solution of eosin Y. Eosin Y colors eosinophilic structures in different shades of red, pink and orange. The eosinophilic structures include intracellular or extracellular protein. Majority of the cytoplasm is eosinophilic. Red blood cells are stained red in an intense manner [52].

1.11 Film formation and polymer coating

1.11.1 Mechanism of film formation:

In wet state, polymer poses as discrete particles. These particles need to come in close proximity in order to deform, coalesce and finally fuse with one another to form a discrete film. During processing, the surface of the substrate will be wetted with diluted dispersion. Under the prevailing processing conditions water will be evaporated and the polymer particles will come in close contact with each other. This process is largely supported by the capillary action of the film of water bordering the particles. Coalescence is achieved completely when the adjacent particles are capable to mutually diffuse into one another [53].

The process of film formation from polymeric solutions is relatively easy since the polymer is present in dissolved state. The surface of the substrate is scattered by sprayed droplets and as the solvent evaporates, the polymer chains diffuse with each other, going through transition from gel state to the formation of film with prolonged drying. Polymer chain interpenetration occurs at a specific concentration which is proportional to the reciprocal of the intrinsic viscosity of the solution [54]. The intrinsic viscosity is basically the increase in relative viscosity due to the dissolution of polymer and thus indicates the hydrodynamic interactions occurring between the polymer and the solvent [55].

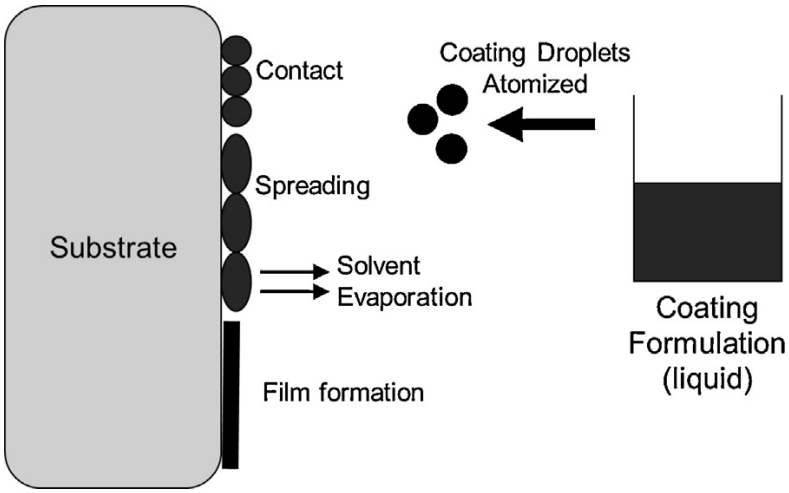

Figure 1.9: Overview of the film formation process [55]

1.11.2 Plasticizers:

Plasticizers are materials with a relatively lower molecular weighthaving the capacity to modify the physical properties of a polymer making it softer and pliable in order to enhance its function as a coating material. Generally, a plasticizer lowers the glass transition temperature. The plasticizer molecules intervene themselves between two polymer strands thus reducing the polymer-polymer interactions to a great extent. Moreover, polymer-plasticizer interaction is regarded to be stronger than polymer-polymer interaction. This is believed to be the mechanism of action of plasticizers. Therefore, the polymer strands attached to plasticizers have a greater chance to slide against each other which in turn transforms polymeric film into a more pliable material [53] [54].

1.11.3 Film coating by polymers:

Conventional film coating, also referred as non-functional coating is used for variety of reasons which includes improvement in product appearance, handling, prevention from dust, enhancements in physical characteristics etc. On the other hand, functional film coating is done with a purpose to achieve a modified and controlled release aspect on the dosage form of a drug [53] [54].

1.12 References

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Chapter Two

Literature Review

2.1 Turmeric (*Curcuma longa*), its phytochemistry and uses:

India is considered as the origin of the plant Curcuma longa belonging to Zingiberaceae family. The turmeric plant is spread throughout tropical and subtropical regions of the world and is being cultivated to a great extent in southeast Asian countries where it has found its application as a food additive, preservative and coloring agent [1]. It is considered as a medicinal plant and is widely used in Ayurveda, unani and siddha medicine in recipes of home remedy for several diseases. It is also considered as auspicious and is a part of religious rituals [2].

As of late, turmeric has been established to possess diverse pharmaceutical functions. Anti-inflammatory [3], anticancer [4], anti-ageing [5], neuro-protection in Alzheimer's disease [6] and wound healing are few examples among many others [7] [8].

Phytochemicals such as alkaloids, terpenoids, flavonoids, polyphenols and essential oils have been distinguished and documented as the bioactive components responsible for the pharmacological activities of plant extracts [9]. Among them especially phenolics are bioactive compounds having great health benefits. Clinical trials and epidemiological studies revealed that an inverse correlation exists between the uptake of dietary antioxidants and the occurrence of oxidative stress related diseases. The bioactivity of phenolics may be related to their ability to chelate metals, inhibit lipoxygenase and scavenge free radicals or prevent the adverse effects of reactive oxygen and nitrogen species (ROS/RNS) on normal physiological function in humans [10].

Curcuminoids namely curcumin (also known as 1,7-bis-(4-hydroxy-3-methoxy-phenyl)-hepta-1,6-diene-3,5-dione), demethoxycurcumin (1-(4-Hydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-hepta-1,6-diene-3,5-dione), and bisdemethoxycurcumin (1,7-Bis-(4-hydroxyphenyl)-hepta-1,6-diene-3,5-dione) are present in turmeric at around 3-5 % [11]. Demethoxycurcumin and bisdemethoxycurcumin are basically desmethoxy and bis-desmethoxy derivatives of curcumin. They are responsible for the characteristic yellow color of turmeric.

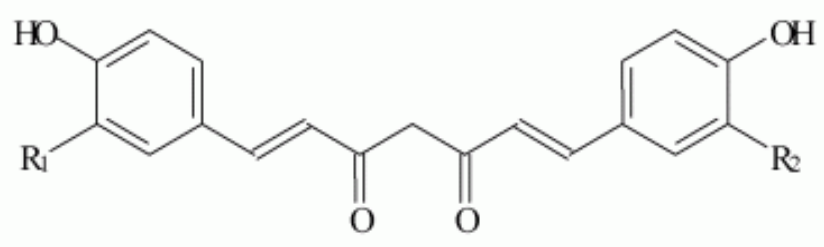

Figure 2.1: Structure of curcuminoids

In the figure 2.1, when $R_1 = R_2 = OCH_3$, it is curcumin. When $R_1 = OCH_3$ and $R_2 = H$, it is demethoxycurcumin while $R_1 = R_2 = H$ is the structure of bisdemethoxycurcumin.

Curcumin, the principal bioactive component of turmeric is claimed to have a broad spectrum of actions on biological systems [12]. Curcumin exhibit a keto-enol tautomerism and has anti-oxidative properties. Being an oil soluble pigment, it is practically insoluble in water at acidic and neutral pH, but slightly soluble in alkali. It is quite stable at high temperatures and also in acids, but unstable in alkaline environment undergoing rapid decomposition at pH values above neutral and is light sensitive, especially when occurs in solutions [2].

The capability to undergo several biochemical alterations is a crucial property that curcumin possess to inhibit cell damage. Curcumin also targets various cell-signaling pathways and this marks its utilization in cases with neoplastic disease. The compounds used as chemo-preventive drugs are divided into two categories: suppressing agents and blocking agents. Suppressing agents blocks the carcinogenic progress in cells while blocking agents prevent the activation of carcinogenesis. Curcumin possess both these abilities and demonstrates a multiple mechanism of action. Previous reports about curcumin propose the fact that it induces programmed cell death in cancer cells only without causing harm to the healthy cells and also inhibits the process of developing new blood vessels from preexisting ones. Natural curcumin has three OH groups that plays a vital role in the anti-cancer activity [13].

2.2 Extraction of bioactive components from Turmeric:

Extraction is the first and an important stair in the preparation process of plant formulations.
It is often a tiresome and time consuming process. Moreover, to extract pigments completely we need to employ several steps along and may require a mixture of different solvents.

Conventional extraction methods mainly rely on the extracting power of solvents. It lists soxhlet extraction, maceration and hydro-distillation [14]. Maceration is a traditional method of extraction. It is inexpensive is usually done in a closed container with grinded plant material [15]. Soxhlet extraction was first projected in 1879 in order to extract lipid. But it became a popular method to extract variety of bioactive compounds from plants with high yield percentage and acts as a benchmark to judge other the efficiency of other conventional and non-conventional extraction processes. Hydro-distillation can be classified into three categories: water distillation, water and steam distillation and direct steam distillation. Since hot water and steam are used as the solvents, it eliminates the separation step of solvent and extract as oils and hydrophobic compounds like curcumin are separated mechanically in condenser [16].

Although in soxhlet extraction the material is extracted continuously but is generally time-consuming than maceration. It takes from a few hours up to days to complete the extraction and also take up large volumes of solvent. Furthermore, the extract constantly receives a high temperature which corresponds to the boiling point of the solvent being used and thus can damage thermo-labile compounds. Acetone and methanol are regarded as ideal solvents for extraction of pigments [2].

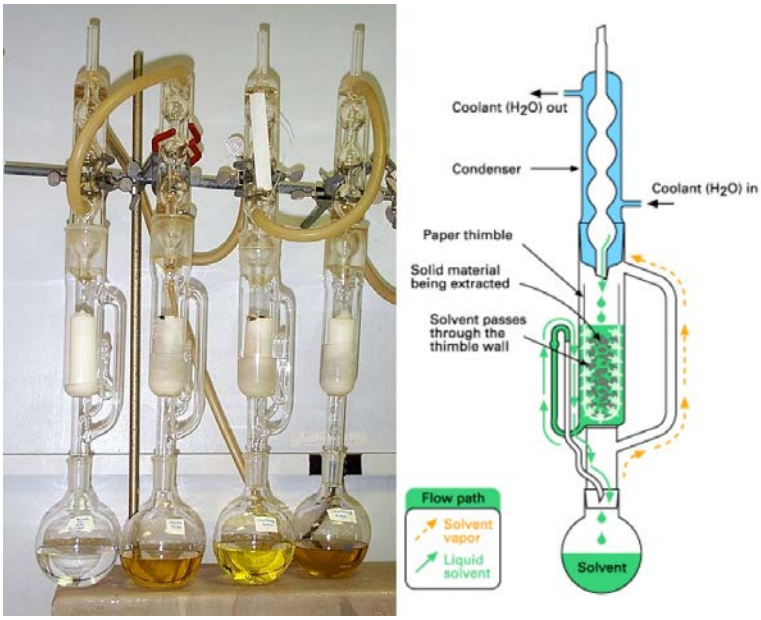

Figure2.2: Soxhlet apparatus [2]

The curcuminoids are not soluble in water and thus nonpolar solvents should be used in their extraction process [2]. The JECFA specifications monograph for Curcumin (FNP 52 Add. 9, 2001) lists acetone, methanol, ethanol, and isopropanol as suitable solvents. Also the European Commission Directive 95/45/EC lists the following solvents as suitable for the extraction: acetone, carbon dioxide, ethyl acetate, dichloromethane, n-butanol, methanol, ethanol, and hexane. Carbon dioxide, as a supercritical fluid, (ADI NS) is recognized by JECFA as an extraction solvent. It is also a carbonating agent, propellant, preservative and freezing agent. In the EU it is an approved solvent in the manufacture of curcumin (Directive 94/36/EC) [2].

Parameters like temperature, mixing time, particle size and solvent to meal ratio has an impact on the curcumin yield from turmeric and the experimental values of the yield ranges between 4.49 and 12.89%. It was observed that the percentage of curcumin extracted first increased and then decreased with the rise in temperature. This can be due to increase in solubility with the increase in temperature but when the temperature reaches beyond 60°C the yield decreases because of thermal degradation. Particle size disclosed a typical pattern of initial decrease and then increase with the increase in size. The fall in yield can be assumed due to the decrease in surface area

when size increases but its further rise can be due to better leaching of pigment. The maximum curcumin yield was achieved when temperature, particle size, mixing time and solvent to meal ratio were 60°C, 0.42 mm, 30 min and 50, respectively [17].

Modern extraction methods use less hazardous volatile organic solvents. It utilizes safer and renewable chemicals preventing pollution and developing pure products with fewer derivatives. The protocols followed also aims to save energy [15]. Common advance extraction methods are microwave-assisted extraction, pressurized liquid extraction, supercritical fluid extraction, enzyme-assisted extraction and ultrasound-assisted extraction [15]. Curcumin extraction yield using Soxhlet method (6.9%) was considerably higher than those obtained from microwave-assisted (3.72%), ultrasound-assisted (3.92%) and enzyme-assisted (4.1%) extractions Good extraction methods should extract targeted compounds with high degree of specificity and should recycle the solvents [18].

2.3 Antimicrobial activity

From the perspective of wound healing, bacterial infection is one of the major worries in its treatment. Chronic wounds generally occur due to bacterial infection. The clinical studies of colonization of wounds and its associated infections describe the phases of bacterial interaction with a wound. These stages are contamination, colonization, local infection (as a result of high degree of colonization), spreading out of invasive infection, and septicemia. Each of them produces several effects on the wound, making it hard to understand the role of bacteria in disrupting the wound healing process [19].

Bacteria secrete extracellular polysaccharide matrices forming biofilms that provide protection to them by different ways such as increasing infection rates, substrate accessibility, metabolic efficiency and resilience towards environmental stress. These biofilms also help to inhibit various antibacterial agents. Chronic wounds and retardation in wound healing are mostly marked by prolonged inflammation, erroneous epithelialization and inadequate matrix remodeling. Thus measures need to

be taken to inhibit the growth of harmful microorganisms to eliminate all the possible hurdles that bacteria may cause in the healing process [20] [21].

The antibacterial report on aqueous extract of turmeric exhibited minimum inhibitory concentration (MIC) values ranging from 4 to 16 mg/ml and minimum bactericidal concentration (MBC) values between 16 to 32 mg/ml against S. epidermis ATCC 12228, S. aureus ATCC 25923, Klebsiellapneumoniae ATCC 10031, and E. coli ATCC 25922 [23]. On the other hand, its methanol extract reported lower MIC values of 16 μg/ml and 128 μg/ml against Bacillus subtilis and S. aureus respectively [22]. Addition of 0.3% (w/v) of aqueous turmeric extract to cheese caused a fall in bacterial counts of Salmonella typhimurium, Pseudomonas aeruginosa, and E. coli 0157:H7. Moreover, Staph. aureus, B. cereus, and Listeria monocytogenes contamination was decreased even after 14 days of cold storage [24].

The stability and assembly of FtsZ protofilaments as a crucial factor for bacterial cytokinesis are introduced as a possible drug target for antibacterial agents. Curcumin suppressed the B. subtilis
cytokinesis through elicitation of filaments. It also causes noticeable suppression of the formation of cytokinetic Z-ring in B. subtilis without disassembling the segregation and organization of the nucleoids [25]. It is established that one of the probable antibacterial mechanisms of curcumin to suppress the bacterial cell proliferation is by inhibiting the assembly dynamics of FtsZ in the Z-ring [25]. Stamping down FtsZ polymerization could inhibit the FtsZ assembly which leads to the disruption of prokaryotic cell division [26]. Moreover, Curcumin also has a suppressing effect on NF-κB activation and hence inhibits the release of IL-8 and cell scattering causing a step-down in the inflammation of gastric tissue. It also inhibits the IκBα degradation, the activity of NF-κB DNA-binding and IκB kinase α and (IKK α and β) [27]. The synergism of the combination of curcuminoids and ampicillin showed marked reduction in the MIC of ampicillin against either clinical or S. aureus ATCC 25923 strain [28]. Curcumin also portrayed a synergistic effect when combined

with some other antibiotics such as oxacillin, ampicillin and norfloxacin against methicillin-resistant S. aureusstrain (MRSA) [29] [30].

2.4 Antioxidant property:

Reactive oxygen species (ROS) including superoxide(O_2^-), hydrogen peroxide (H_2O_2), hydroxyl (OH^-), nitric oxide (NO) are responsible for oxidative stress in the cells of human body. Due to them each cell encounter about 10,000 oxidative hits per second. When the production of ROS is more than the antioxidant defense of the cells, free radicals start attacking cell proteins, lipids and carbohydrates causing the growth of degenerative diseases [31].

Majority of the antioxidants manufactured cause unsuitable side effects when consumed in vivo. So a trend to find naturally occurring non-toxic antioxidants to supplement processed food (for value addition and extension of the shelf life) or pharmaceuticals is becoming popular. It is a common belief that “green medicine” guarantees safety and effectiveness without any adverse side effects and thus can replace synthetic antioxidants that are being limited due to their cost and toxic nature [32] [33].

The antioxidant effect of curcumin is determined by the keto-enol tautomerization depicted in figure 2.3. Delocalization of the carbonyl oxygen’s electrons makes the enol group to function as a hydrogen radical donor and thus actively participates in the process of scavenging the free radicals. This property highlights curcumin as an oxidative-stress reducing agent including lipid peroxidation in diseases like Parkinson, Alzheimer and HIV as some of the examples [34].

In Alzheimer disease, a chronic inflammation occurs because of activated macrophages and an increased amount of cell signaling proteins. Free radicals are released Due to inflammation there is a release of free radicals which causes cell damage and cell death. Studies have shown that non-steroidal anti-inflammatory drugs (NSAID) decrease the risk of Alzheimer but long-term use of them will intoxicate renal function including liver and gastrointestinal area. Curcumin can be an

excellent alternative to NSAIDs because of its high anti-oxidant activity, scavenging free radicals without intoxicating the organs [35].

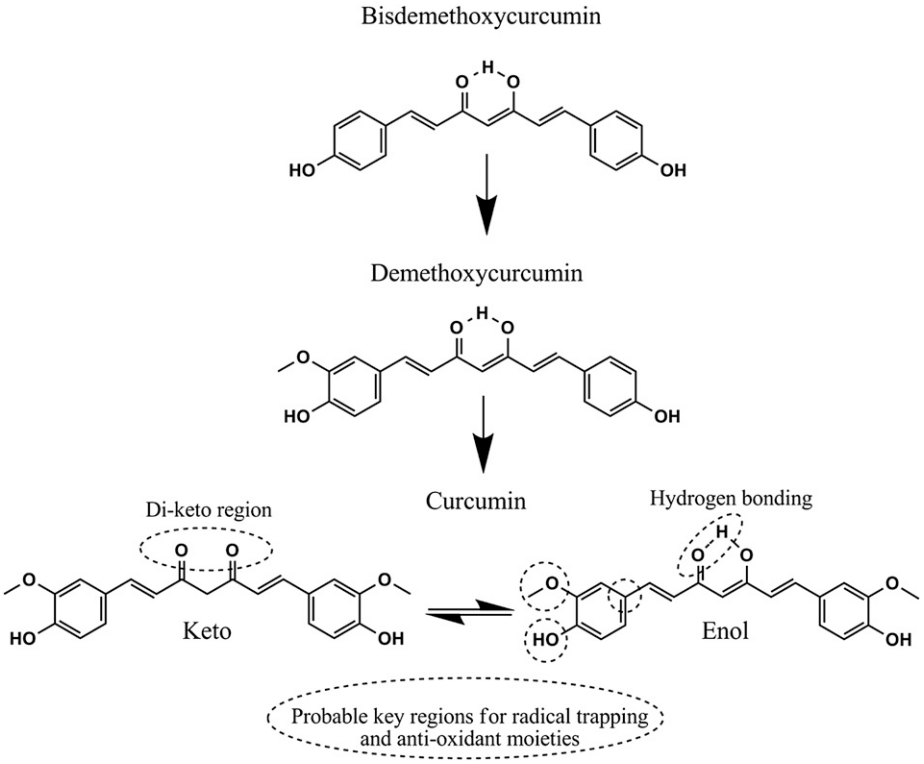

Figure 2.3: Crucial sites of curcumin involved for its antioxidant property and the transformation from bisdemethoxycurcumin to curcumin [36]

Solvent extraction is preferred over steam and soxhlet extraction for the extraction of turmeric because sit does not require heat. Application of heat can damage the antioxidant activity. Curcumin extracted at 50 ^{0} C showed the highest antioxidant activity with 24.968 as its IC50 value while the lowest activity was observed for the curcumin extracted at 70 ^{0} C having anIC50 value of 111.93 [37].

2.5 Nanoparticle and its synthesis

Silver is claimed as an antibacterial agent. Traditionally it is used as one of the most commonly used antibacterial substances before the discovery of antibiotics. Silver was used in sutures to inhibit postoperative inflammation and infections. Nanoparticles has been reported to enhance the antibacterial properties of silver. The increased surface area due to nano-structures induces an increased rate of interaction

between the test subjects and the ionic silver which in turn lowers the minimum inhibitory concentration. This causes incorporation of lower concentration of the material into a healing systems to experience the same degree of effect [19].

The antimicrobial effect of silver is due to blockage of respiratory enzyme pathways, alteration of microbial DNA and the cell wall [39]. The therapeutic efficacy of silver nanoparticles is much greater than conventional silver compounds. The nanoparticles exert their antimicrobial property by interacting with the sulphur-containing proteins present in bacterial cell membrane as well as with phosphorous containing DNA [40]. In addition, silver nanoparticles based antimicrobials have many advantages due to their thermal stability, health and environmental safety [41]. For developing silver nano-products, chemical reduction method can be used. However, using chemicals such as hydrazine hydrate, dimethyl formamide, ethylene glycol, etc. in the reduction process causes toxicity or biological hazards **[38].**

2.6 Wound healing

Wound healing is a complex process necessitating a combination of actions of various tissues and cell lineages and has been thoroughly researched since ages. This controlled process is characterized by four discrete but overlapping stages: hemostasis, inflammation, proliferation and remodeling. Keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets cells are important cells in wound repairing process [42] [43]. Hemostasis is defined as the release of blood components at the place of injury, permitting the platelets to make contact with the exposed collagen and other components of the extracellular matrix. This in turn triggers the discharge of clotting factors, essential growth factors and cytokines such as platelet-derived growth factor (PDGF) and transforming growth factor (TGF-ß) from the platelets. During hemostasis, platelets attach to the injury site and aggregate. It is then followed by the activation of coagulation cascade which ensues the establishment of platelet clots in a protein mesh composed of cross-linked fibrins. During inflammation, neutrophils enter the injured area and start phagocytosis to remove undesired substances including bacteria and unhealthy tissue. Macrophages also help in the

phagocytosis process by further releasing PDGF and TGFß during inflammation. The sheer number of growth factors and cytokines involved in the overall wound healing task, including fibroblast growth factor, vascular endothelial growth factor, granulocyte-macrophage colony-stimulating factor, platelet-derived growth factor, connective tissue growth factor, interleukin, and tumor necrosis factor-alpha, indicates the complexity and fragility of the process [44]. The cytokines and growth factors that are being released during the initial phases (hemostasis and inflammation phase) play as chemical signals capable of controlling matrix production, cell migration, differentiation, and enzyme expression resulting in the increase of new blood supplies and connective tissue cells at the damaged area. Once the inflammation phase is over, fibroblasts migrate to the injury site and begin the proliferative phase with the liberation of fresh extracellular matrices. These matrices are then cross-linked with each other and reorganized during the remodeling stage.

An ideal wound dressing material should have the capability to maintain moisture, act against microorganisms, be nontoxic in nature and non-adherent. Modern wound dressing theory encourages to maintain a dynamic equilibrium between exudate absorption and optimal moisture at the wound surface. Also it should be able to permeate gases so that the wound gets enough oxygen tension [45] [46].

2.7 Polymer encapsulation

Low bioavailability, poor aqueous stability, low permeation capabilities and lack of appropriate delivery systems limits the chemotherapeutic effect of curcumin. Due to loss, inactivation and/or degradation during transport from site of administration to target site, phytoceuticals are required in large doses to achieve any therapeutic effects. This frequently results in selective or functional toxicological complications [47]. Moreover, plants extracted with non-aqueous/non-polar solvent are generally insoluble or sparingly soluble in water [48]. When stored or delivered by surfactants like dimethylsulphoxide and other solubilizing agents such as alcohol, the concentration and potency of these phytoceuticals in solution decreases to a great extent due to their poor stability [49].

An acceptable oral administration of such phytoceuticals has been a concern because of inactivation and degradation by acids and degrading enzymes in the gastrointestinal tract [50]. Functional carbohydrate polymers for e.g. starch has been used extensively as pharmaceutical excipients for ages. Chitosan is also a better alternative for controlled release and targeted delivery of drugs, proteins and genes because of its impressive physical, chemical and biological properties making it a technologically exceptional biopolymer in oral to gastrointestinal targeted delivery [50]. Development of oral drug delivery carriers for targeted, controlled and sustained release is often driven by the bioavailability, biocompatibility and biodegradability of the carriers. Chitosan carriers are often leaky to encapsulated drug as it dissolves easily in acidic conditions. Polyelectrolyte complexation of chitosan with other biodegradable polymers such as alginate or synthetic polyethyleneglycol, can shield the carrier from degrading enzymes and acids reducing the leakages of entrapped drug and thereby increasing the retention effect of the carriers [51].

Polyvinyl alcohol(PVA) is a water soluble synthetic polymer. It has less toxicity and possess excellent wound dressing properties like high tensile strength and flexibility. Polyvinyl alcohol has excellent film forming, emulsifying and adhesive properties. It is also resistant to oil, grease and solvents and has high oxygen and aroma barrier properties [52] [53]. It can also be used as a stabilizer to nanoparticles.

Chitosan is a natural polymer composed of poly(β-(1-4)-2-amino-2-deoxy-D-glucose). It is one of the structural polysaccharide which is abundantly found in nature. Chitosan interacts very easily with bacterium and binds to DNA, glycosaminoglycans and most of the proteins thereby enhancing the antimicrobial effect of silver nanoparticles [54].

Gelatin and poly (vinyl alcohol) (PVA) can be cross linked with glutaraldehyde. In the case of gelatin, the glutaraldehyde reacts with each e-NH_2 functional group of adjacent lysine residues, while for PVA, it reacts with two adjacent hydroxyl groups, forming acetal bridges. Thus it can be considered possible to cross link adjacent macromolecules of gelatin and PVA using glutaraldehyde [55].

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Chapter Three

Objectives and proposed plan of work

3.1 Objectives:

Since ages, turmeric has found its use in medicinal fields. Although the goodness of turmeric is claimed traditionally, providing proper scientific data will not only support the statements making it more evident but will also search for possible ways to enhance its potential.

Bioavailability of the bioactive component in turmeric, the curcumin, is very low and causes a hindrance in its therapeutic activities especially in in-vivo models. This can be increased by reducing system systemic elimination. Synthesis of nanocomposites will increase the solubility of curcumin and help in penetration to cells to a greater extent. Also the amount of phytochemicals extracted and its dependence in pharmacological actions is to be studied to make the extraction process less time consuming and more cost efficient.

In-vitro evaluation of biological activities of turmeric is to be done which includes its potential against breast cancer cell line MCF-7. Turmeric is more popularly used in topical applications rather than oral administration. After initial screening of turmeric extract, preparation of ointment and polymer encapsulation will provide a platform for its topical use. Being reported as a wound healing agent, a proper vehicle of administration of bioactive components will help to exploit its wound healing property in an optimized way. If used as an anticancer or wound healing agent, it will eliminate the use of synthetic drugs that generally toxic and have adverse side effects on health.

3.2 Proposed plan of work:

- The work was initiated by extracting bioactive components from turmeric rhizome by solvent extraction and preparing samples of crude extract to carry out assays used in this work.
- At first, quantitative phytochemical analysis was carried out to establish the best solvent for its extraction process.
- Preparation of nano-composite using turmeric extract and silver nitrate
- Comparative analysis of antimicrobial effect between different solvent extracted samples and the synthesized nano-composite.
- Evaluation of antioxidant activity of crude extracts by DPPH assay.
- Cytotoxicity assessment by MTT assay on PBMC (which was separated from whole blood) and MCF-7 (a breast cancer cell line).
- Ointment preparation using extract, its characterization.
- Implication of in-vitro and in-vivo methods to study the wound healing potential of the turmeric ointment prepared.
- Polymer encapsulation of the extract to achieve sustained release of phytoceuticals and enhanced wound dressing applications.

Chapter Four

MATERIALS AND METHODS

4] METHODOLOGIES USED AND ITS CORRESPONDING MATERIALS REQUIRED

4.1 Preprocessing of turmeric

Material required: Raw turmeric

Before the extraction process, raw turmeric was washed with water, peeled, cut into small pieces and was divided into two parts- one sundried for two weeks (part A) and the other was kept in the incubator at 37 °C for two days (part B) to reduce the moisture content in the turmeric to a negligible amount. Then it was crushed into coarse powder form using mortar and pestle.

4.2. Extraction of the bioactive components

Materials required: Methanol, benzene, acetone, 2-propanol, ethanol, and acetonitrile.

30 grams of powdered turmeric from part A and part B was macerated with 200 ml of methanol for 3 days allowing enough time for the bioactive components to solubilize in the solvent. This was followed by digestion. Digestion was performed by adding fresh 10 grams of powder in the solution with gentle heating to increase the concentration of the bioactive components in the solution and is left for another one day. Similar process is followed for the turmeric powder from part B with solvents namely benzene, acetone, 2-propanol, ethanol, and acetonitrile.

4.3 Sample preparation

Material required: Whatman No.1filter paper

Instruments required: Rotary evaporator ((Manufacturer:IKA), lyophilizer (Manufacturer: Biobas)

The crude extracts were filtered using Whatman No.1filter paper and concentrated using rotary evaporator. Then the highly concentrated solutions were lyophilized to achieve the final prepared samples which were stored at -20 °C for further use. Samples (i.e. the final product after lyophilization) were named from A to F depending on the solvent used in the extraction process where A, B, C, D, E, F corresponds to extraction by benzene, acetone, 2-propanol, ethanol, acetonitrile and methanol respectively.

4.4. Phytochemical analysis

4.4.1 Total phenolic content (TPC)

Materials required: FolinCiocalteu reagent (FCR), gallic acid, sodium carbonate, distilled water

Instrument required: UV-visible Spectrophotometer (Agilent Technologies, Cary 60)

TPC of each lyophilized samples were calculated from the calibrated standard curve using gallic acid by spectrophotometric analysis using FolinCiocalteu reagent (FCR). In this process, 0.5 ml of extracts (of concentration 1000 µg/ml) or gallic acid (of different concentrations from 0-500 µg/ml) were mixed with 3 ml of distilled water. Then 0.25 ml of FCR was added followed by 0.75 ml of saturated aqueous sodium carbonate solution. Lastly, 1 ml of distilled water was added and the mixture was incubated for 30 minutes in 37 °C. Absorbance was taken at 765 nm against a selected blank using UV-visible Spectrophotometer [1]. A standard curve of gallic acid was prepared at-first as reflected in Figure 1. Phenol content of different samples was calculated in gallic acid equivalent.

4.4.2 Total flavonoid content (TFC)

Materials required: sodium nitrite, aluminum chloride, sodium hydroxide, quercetin

Instrument required: UV-visible Spectrophotometer (Agilent Technologies, Cary 60)

TFC of each extracted samples are calculated from the calibrated standard curve using quercetin by spectrophotometric analysis. In this process, 0.5 ml of extracts (of concentration1000 µg/ml) or quercetin (of different concentrations from 0-500 µg/ml) were mixed with 0.5 ml of distilled water. Then 0.3 ml of 5% sodium nitrite was added and kept at room temperature for 5 minutes after which 0.3 ml of 10 % aqueous solution of aluminum chloride was added. 5 minutes later 2 ml 1 molar sodium hydroxide solution was added and test tubes were shaken before taking the absorbance at 510 nm against a selected blank using UV-visible Spectrophotometer.[2]

A standard curve of quercetin is prepared at-first as reflected in Figure 2. Flavonoid content of different samples was calculated in quercetin equivalent.

4.5. Nanocomposite synthesis

Materials required: Dimethyl sulfoxide (DMSO), silver nitrate

Instrument required: Magnetic stirrer (Manufacturer: REMI equipment) , centrifuge machine (Manufacturer: REMI equipments), Lyophilizer (Manufacturer: Biobas), FTIR spectrometer,

Field Emission Scanning Electron Microscope (Model: INSPECT F50)

As sample F has highest phenol and flavonoid content we choose it for further study. 100 mg of sample F was dissolved in 20 ml of dimethyl sulfoxide and added to an aqueous solution of silver nitrate (0.1 % w/v) dropwise under constant stirring condition. Stirring was continued for 6 hours resulting in the formation of a clear brown colored solution. Solution was centrifuged at 6000 rpm for 20 minutes. Supernatant was discarded and the pellet so formed was washed with distilled water before collecting it in a petri plate for freeze drying. The nanocomposite (sample G) synthesized using the turmeric extract was characterized by FTIR and FESEM.[3]

4.6. Antibacterial assay

Materials required: Nutrient broth, Agar agar, streptomycin, dimethyl sulfoxide (DMSO)

Test Microorganisms: Standard strains of E. coli and S. aureus

To check the potency of turmeric extracts against selected bacterial strains, 50 mg of each of the lyophilized samples and 20 mg of the synthesized nanocomposite were dissolved in 1 ml of DMSO, DMSO being the standard solvent for each samples. Both well-diffusion and disc-diffusion methods were used to determine the antibacterial activity against E. coli and S. aureus. DMSO was used as the control and streptomycin as the standard. Plates are incubated for 24 hours and the zone of inhibition was measured. Then, the mean value of two different methods was taken into account.

4.7 Antioxidant activity – by DPPH assay

Materials required: 2,2-diphenyl-1-picrylhydrazyl (DPPH), methanol

Instrument required: UV-visible Spectrophotometer (Agilent Technologies, Cary 60)

Stock solution of ethanolic extract (i.e. sample D) was prepared at 0.2 mg/ml concentration in 1% DMSO. Then it was serially diluted into four concentrations: 100 µg/ml, 50 µg/ml, 25 µg/ml and 12.5 µg/ml. A solution of 0.003 % DPPH in methanol was prepared. 20 µl of test solutions were added to 3ml of DPPH solution and shaken vigorously. The mixture was kept at room temperature in the dark and allowed to stand for 30 minutes. Absorbance was measured at 515 nm using UV spectrophotometer since DPPH shows maximum absorbance value at 515 nm. Standard antioxidant used was citric acid and water as the control. The potentialilty to scavenge the DPPH radical was determined using the following equation [4] [5].

$$\text{DPPH Scavenged (\%)} = ((A_{control} - A_{sample})/A_{control}) \times 100$$

where, A_x is absorbance of x at t= 30 min. All the tests were performed in triplicates and a calibration curve of % DPPH scavenged versus concentration was plotted. Values were presented as mean ± S.D.

4.8 Separation of PBMC:

Materials required: Whole blood, saline solution, HiSep LSM 1077, RPMI 1640, Fetal Bovine Serum (FBS), Penicillin-Streptomycin

Fresh blood was collected in EDTA vials and normal 0.9 % saline solution was used for 1:3 dilution of whole blood. 3 ml of HiSep LSM 1077 (a density gradient medium) is taken in a falcon tube and 12 ml of diluted blood was layered carefully over it. The tube was centrifuged at 2000 rpm for 15 minutes. This causes 4 layers to form. The topmost layer is of plasma thrombocytes and is followed by the white layer of PBMCs. This layer was taken carefully using a pipette without any RBCs or thrombocytes. It was washed 3 times using RPMI 1640 media and suspended in the same media with 10 % FBS and 2 % penicillin-streptomycin.

4.9 Cytotoxicity test – MTT assay

Materials required: RPMI 1640, DMEM, FBS, penicillin-streptomycin, MTT, PBS

PBMC was cultured in RPMI 1640 whereas MCF-7 was cultured in DMEM. Cells were added on 96 well plates. After 24 hours, cells were treated with different

concentrations of turmeric extract in 1 % DMSO and cisplatin. Cisplatin is used as the standard anti-cancer drug against MCF-7 and concentration used was 50 µg/ml. Concentrations of turmeric extract 50, 100, 200 and 400 µg/ml were named as $T.E_1$, $T.E_2$, $T.E_3$, and $T.E_4$ respectively. Then the plates were incubated at 37 ^{0}C and 5 % CO_2 for 24 hours. After that, 10 µl of MTT of concentration 5 mg/ml in PBS was added to each well and incubated for another 4 hours. Then MTT along with the media was removed carefully and 100 µl of DMSO per well was added to dissolve the formed formazan. The absorbance was taken at 590 nm and % viability was calculated [6].

$$\% \text{ viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

4.10 Ointment preparation:

Materials required: Beeswax (Loba Chemie Pvt. Ltd., CAS number: 8012-89-3), liquid paraffin [heavy] (Merck), propylene glycol (Loba Chemie Pvt. Ltd., CAS number: 57-55-6)

The beeswax was melted and the ointment base i.e. the vehicle was prepared consisting of beeswax, liquid paraffin (heavy), propylene glycol in the ratio of 1:3:2. Turmeric ointment was prepared by incorporation turmeric extract in the ointment base in 1% concentration.

0.1 gm of each ointment was dispersed in 10 ml of distilled water and the pH was recorded. Homogeneity of test ointments were tested by visually inspecting them and formation of lumps was checked.

4.11 Skin irritation test:

6 animals were divided into 2 groups A and B with 3 animals in each group. Dorsal hair was removed and on one side of the depilated back ointment was applied while the other side was kept blank serving as the control. Group A received ointment base and in group B turmeric ointment was given. Application of ointments was done once daily for 5 days. Dermal toxicity was checked by observing any occurrence of erythema or edema at the site of application of ointments.

4.12 Wound healing activity:

4.12.1 Experimental animals: Healthy Swiss Albino female mice weighing between 18-22 grams were used here. Acclimatization of animals to laboratory conditions was done for 2 days before starting the experiment. The mice were housed in polypropylene cage and wielded in standard laboratory conditions of temperature 20-24 ^{o}C and 12 hours of light-dark cycle. Standard diet was provided with water ad-libitum throughout the experiment.

4.12.2 Wound creation and treatment protocol:

Materials required: Ethanol, diethyl ether, formaldehyde, Nebasulf

Instruments required: Surgical blade No. 10, Suture No. 1/0, forceps, scissors

The dorsal fur of mice was removed and the area to be wounded is cleaned with 70% ethanol. The animals were divided into 2 groups A and B corresponding to incision and excision model respectively. Each group comprises of 4 subgroups I, II, III and IV with 3 mice in each of them. Group I was challenged with no treatment given. Group II was the control group in which standard drug nebasulf (Bacitracin +Neomycin +Sulphacetamide sodium) was given. Group III and IV were treated with ointment base and turmeric ointment respectively. Prior to the wounding process, animals were anesthetized using anesthetic ether i.e. diethyl ether. Treatment was done daily and continued for 7 days.

4.12.2.1 Incision wound model: A paravertebral incisions of 1 cm long was made using surgical blade No. 10 through the entire thickness of skin on the depilated back of mice. After the incision, interrupted surgical sutures No 1/0 were applied to the parted skin at an interval of 0.5 centimeter. On 5th day sutures were removed and on 6th day the tensile strength of cured skin at the site of wound was measured.

Measurement of tensile strength:

Under ether anesthesia two Allis forceps were fixed to the skin 0.5 mm away from the incision on each side of the injury. One forceps was held fixed at a position while the other forceps was freely hanged to which a container was attached. Water was added slowly to the container until the wound started to open and the amount of water needed to do that was noted down. Total weight required (i.e. weight of forceps + container + water) gave an indirect measure of the tensile strength of wounds from which % breaking strength of a group with respect to control group (i.e. Group II) was calculated [7].

$$\% \text{ breaking strength} = \frac{\text{Wound tensile strength of a group}}{\text{Wound tensile strength of control group (i.e. Group II)}} \times 100$$

4.12.2.2 Excision wound model: Using forceps and pointed scissors circular excision wounds of about 8 mm radius were created to full thickness of the sink under aseptic conditions. The wound area was marked immediately on a transparent polythene and the area was calculated. Observations were taken on the subsequent post wounding days and percentage of wound closure was calculated.[8]

$$\% \text{ Wound contraction} = \frac{(\text{Initial wound size} - \text{Specific day wound size})}{\text{Initial wound size}} \times 100\%$$

Measurements were done in triplicates and values are presented as mean ± S.D.

After 7 days, wound areas were excised and fixed in 10 % formalin. Histopathological examination with H and E staining and biochemical parameters estimation were carried out by using the tissue specimens isolated from the healed skin of each groups of mice to determine the healing property of turmeric extract.

4.12.3 Biochemical parameters estimation:

4.12.3.1 Tissue sample preparation:

Materials required: Acetone, HCl, NaOH

Around 250 mg of excised wet granulation tissue was defatted using acetone for 4 days. Acetone was changed two times in a day. After defatting it was dried at 50 ^{0}C for 24 hours. 40 mg of the dried granulation tissue was kept in glass stopper test tubes. To each test tube 1000 µl of 6 N HCl was added and kept in boiling water bath for 24 hours in two days (12 h each day) for hydrolysis. The hydrolysate was cooled to room temperature. Excess of acid was neutralized by 10 N NaOH using phenolphthalein as the indicator. Neutral hydrolysate was diluted to a concentration of 20 mg/ml with distilled water. The final hydrolysate was used for the estimation of hydroxyproline, hexosamine and hexuronic acid following the standard curve prepared using a proper substrate for each of them. [9]

4.12.3.2 Estimation of hydroxyproline:

Materials required: $CuSO_4$, H_2O_2, H_2SO_4, L-hydroxyproline (Loba Chemie Pvt. Ltd., CAS number: 51-35-4)

L-hydroxyproline was used as a standard substrate for to determine the hydroxyproline content in the tissues. 300 µl of different samples i.e. tissue hydrolysates or standard solutions of L-hydroxyproline were mixed with 1ml of 0.01M $CuSO_4$. This was followed by the addition of 1ml of 2.5 N NaOH and 1ml of 6% H_2O_2. The solutions were mixed and shaken frequently for 5 minutes. Then test tubes were incubated at 80 ^{0}C for 10min with frequent vigorous shaking. After cooling the solutions, 1.2 ml of 3 N H_2SO_4 was added with agitation. Finally, 2 ml of Ehrlich's reagent was added and the samples were incubated at 75 ^{0}C for 15 minutes. Test tubes were then cooled by placing them in running stream of water. Absorbance was measured at 540 nm against the blank. Standard curve was prepared with L-hydroxyproline as the substrate and the amount of hydroxyproline in the samples was calculated using it. [9] Data is expressed as mean ± S.D.

4.12.3.3 Estimation of hexosamine:

Materials required: Sodium nitrite ($NaNO_2$), acetic acid, ammonium sulfamate, HCl, indole (Loba Chemie Pvt. Ltd., CAS number: 120-72-9), ethanol, Glucosamine hydrochloride or 2-amino-2 deoxy-D- glucose, chitosmine (Loba Chemie Pvt. Ltd., CAS number: 66-84-2)

Glucosamine hydrochloride was used as a standard substrate to determine the hexosamine content in the tissues. The acid hydrolysates of different tissue samples or standard solutions of Glucosamine hydrochloride is subjected to deamination of hexosamine for determining the amount of hexosamine. In order to do deamination, 0.5ml of 5% sodium nitrite solution and 0.5ml of 33% of acetic acid solution was added to 0.5ml of test samples. The test tubes were shaken and allowed to stand for 10 minutes to complete the deamination process. The excess of nitrous acid was removed by adding 0.5ml of 12.5% ammonium sulfamate solution. The mixture was shaken frequently for 30 minutes. This was followed by indole reaction in which 2ml of 5% HCl and 0.2ml of 1% solution of indole in ethanol was added to 2ml of the sample solutions. The tubes were immersed for 5 minutes in a boiling water bath until an intense orange color is seen with slight turbidity. Turbidity was removed by adding 1 ml of ethanol followed by shaking. The absorbance of the solutions was determined by using UV visible spectrophotometer at 492 and 520 nm. The increase in the difference in absorbance values at 492 and 520 nm was considered as the measure of the amount of hexosamine present in the solutions. The hexosamine content in each tissue hydrolysate was calculated in µg from the calibrated standard curve of Glucosamine hydrochloride. [9] Data is expressed as mean ± S.D.

4.12.3.4 Estimation of hexuronic acid:

Materials required: Borax, sulfuric acid, carbazole (Loba Chemie Pvt. Ltd., CAS number: 86-74-8), D-Glucurono-6,3-lactone (Tokyo Chemical Industry Ltd., CAS number: 32449-92-6)

D-Glucurono-6, 3-lactone was used as a standard substrate to determine the hexuronic acid content in the tissues. 2.5ml of 0.025 M Borax in concentrated sulfuric acid was added in stoppered test tubes and cooled to 4 ^{0}C. 0.2ml of the different tissue hydrolysates or standard solutions of D-Glucurono-6, 3-lactone was diluted with 0.5ml of distilled water. These 0.5ml of diluted solutions were layered carefully on Borax-sulfuric acid mixture. After closing the test tubes they were shaken slowly and then vigorously, while maintaining the temperature of 4 ^{0}C. Then the test tubes were kept in a boiling water bath for 10 minutes followed by cooling to room temperature. Afterwards, 0.1ml of 0.1% carbazole reagent in absolute ethanol was added to each test tube, shaken and heated in a boiling water bath for

15 minutes. Bringing the solutions to room temperature, color intensity was measured at 530 nm against a selected blank. The content of hexuronic acid in the test solutions was determined from the standard curve prepared from D-Glucurono-6, 3-lactone. [9] Data is expressed as mean ± S.D.

4.13 Film formation and its characterization

4.13.1 Polymeric film formation and coating:

Materials required: Gelatin, polyvinyl alcohol (PVA), chitosan, glutaraldehyde, curcumin

Stock solutions of 5 % PVA, 0.5 % chitosan and 5 % gelatin was made. The biopolymer gelatin was hydrated for 30 minutes and then dissolved at 55 ^{0}C (Sobral et al., 2001) by stirring until dissolution. Similarly, PVA was first homogenized with distilled water for 30 minutes and then dissolved at 80 ^{0}C under stirring condition for 5 hours to obtain a homogeneous solution [10]. Chitosan was dissolved in 1 % acetic acid at 60 ^{0}C. 2 % glutaraldehyde solution was prepared which was used as the crosslinking agent.

Blends of PVA plus chitosan and PVA plus gelatin were prepared and cross-linked by mixing equal volumes of two polymeric solutions at varying concentration followed by addition of glutaraldehyde solution. 25 g of glycerol per 100 g of polymers was used as a platicizer. In total 6 blends were formed. They are named from S1 to S6 where:

S1: 0.5 % chitosan + 5 % PVA

S2: 0.5 % chitosan + 2.5 % PVA

S3: 5 % PVA + 5 % gelatin

S4: 5 % PVA + 2.5 % gelatin

S5: 2.5 % PVA + 5 % gelatin

S6: 2.5 % PVA + 2.5 % gelatin

Gauze bandages were pre-treated with water and ethanol and dried at 37 ^{0}C in the incubator prior to the coating process. The blends were transferred into petri plates containing pre-treated gauze bandages and one batch was dried in incubator (subscripted with I, for e.g. $S1_I$) while the other one was lyophilized (subscripted

with L, for e.g. $S1_L$). The thickness of the sample films can be controlled by varying the ratio of dry mass of film forming solution (FFS) to container area. [10] To prepare drug loaded films, extract was mixed with the blends at an amount such that each film contained 50 mg of the extract.

4.13.2 Folding endurance:

The test was performed to find the flexibility of film which is needed to handle the film easy, comfortable and for secured application of film on the wound. Flexibility of the films were determined by repeatedly folding a film at same place till it breaks or folded upto 300 times manually. The number of times a film could be folded at one position without breaking suggests its folding endurance. [11]

4.13.3 Dressing pH:

All the films were separately immersed in saline solution till it reached equilibrium. After removal of films the pH of the normal saline solution was recorded. This is considered as the dressing pH of the film under consideration using digital pH meter. All data were recorded in triplicate and average values were noted. [12]

4.13.4 Microbial penetration:

The ability of the polymeric scaffolds to prevent microbial penetration was investigated by tightly fixing the films on top of test tubes to close the openings. All the tubes contained 5 ml of nutrient broth. In the negative control a test tube was closed tightly with cotton ball while an open test tube serves as the positive control. All the tubes were kept in an open environment for 5 days. The cloudiness of the nutrient broth after the experiment indicated the extent of microbial contamination.[11]

4.13.5 Hemocompatibility test:

This was performed on the basis of the ASTM standard. It determined the extent of hemolysis due to the presence of the sample in blood. Blood was collected in EDTA vials to avoid clotting and diluted with normal saline in a proportion of 4:5. For checking hemolysis, 0.2 ml of diluted blood was added to 9.8 ml of distilled water and incubated at 37 ^{0}C for 60 minutes after which the OD was measured in a spectrophotometer at 545 nm. Distilled water causes large-scale rupture of RBCs

and thus the OD of this solution was considered as positive control (OD (+)). For the negative control, 0.2 ml of diluted blood was further diluted to 10 ml of normal saline and was incubated at 37 ◦C for 60 min. Normal saline solution causes least rupture of RBCs. The OD of this solution was measured in a spectrophotometer at 545 nm and was assigned as OD (-). Similar procedure was followed with all the samples and their respective OD values were noted. The samples having a dimension of 1 cm × 1 cm was taken in falcon tubes containing 9.8 ml of normal saline and incubated at 37 ^{0}C for 30 minutes in order to reach temperature equilibrium. 0.2 ml of diluted blood was then added to the tubes, mixed gently and incubated for another 60 minutes. Finally, solutions were centrifuged at 2000 rpm for 5 minutes and their OD i.e. OD (sample) was recorded. [13]

$$\% \text{ Hemolysis} = \frac{[OD\ (sample) - OD\ (-)]}{[OD\ (+) - OD\ (-)]} \times 100$$

4.13.6 Preparation of simulated wound exudate (SWE):

This fluid simulates wound fluids and provides a platform for various in-vitro assays related to the wounds. It was prepared by dissolving 0.68 g NaCl, 0.22 g KCl 2.5 g of NaHCO3, and 0.35 g of NaH2PO4 in 100 mL of distilled water.[14]

4.13.7 Water absorption capacity:

The membrane samples were cut into 1×1 cm^2 and their weights were noted. The swelling index was determined by immersing the scaffold in SWE at room temperature allowing them to swell and then measuring their weights at an interval of every 1 hour. The readings were taken until an equilibrium state was reached. The surface moisture of scaffold was soaked with filter paper to remove excess water before taking their weights. The swelling index (S.I) or % water absorption capacity was calculated using the formula:

$$S.I = \frac{(W_W \_ W_D)}{W_D} \times 100$$

where W_D = weight of dry membrane i.e. at time t=0, W_W = weight of swollen membrane after immersion at a particular time. [15]

4.13.8 Release study:

Drug loaded samples were cut into 2 × 2 cm^2 size and immersed in 8 ml of SWE. In order to study the release profile from the membranes, OD of SWE was recorded periodically at 425 nm. The change in the value of O.D between two time intervals estimates the release efficiency of the scaffolds.

4.14 References

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Chapter Five

RESULTS AND DISCUSSION

5.1 Phytochemical analysis of turmeric extracts

On the first trial of extraction with methanol, both the samples designated as part A and part B showed almost same results of phytochemical analysis. So turmeric powder from part B was chosen henceforth for the extraction process by different solvents due to its shorter preparation time.

5.1.1 Total phenol content (TPC)

The Folin–Ciocalteu reagent (FCR) is sensitive to polyphenols. It produces a blue color complex on reaction. The principle of F-C assay is based on the transfer of electrons (which act as reducing equivalents) in an alkaline medium from phenols to phosphomolybdic/phosphotungstic acid complexes and this responsible for the formation of blue colored complexes.

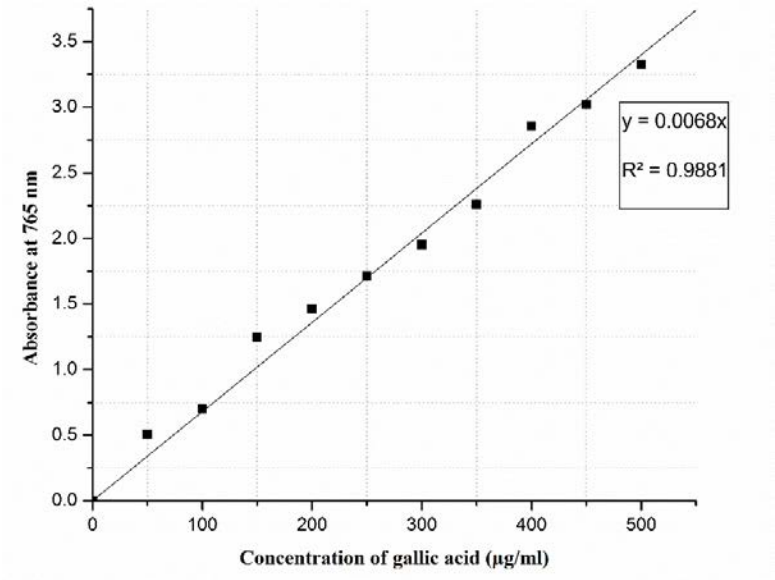


Figure 5.1: Linear curve of Absorbance vs Concentration of standard graph of Gallic acid

Table 5.1: Total phenol content expressed in gallic acid equivalent (mg GAE /g of extract)

Sample	TPC (mg GAE / g)
A	248.79
B	268.64
C	273.12
D	319.18
E	332.91
F	398.68

From Table 5.1 it is observed that methanolic extract showed the highest phenol content while benzene extract has the lowest value among others. Total phenol content varied significantly with the solvent used during the extraction process.

5.1.2 Total flavonoid content (TFC)

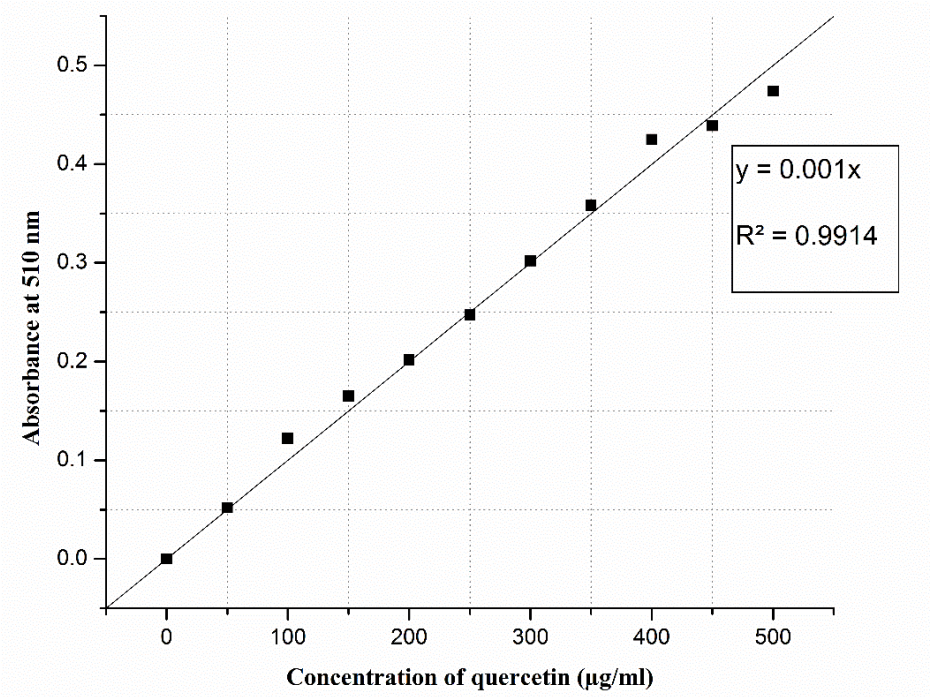


Figure 5.2: Linear curve of Absorbance vs Concentration of standard graph of Quercetin

Table 5.2: Total flavonoid content expressed in quercetin equivalent (mg QE / g of extract)

Sample	TFC (mg QE / g)
A	164.00
B	302.00
C	174.00
D	210.00
E	124.00
F	296.00

From Table 5.2 it is observed that Acetone extract has the highest flavonoid content followed by methanol whereas acetonitrile extract witnessed the least value. Again, the solvent used in the extraction greatly influenced the flavonoid content.

5.2 Characterization of the synthesized nanocomposite

5.2.1 Solubility

Decrease in the particle size of active ingredients to a nano level has established some betterment in its efficacy, solubility, and bioavailability. Stabilizers or surfactants to nano-composite were not used. The suspension of the synthesized nanocomposite in water formed a very fine dispersion and seemed to be soluble unlike the turmeric extract which is practically insoluble in water. The difference in the solubility is portrayed in figure 5.3 given below.

A B

Figure 5.3: Aqueous suspension of extract (A) and its nano-composite (B)

Undissolved clumps of the extract are distinctly visible in the suspension while the nano-composite dissolved with no noticeable precipitation.

5.2.2 FESEM analysis

The morphology of the nanocomposite was studied by FESEM as reflected in figure 5.4. Two types of structure were found- spherical and cubical. The nanoparticles were well dispersed and the particle size varied from 20 nm to 400 nm. Agglomeration is not visible.

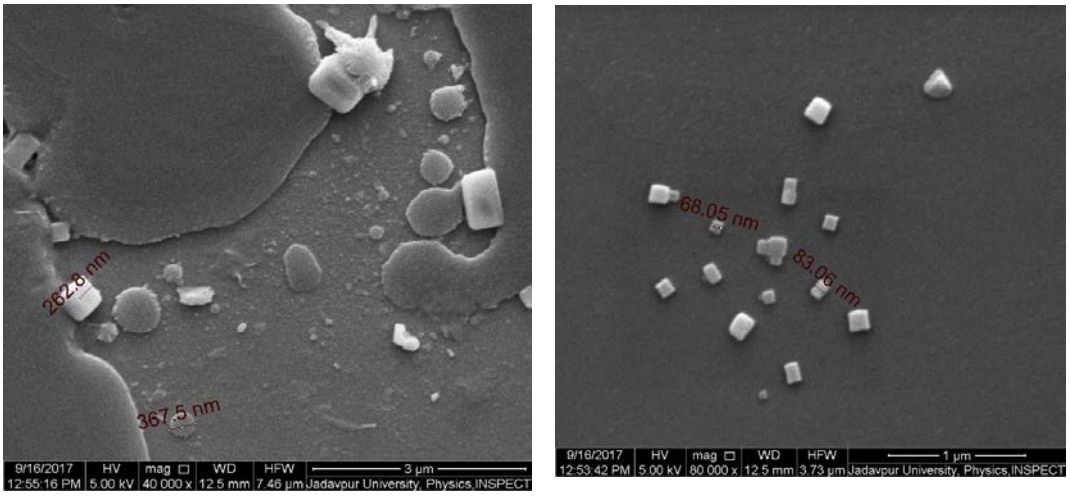


Figure 5.4: FESEM images of the nanocomposites

5.2.3 FTIR analysis of turmeric extract and its nanocomposite

FTIR result of turmeric extract (sample F) and its comparison with the synthesized nanocomposite predicts the presence of curcumin in both the samples. The peak at 3338.78 $cm^{-1}$of turmeric extract corresponds to the alcohol/phenol O-H bond stretching and it gets shifted to 3523.95 in nanocomposite. Similar instances have been found where the peak values (in cm^{-1}) 2929.87, 1412.54, 1276.88, 1207.44, 1101.35, and 987.55 gets shifted to 2926.01, 1436.97, 1278.81, 1128.36 and 952.84 respectively. These peaks correspond to C-H stretch, aromatic C=C, enol C=O, phenol C=O, enol C-O-C and benzoate trans C-H vibration respectively. Shifting indicates the formation of co-ordination bond between silver atoms and electron rich groups present in the extract and thus claims a probability of their involvement in nanocomposite synthesis.

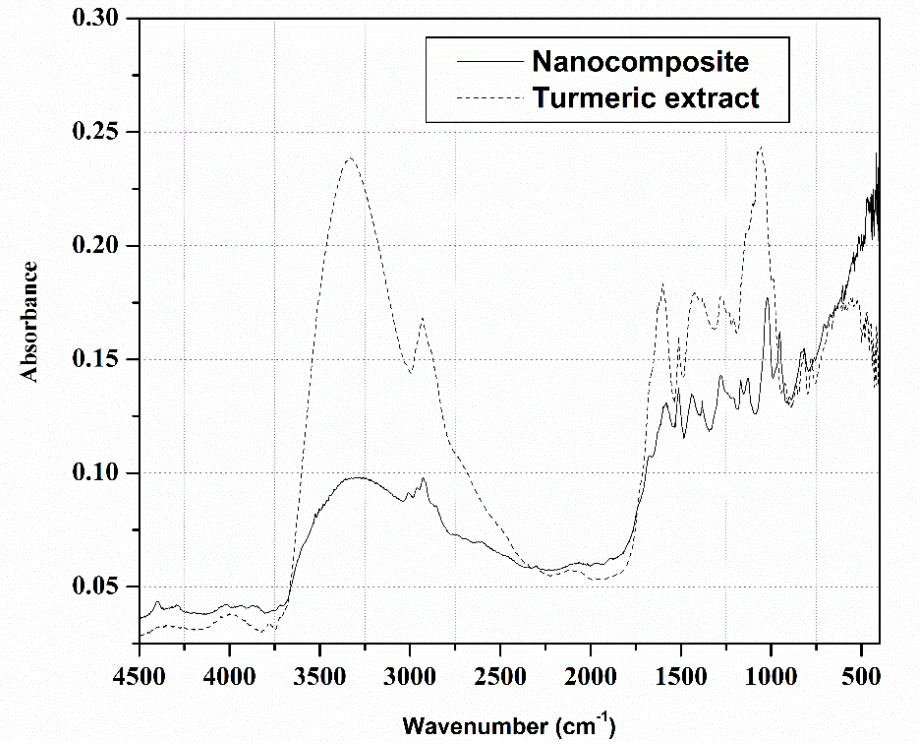


Figure 5.5: FTIR image of turmeric extract and its nanocomposite

5.3 Antibacterial activity

Methanolic extract showed the highest antibacterial activity while benzene extract had the least potency against the selected strains of bacteria. It was observed that the total content of phenols plus flavonoids directly influenced the % inhibition, i.e. more the value of summation of TPC and TFC, more was the zone of inhibition. The synthesized nanocomposite showed high antibacterial activity even at much lower concentration than the extracts, thus claiming to enhance the activity of the bioactive components present in the extracts.

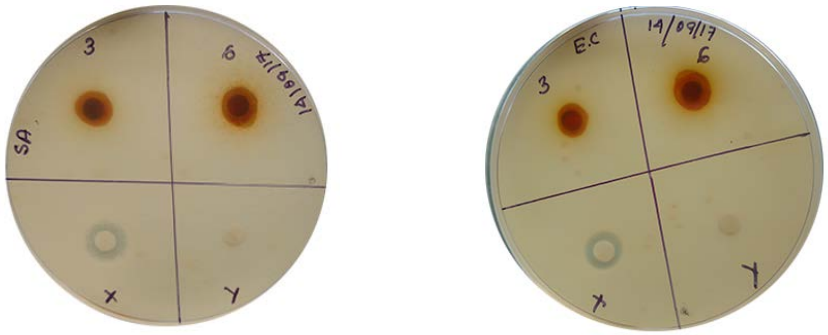


Figure 5.6: Antibacterial activity of turmeric extracts showing zone of inhibition against S. aureus and E. coli respectively

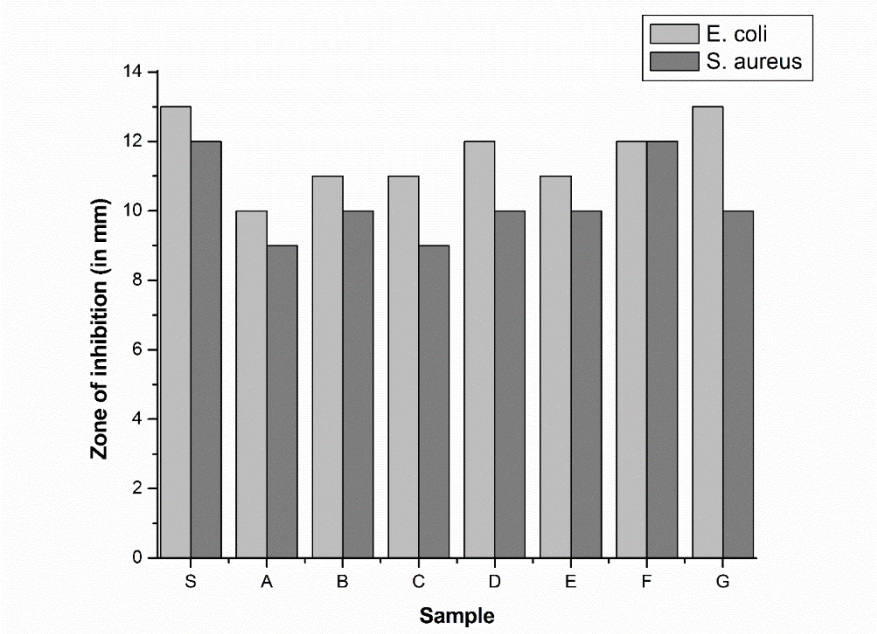


Figure 5.7: Comparative analysis of zone of inhibition of different samples against different strains

5.4 Antioxidant activity – by DPPH assay

Variation in antioxidant activity of turmeric extract with the change in concentration is shown in the graph in figure 5.6 and compared with the chosen standard citric acid. The % free radical scavenging activity for each concentration is calculated and the antiradical dose that cause 50% inhibition i.e. the IC_{50} value is estimated.

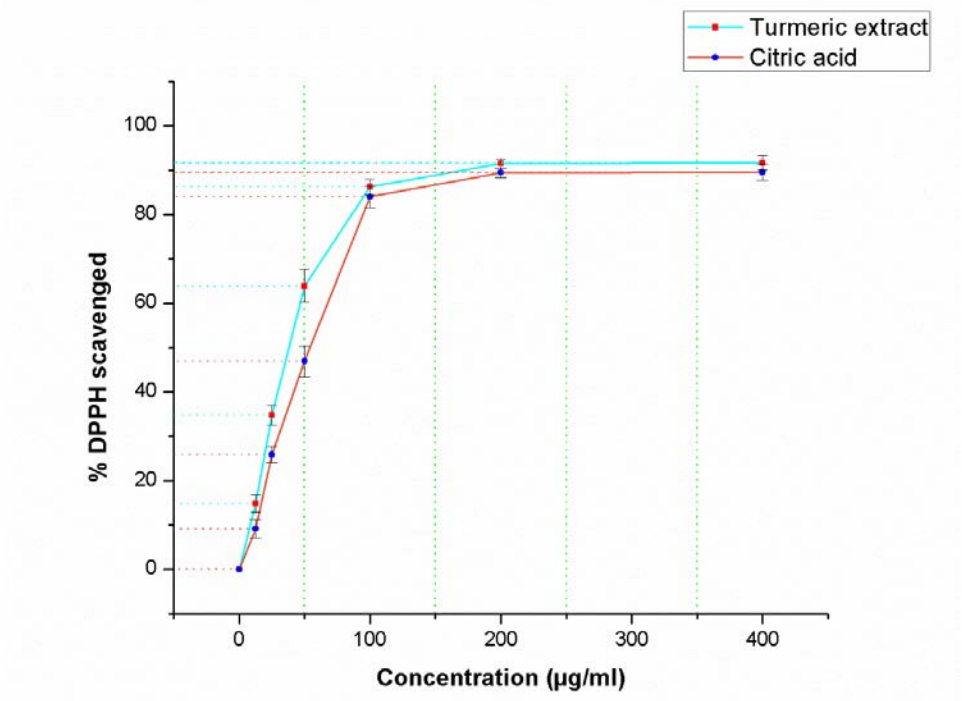


Figure 5.8: Antioxidant activity of turmeric extract and its comparison with citric acid

It was interpolated that the IC_{50} of turmeric extract was 37.09 µg/ml while that of citric acid was 53.51 µg/ml. This IC_{50} claims turmeric to be a good antioxidant capable of inhibiting cellular damages due to reactive oxygen species (ROS).

5.5 Cytotoxicity test - MTT assay:

Turmeric extract seemed to increase the proliferation of PBMCs whereas selectively inhibiting the growth of MCF-7. IC50 value on MCF-7 was 96 µg/ml. This justifies the anticancer activity of turmeric without harming normal body cells.

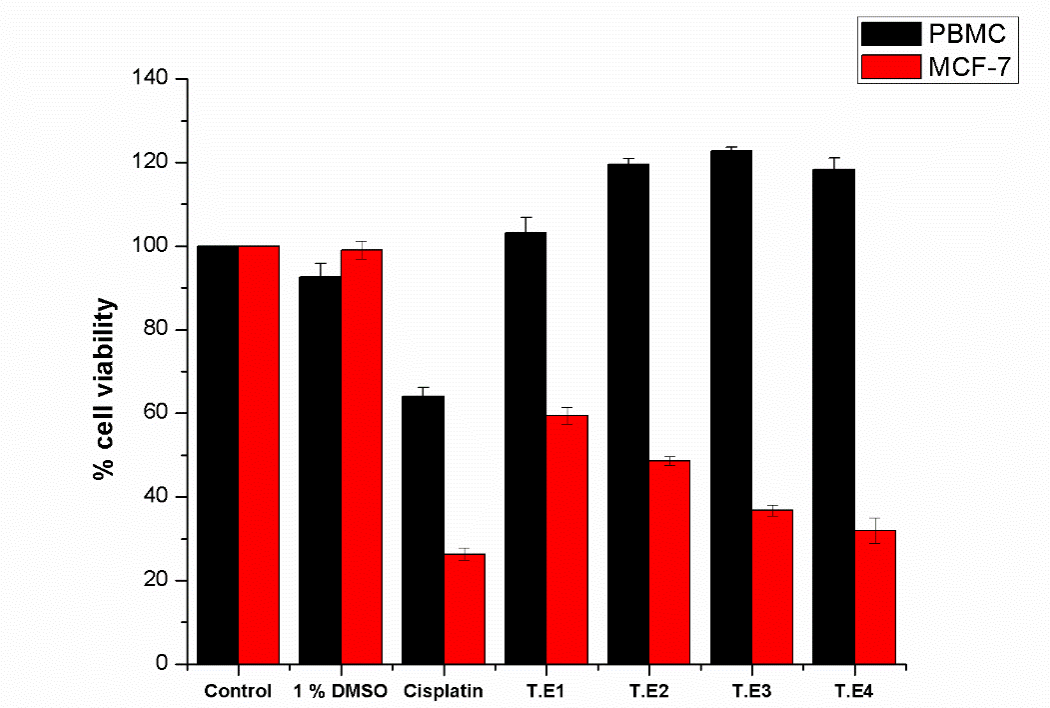


Figure 5.9: Comparative study of dose-dependent effect of turmeric extract on PBMC and MCF-7

5.6 Evaluation of Ointments:

pH of the ointment base was noted as 6.84 while that of the turmeric ointment was 6.96. Both the test ointments showed high degree of homogeneity with the absence of any lumps.

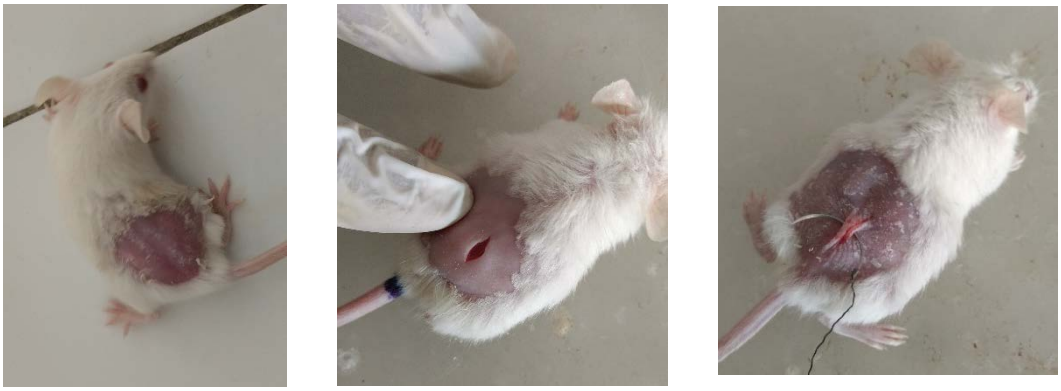
5.7 Skin irritation test:

Both the groups A and B showed absence of any irritation or inflammation on the entire dorsal region indicating their nontoxic nature on topical application.

5.8 Incision wound model:

On 6th day, animals were observed and noted that those treated with turmeric ointment showed excellent healing of wounds compared to the other treated groups whereas group I (the untreated group) showed decreased rate in their healing process.

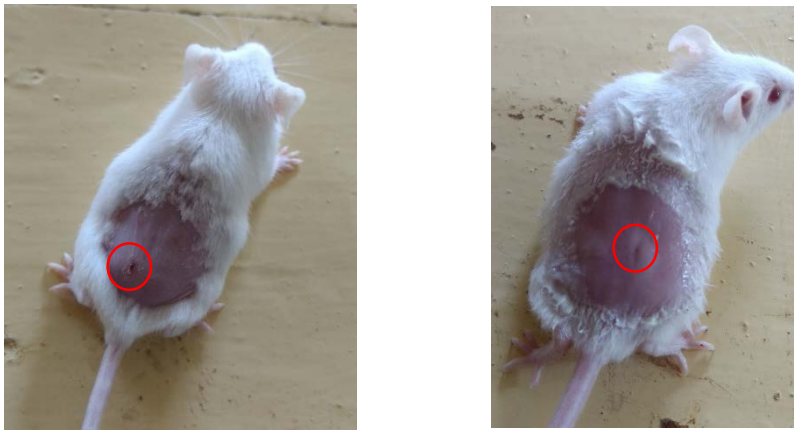
Day 1:



A B C

Figure 5.9: On the first day, hair was removed (A), an incision wound was created (B) and surgical sutures were applied (C)

Day 6:



A B

Figure 5.10: Comparison of incision wound (marked by red circles) on 6th day between group I (challenged) and group IV (turmeric ointment treated)

Tensile strength is the force required to open up the incision. It indicates the resistance to breaking of the repaired tissues under tension and is equal to the

maximum force applied per unit of cross-sectional area of the repaired tissue before its breaking point. As the tensile strength increases, quality of the repaired tissues becomes better.

Table 5.3: Wound tensile strength of different groups and comparison of their % breaking strength

Group	Treatment	Tensile strength (in g)
I	No treatment	163.86 ± 4.81
II	Nebasulf	201.75 ± 6.04
III	Ointment base	192.91 ± 7.16
IV	Turmeric ointment	220.47 ± 5.31

Table 5.3 depicts the tensile strength (in grams) of different groups of animals. Data is presented as mean ± s.d. Breaking strength percentage of group II was considered as 100 % and is calculated for other groups with respect to group II.

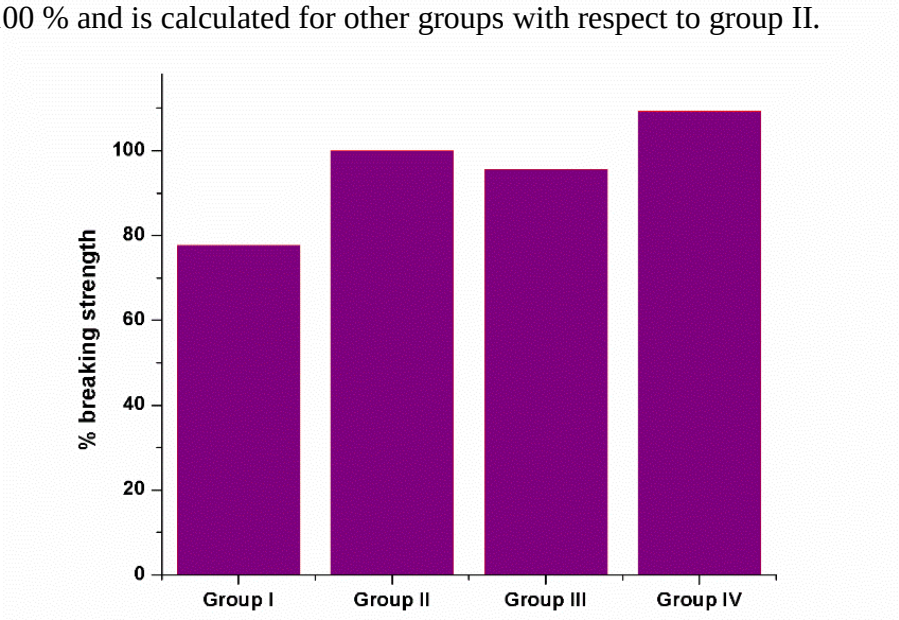


Figure 5.11: Comparison of % breaking strength among different groups

Application of turmeric ointment significantly increased the % breaking strength to 109.28 % while it exhibited a value of 77.75 % and 95.62 % in group I and III respectively. This also indicates that the ointment base itself has some wound healing property.

5.9 Excision wound model:

Monitoring the wounds daily by visual examination established the absence of any kind of infection at the site of injury.

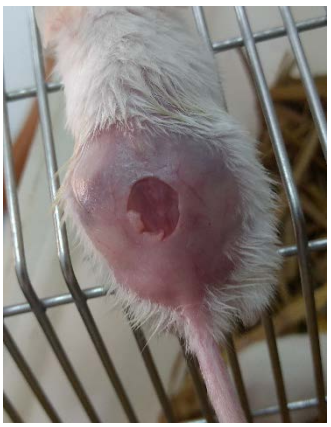


Figure 5.12: Excision wound at Day 1

After the treatment with any samples, dressing was important to prolong their action and giving sufficient time for the drugs to actively participate in the healing process.

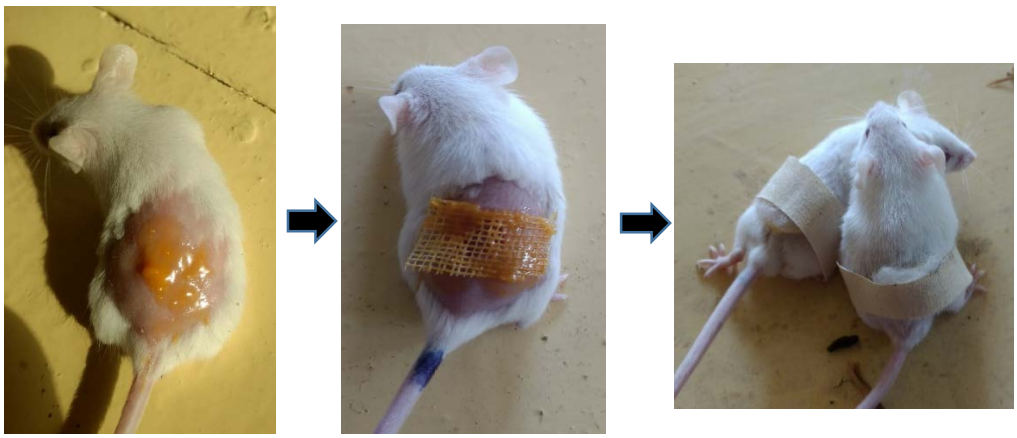
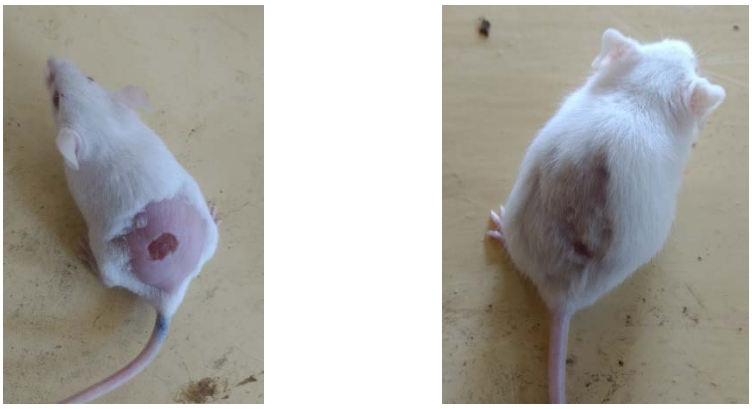


Figure 5.13: Topical application of turmeric ointment and dressing of the wound

Table 5.4: Wound area of different groups of animals on 1st, 4th, and 8th day

Group	Treatment	Wound area (in mm^2)		
		Day 1	Day 4	Day 8
I	No Treatment	204 ± 2.67	122.67 ± 2.22	73 ± 2.67
II	Nebasulf	204.33 ± 2.89	99.67 ± 1.11	47.67 ± 2.22
III	Ointment base	202.67 ± 1.11	103.33 ± 2.44	49.33 ± 1.78
IV	Turmeric ointment	203 ± 2	91.67 ± 1.56	36.67 ± 1.56



A B

Figure 5.14: Progression of wound healing in turmeric ointment treated animals. A and B indicate the wound on 4th and 8th post wounding day

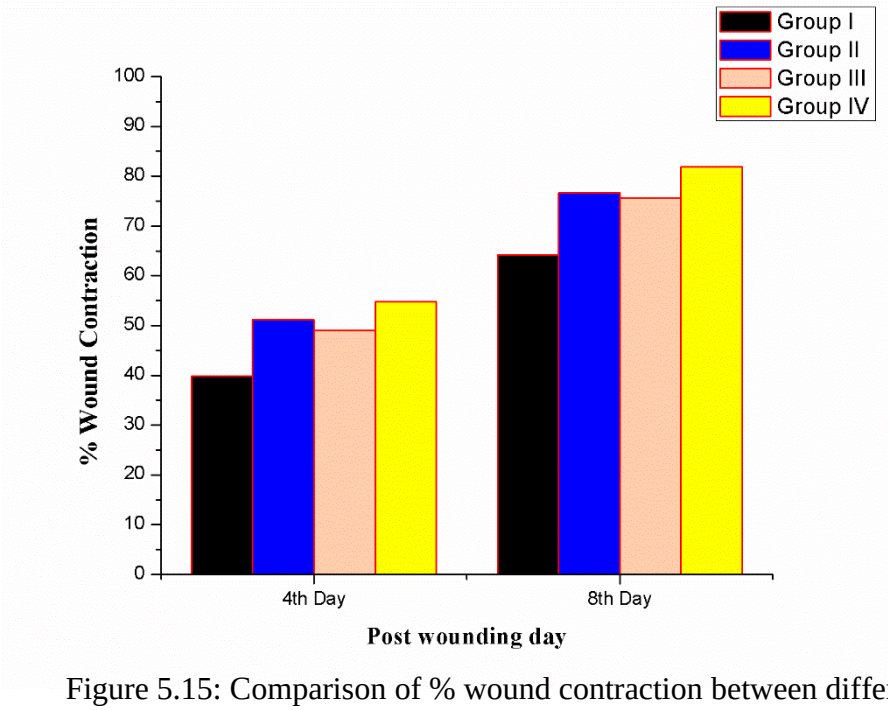


Figure 5.15: Comparison of % wound contraction between different groups on 4th and 8th post wounding day

Among all the groups, turmeric ointment treated animals (i.e. group IV) showed increased enhancement in the progression of wound healing with wound contraction of 54.84 % and 81.94 % on day 4 and day 8 respectively whereas challenged group exhibited a contraction of 39.87 % and 64.21 % only. The ointment base also showed some healing properties and its curative potential was quite comparable with the standard drug used here.

5.10 Histopathological examination:

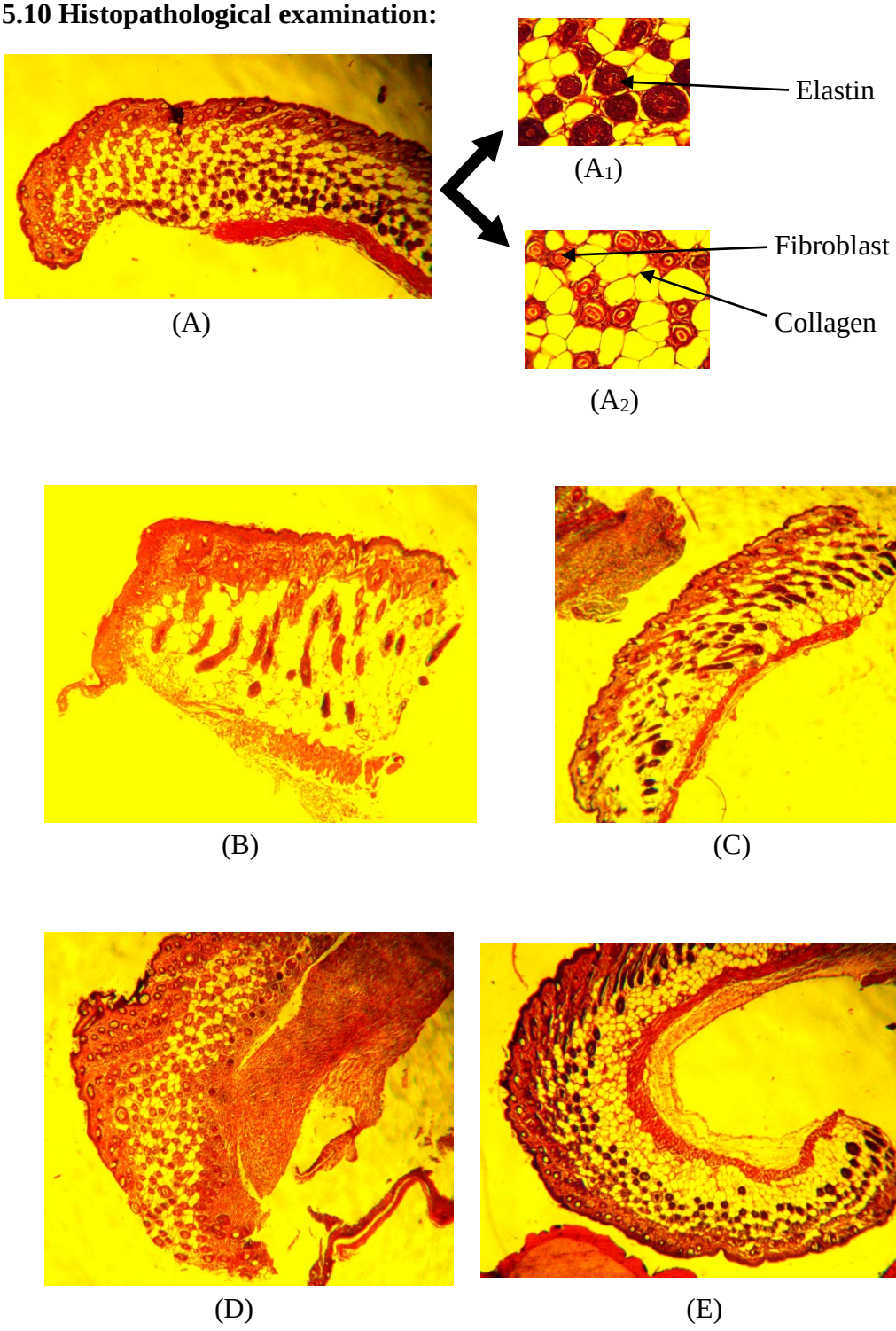


Figure 5.16: Histopathological examination of excised tissues stained with H&E. (A), (B), (C), (D) and (E) represent intact tissue and tissues from group I, II, III and IV respectively. (A_1) and (A_2) are the enlarged view indicating elastin, fibroblast and collagen.

The above figure compares tissues of different groups. Intense collagen, fibroblasts and elastin is observed in (A). Tissue from group I i.e. (B) showed delayed healing than other groups with less collagen observed. The collagen content in group II, III and IV i.e. standard, vehicle and turmeric ointment treated respectively is almost similar to that of the intact tissue indicating healthy healing process in treated animals. Also the number of fibroblasts and inflammatory cells in the dermal layer is more in treated animals than the challenged group.

5.11 Tissue biochemical parameters estimation

5.11.1 Estimation of Hydroxyproline:

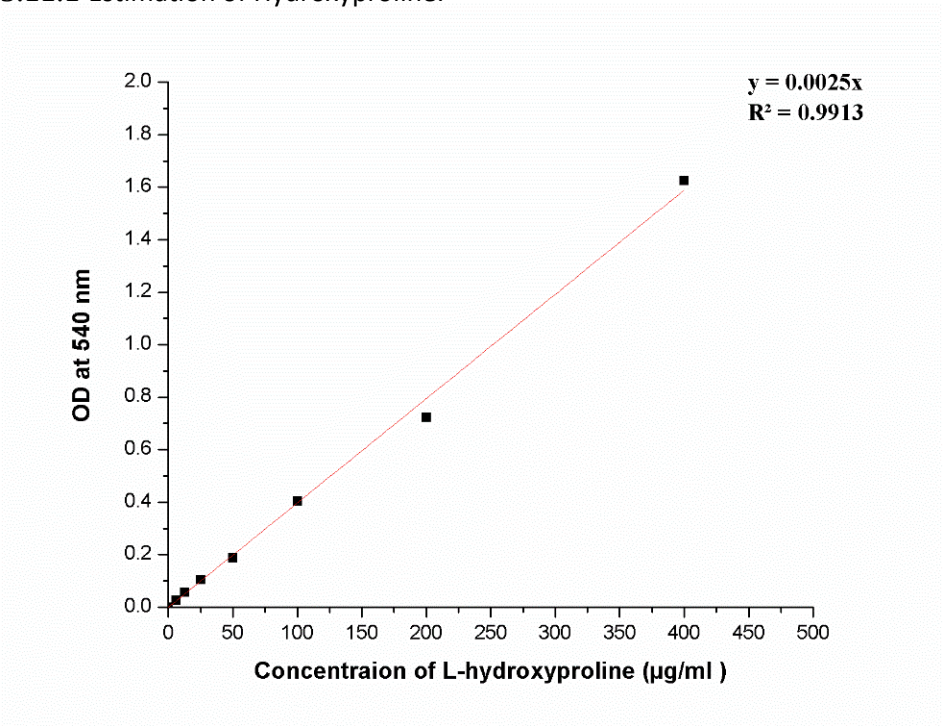


Figure 5.17: Linear standard curve of L-hydroxyproline

From the standard curve, the hydroxyproline content in the tissues of different groups were calculated. Data is expressed in µg equivalent of hydroxyproline per mg of tissue.

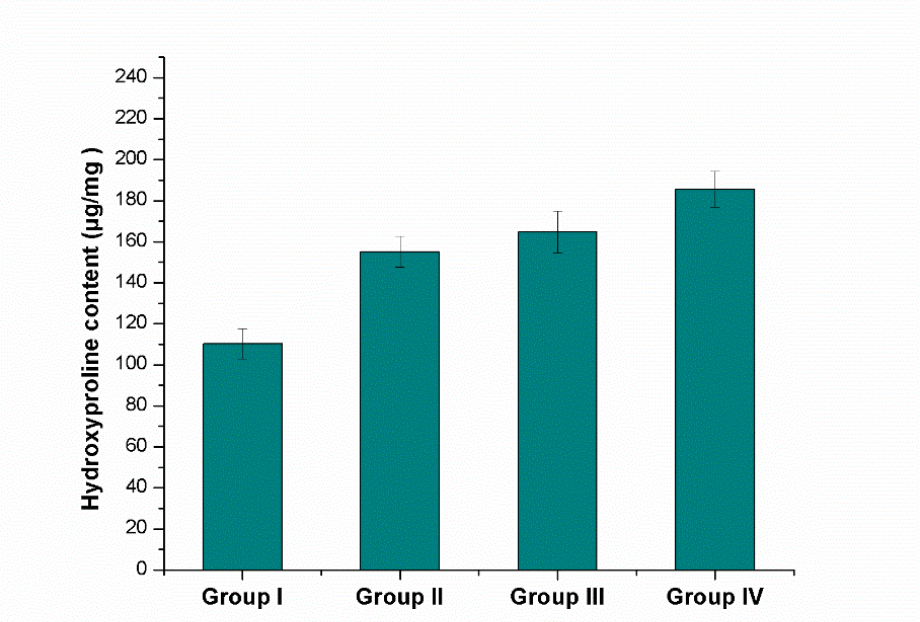


Figure 5.18: Comparison of hydroxyproline content among different groups

There is a significant difference in hydroxyproline content when compared between the challenged and turmeric ointment treated groups. The content in group IV is 185.52 ± 8.76 µg per mg of tissue whereas group I exhibited a value of 110.35 ± 7.38 µg per mg of tissue. This increase indicates the presence of more collagen and thus better healing process in group IV than others.

5.11.2 Estimation of hexosamine:

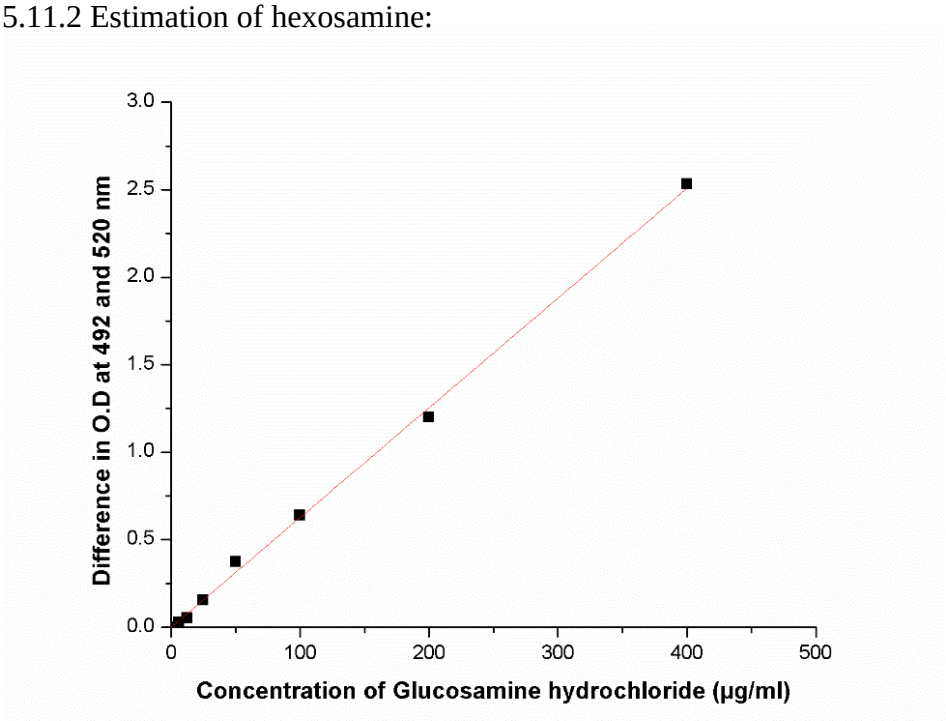


Figure 5.19: Linear standard curve of Glucosamine hydrochloride

From the standard curve, the hexosamine content in the tissues of different groups were calculated. Data is expressed in µg equivalent of Glucosamine hydrochloride per mg of tissue.

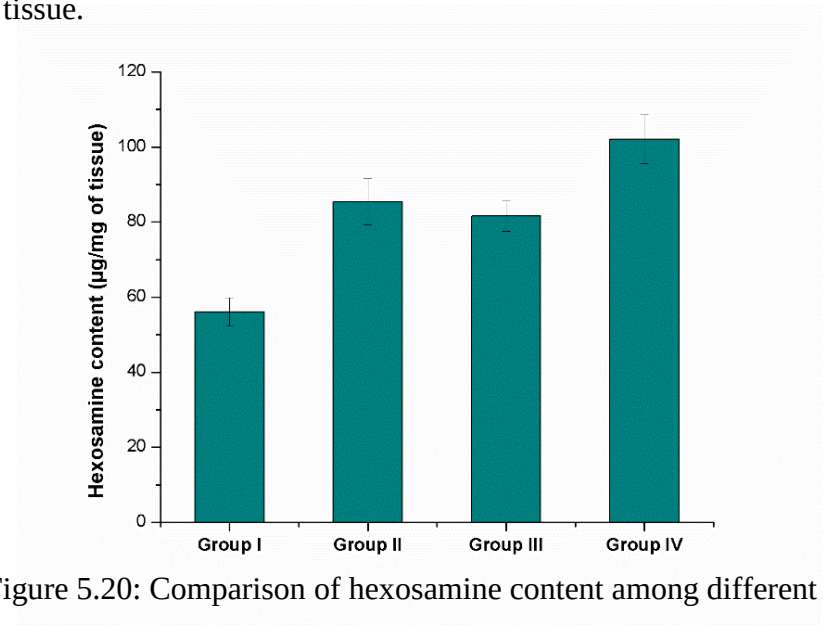


Figure 5.20: Comparison of hexosamine content among different groups

Although only a slight change is observed in case of group II and group III, a typical rise is followed for group IV animals with hexosamine content of 102.08 ± 6.49 µg equivalent of glucosamine hydrochloride per mg of tissue. Group I portrayed the least value of 56.12 ± 3.81 among all the groups.

5.11.3 Estimation of hexuronic acid:

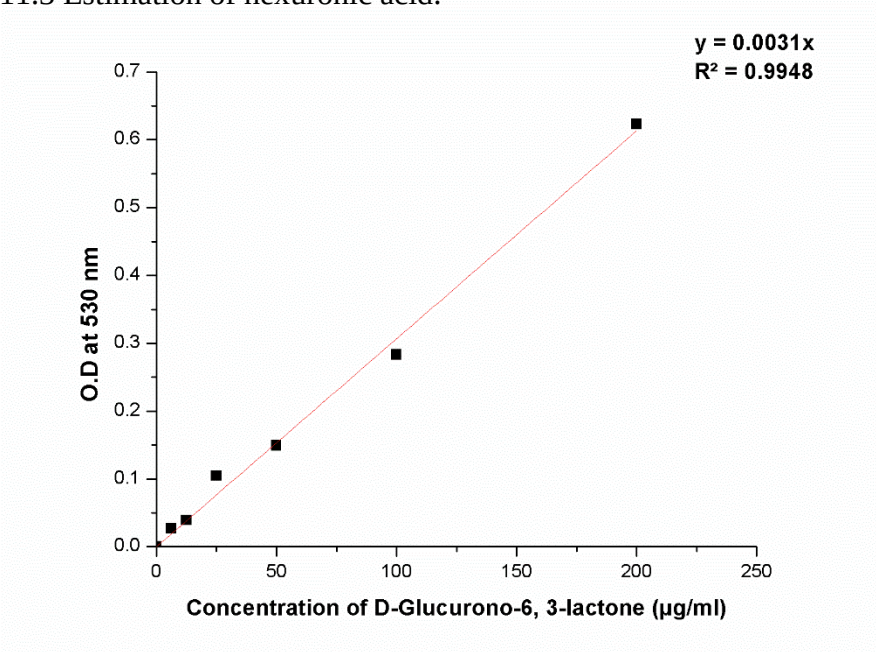


Figure 5.21: Linear standard curve of D-Glucurono-6, 3-lactone

From the standard curve, the hexuronic acid content in the tissues of different groups were calculated. Data is expressed in µg equivalent of D-Glucurono-6, 3-lactone per mg of tissue.

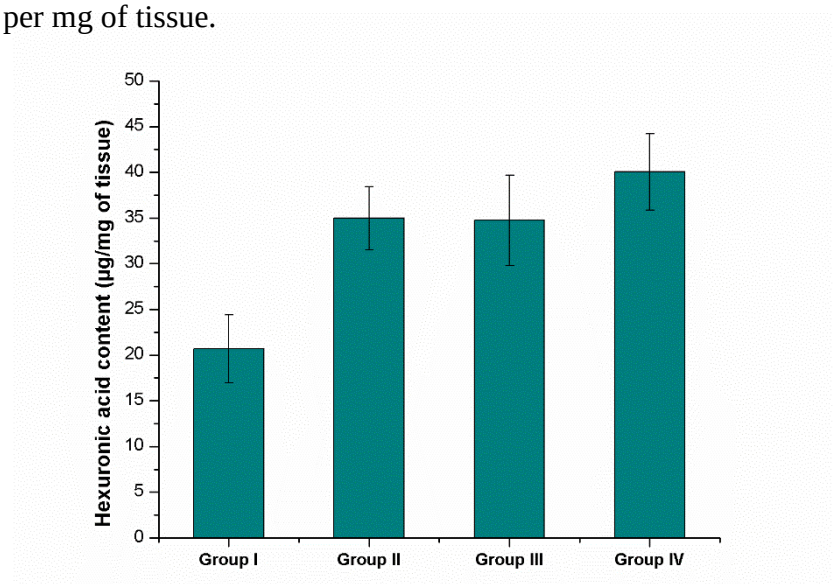


Figure 5.22: Comparison of hexuronic acid content among different groups

Hexuronic acid content of group I animals is 20.68 ± 3.71 µg equivalent of D-Glucurono-6, 3-lactone per mg of tissue which is much lower than group IV with a value of 40.06 ± 4.16 justifying higher healing potential of turmeric than other treatments used here.

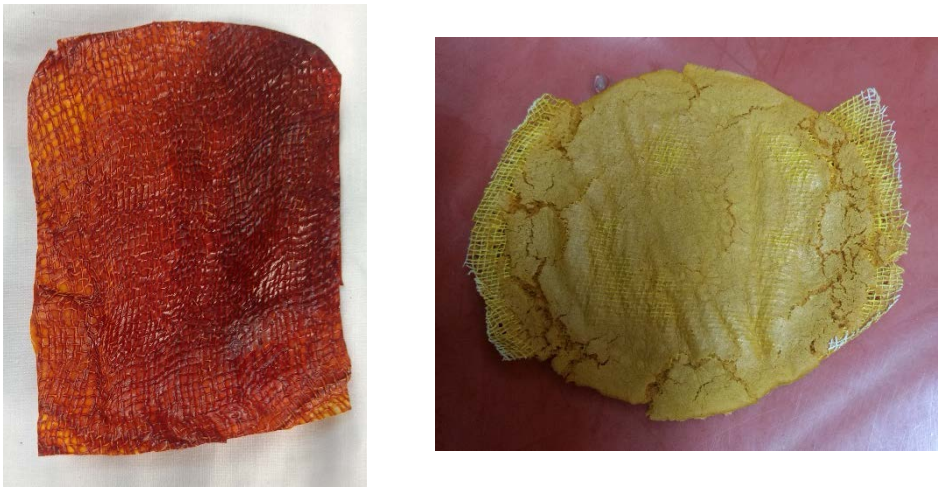
5.12 Polymeric encapsulation

5.12.1 Polymer coating:

The coating of gauze by extract loaded polymeric blends was achieved by both lyophilization and incubation. It results in the formation of scaffold like structures with pores and drugs encapsulated in it. The scaffolds (S_I) prepared by incubation form 2-D structure films (having negligible thickness) with higher densities when compared to the lyophilized scaffolds (S_L) which are more soft and porous.

All the films did not break upon folding at a same place up to 300 times suggesting a high value of their folding endurance. Group S_I was more flexible than S_L. Dressing pH of different films varied from 5.9 to 6.7 which is in accordance with the pH range (5.25 to 7.9) of wound care products as reported earlier. Dressing pH controls infection at the wound site and accelerates fibroblast proliferation. Since the pH of normal human skin is in between 4.0 to 6.8, a

dressing material maintaining slight acidic environment will increase the healing rate when compared to neutral and alkaline environments.



A B

Figure 5.23: Gauze coated with turmeric extract loaded polymeric blends. (A) and (B) indicate the texture of incubated scaffold i.e. S_I and lyophilized scaffold i.e. S_L respectively.

5.12.2 Microbial penetration test:

The positive control test tube with open head exhibited cloudiness indicating microbial contamination in the media. No other test tubes reported such result and similar clarity of the solutions with the negative control confirms inhibition of microbial penetration through the films and further contamination.

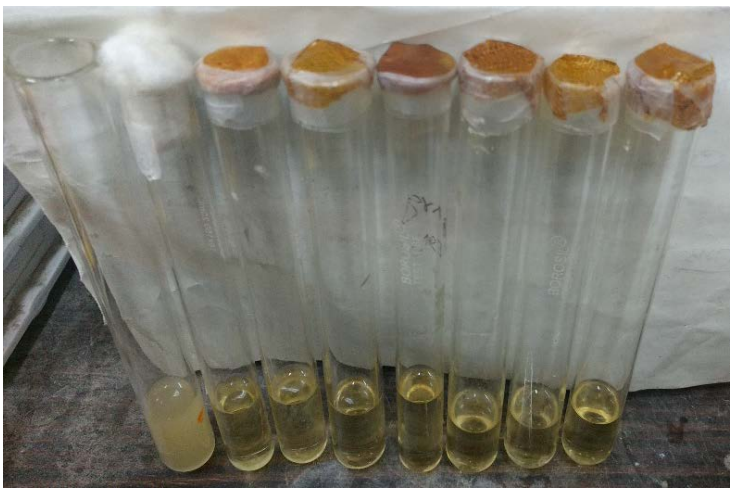


Figure 5.24: Observation of microbial contamination in the test tubes

Secondary microbial infection is a major concern in wounds and thus these scaffolds can be used as wound dressing materials to enhance the healing process.

5.12.3 Hemocompatibility:

The % hemolysis for all the scaffolds varied from 0.86 % to 2.09 %. As per the accepted norm, % hemolysis less than 5 of the test material is considered highly hemocompatible and a value between 5 to 10 as hemocompatible whereas greater than 10 implies its non-hemocompatibility nature. [….3.9////] Thus the materials are declared to be highly hemocompatible and hence can be exploited as wound dressing materials.

A B C D

Figure 5.25: Hemolysis in distilled water (A), saline (B), $S1_L$ (C) and $S3_L$ (D)

5.12.4 Water absorption capacity:

Water absorption capacity of a wound dressing material is of utmost importance as it measures the efficiency of the material to absorb wound exudates. [….3.5////] The pH of simulated wound exudate (SWE) prepared was recorded as 8.14 which is in accordance with chronic wounds (as chronic wounds exist in an alkaline environments and open wounds generally have a neutral to alkaline pH ranging from 6.5 to 8.5).

Table 5.5: Swelling index of incubated scaffold

Scaffold	Swelling index or % water absorption		
	t=30 minutes	t=1 hour	t=2 hours
$S1_I$	54.79	53.16	53.02
$S2_I$	32.81	34.73	34.79
$S3_I$	66.17	68.12	72.54
$S4_I$	82.83	94.47	96.25
$S5_I$	24.19	25.14	24.97
$S6_I$	48.26	53.52	55.90

Highest swelling index is observed in $S4_I$ i.e. blend of 5 % PVA and 2.5 % gelatin. All the films almost reached their maximum water absorption capacity within 1 hour of exposure to aqueous environment.

Table 5.6: Swelling index of lyophilized scaffolds

Scaffold	Swelling index or % water absorption			
	t=1 hour	t=2 hours	t=3 hours	t=4 hours
$S1_L$	214.90	160.66	156.72	153.84
$S2_L$	95.21	89.62	87.14	88.27
$S3_L$	122.16	132.04	136.62	142.97
$S4_L$	205.85	248.88	262.47	264.69
$S5_L$	335.17	338.52	340.68	346.73
$S6_L$	122.17	132.04	138.53	139.70

$S5_L$ i.e. blend of 2.5 % PVA and 5 % gelatin showed highest water absorption capacity, followed by $S1_L$ i.e. blend of 0.5 % chitosan and 5 % PVA. All the samples reached their equilibrium water content within 3 hours of exposure to aqueous environment whereas chitosan scaffolds took only 1 hour to swell maximally with the release of water in the subsequent hours. This observation makes chitosan PVA scaffolds useful in wound dressing because initially it is required to absorb wound exudates but later a release of little amount of exudates will keep the wound moist enhancing the healing process.

It can be concluded that drying by lyophilization rather than incubation increases swelling capacity by several folds. Thus a more porous structure is obtained on

lyophilization which can be useful for cell proliferation marking its potential as a scaffold for wound healing.

5.12.5 Release profile of the scaffolds:

Table 5.7: O.D. values of simulate wound exudate at different time intervals:

Scaffold	O.D. at 425 nm				
	t=30 mintues	t=2 hours	t=4 hours	t=6 hours	t=8 hours
$S1_I$	0.09	0.17	0.20	0.22	0.27
$S1_L$	0.11	0.27	0.35	0.40	0.51
$S2_I$	0.02	0.08	0.22	0.19	0.23
$S2_L$	0.12	0.24	0.31	0.37	0.42
$S3_I$	0.17	0.42	0.58	0.54	0.59
$S3_L$	0.08	0.17	0.31	0.41	0.58
$S4_I$	0.32	0.49	0.65	0.69	0.71
$S4_L$	0.14	0.25	0.32	0.40	0.51
$S5_I$	0.24	0.41	0.46	0.52	0.52
$S5_L$	0.06	0.15	0.24	0.36	0.48
$S6_I$	0.38	0.50	0.62	0.67	0.72
$S6_L$	0.79	1.81	2.47	2.71	2.82

Incorporation of chitosan with PVA resulted in more sustained release for a period of 8 hours than gelatin. In general, the release profile of lyophilized samples was distributed over time whereas incubated samples showed sudden burst of release initially till 2-4 hours with the fall in its release rate henceforth.

Chapter Six

CONCLUSION AND FUTURE SCOPE

6.1 Conclusion

Turmeric has shown significant content of phenols and flavonoids which can be claimed to be responsible for its biological activities. Total phenol content varied significantly with the solvent used during the extraction process. The purpose of increasing solubility with the synthesis of nanocomposite was achieved and its FESEM analysis showed particle size varied from 20 nm to 400 nm with no agglomeration. Crude turmeric extract showed appreciable zone of inhibition against both E. coli and S. Aureus whereas its silver nanocomposite showed same extent of inhibition at a much lower concentration. Turmeric extract possess good antioxidant activity with IC_{50} value of 37.09 μg/ml indicating its therapeutic potential and it was established by the evaluating the anticancer effect against MCF-7 cell line. The turmeric ointment prepared showed no irritation on topical application and claimed to have wound healing property when applied topically on incision and excision wound model and this fact was justified by histopathological examination and estimation of biochemical parameters of tissues. Hydroxyproline, hexosamine, and hexuronic acid content in ointment treated animals were much more compared to the untreated as well as standard drug treated animals and this marks the presence of excess collagen in them. Polymer encapsulation of turmeric extract by chitosan-PVA and PVA-gelatin blends released the bioactive components in a sustained manner and this trend was observed more in chitosan-PVA scaffolds. All the gauzes coated with blends were highly hemocompatible and showed no microbial penetration. The incubated scaffolds showed lesser porosity and water absorption capacity as well as poor release profile than the scaffolds obtained by lyophilization.

6.2 Future scope of work

- Optimization of polymer encapsulation of turmeric extract.
- *In-vivo* and *in-vitro* evaluation of wound healing property of polymer coated gauzes.
- Optimization of shape and size of nanocomposite synthesized using turmeric extract and silver nitrate along with its TEM analysis.
- Cytotoxicity testing of the extract and its nanocomposite against different cancer cell lines to identify its best activity against a particular cancer cells.
- Investigation of action of mechanism involved in the wound healing process with turmeric.
- Study on antimicrobial activity of turmeric against different strains of microbes responsible for hindrance in the healing process.
- Optimization of extraction procedure to make the process less time consuming and more cost efficient.