SCREENING OF PHYTOCHEMICALS FROM *Azadirachta indica* and *Curcuma longa* USING CELL LINE BASED ASSAY FOR WOUND HEALING

Dissertation submitted in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE IN BIOTECHNOLOGY

By

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Under the guidance of

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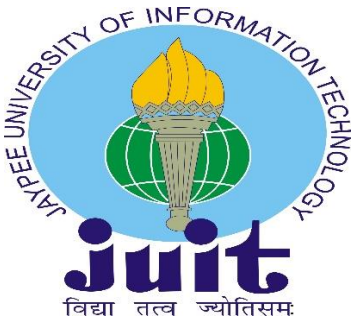

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DECLARATION

I hereby declare that the work reported in the M.Sc. dissertation entitled **"Screening of phytochemicals from *Azadirachta indica* and *Curcuma longa* using cell line based assay for wound healing"** submitted at Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh, India, is an authentic record of my work carried out under the supervision of Dr Udaybanu Malairaman, Dept. of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh-173234, India. I have not submitted this work elsewhere for any other degree or diploma.

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SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the M.Sc. dissertation entitled **"Screening of phytochemicals from *Azadirachta indica* and *Curcuma longa* using cell line based assay for wound healing"** submitted by Shubham Pal (207823) at Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh, India, is bonafide record of his original work has not been submitted elsewhere for any other degree or diploma.

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ABBREVIATION

ECM- Extracellular matrix

ATP- Adenosine triphosphate

DHEA- dehydroepiandrosterone

WHO- World Health Organization

AYUSH- Ayurveda, Yoga and Naturopathy, Unani, Siddha and Homeopathy

RBC- Red blood cells

Ab- Antibody

DNA- Deoxyribonucleic acid

DBMA- 12dimethylbenz(a)anthracene

TNF- Tumor necrosis factor

HIV- Human immunodeficiency virus infection

MRVP- Methyl Red / Voges-Proskauer

ABTS- 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid

DPPH- 2,2-diphenyl-1-picryl-hydrazyl-hydrate

DMSO- Dimethyl sulfoxide

IC50- Half-maximal inhibitory concentration

ABSTRACT

Human health is preserved in big extent by the skin. The spread of wound disease occurs when bacteria enter the body through the skin. Effective wound management is difficult because wound healing is a challenging process which is influenced by various internal and external factors. Due to the side effects of modern medicine and the lower cost of herbal products, natural herbal remedies have now become essential for the management of skin disorders and the treatment of wounds. Ayurvedic texts have described the potential of neem leaves and turmeric rhizomes. The objective of the current study was to evaluate the wound healing ability of the selected plants by using in vitro wound healing assays as scientific evidence of their efficacy is limited therefore it is important to introduce a scientific validation for the medicinal effect of plants used in traditional medicine. MTT assay was performed which revealed that there was 2-fold increase in the cell proliferation when 3T3 fibroblast cell line was treated with neem and turmeric plant extracts.

CHAPTER 1: INTRODUCTION

1. INTRODUCTION

Plants and their products have been used in treatment from ancient times to boost or increase immunity to colds, joint or muscle pains, fevers, and many other illnesses. Despite development in synthetic drugs and antibiotics, plant still occupy an important place in modern as well as traditional system of medicine all over the world. In developing countries, people living in villages rely heavily on herbal treatments. Also, herbal medicines and health foods, derived from plants are becoming more important both in developing nations and in superpower nations as people are getting to know about the immense benefits of plant products. Many herbal medicines have been tested, and they have shown promise in the treatment of allergies, metabolic disorders, and age-related degenerative disorders. Plants has been used as traditional medicines, but it is necessary to establish the scientific basis for traditional plant medicines' therapeutic actions, as these could be used to develop more effective drugs.

1.1 WOUND

A wound disrupts the continuity and integrity of the skin. In general, a wound can be created by mild or severe trauma, or by a pathological process, or by bruising and abrasions, or by burns, and it can even occur during the course of a surgical treatment. [1]

In our daily life, almost everyone is likely to sustain multiple kind of wounds during the course of our lives. Injuries, cuts and bruises, burns, poor circulation, ulcers, and diseases like diabetes can all cause lesions that disrupt the skin's normal function and structure. Simple cuts and bruises in the elderly, and in diabetics or those with circulatory problems can turn into chronic, non-healing wounds that necessitate extensive treatment.

1.2 CLASSIFICATION OF WOUNDS

There are two types of wounds. The acute wound demonstrates normal wound pathophysiology, and healing tends to proceed through the typical phases of wound healing in most cases. In contrast, a chronic wound is physiologically damaged.

Acute wounds

Acute wounds include burns, surgical wounds, scratches, minor cuts and abrasions, and severe traumatic puncture wounds produced by bullet injuries. Acute wounds, regardless of the type of epidermal injury, are supposed to recover in a typical or regular time span. Though, the chosen healing procedure will be different depending on the site, and depth of the injury. In such wounds, healing progresses through natural processes such as inflammation and granulation to final re-epithelization and maturation stage.

Chronic wounds

These kinds of wounds do not follow the normal cycle of healing process and frequently stay in the inflammatory stage for far too long, and sometime they might never heal or take years to do so. Chronic wounds generally develop due to lack of proper arterial blood flow or venous drainage. Chronic wounds are most often induced by endogenous mechanisms, which are caused by a preexisting factor that affects dermis and epidermis tissue integrity. [2]

1.3 WOUND HEALING PROCESS

The process by which tissue repair occurs is known as wound healing process. This process commences right after the injury. All wounds go through a similar repair process of healing, though different tissues differ in their healing process, as well as the time required for complete restoration and the pathway followed for healing, which vary quite differently in different tissues. [3]

Wound healing process has been said to be a dynamic and complex process. This process involves an initial inflammatory phase, followed by re-epithlialization of the wound region, production of granulation tissue, neovascularization, and wound contraction. Interaction between different cell types encourages individual events to be coordinated throughout the wound healing process, allowing for temporal and spatial control. [4]

1.3.1 STAGES OF WOUND HEALING PROCESS

There are four stages to the wound healing process. However, healing process is not always in sequential order, and wounds can often shuffle in between the different stages, depending on the internal and external factors. [5]

Different stages of wound healing process are as following:

A. Coagulation and hemostasis stage.

The stage of hemostasis and coagulation that begins right after the injury and prior to the inflammatory phase is commonly considered as the first of the four stages of normal wound healing. It is a method of protecting the vascular system in order to keep and maintain vital organ functions. Plasma proteins are typically secreted at the injured site, where they coagulate and forms a strong fibrin blood clot. This clot will act as a barrier to limit the blood loss and seals the wound site.

B. Inflammatory stage.

Hemostasis and coagulation stage is followed by the inflammatory stage. Phagocytes produce reactive oxygen species that could last up to a week in acute wounds and much longer in chronic wounds. During this phase, white blood cells and certain enzymes invade the wound site to minimize the risk of infection by removing pathogens and debris; and prepare the wound site for tissue regeneration. During this stage, the normal characteristics of inflammation could be seen: redness, heat, swelling, pain, and functional impairment. [6]

C. Proliferative stage.

The proliferative period is marked by the diffusion, proliferation, and maturation of epithelial cells, dermal fibroblasts, vascular epithelial cells, and progenitor cells that emerge in bone marrow and migrate to the wound area.[7]

In proliferation stage, the wound is reconstructed with fresh granulation tissue made up of collagen tissue and ECM, within which a new blood capillary grows. Granulation tissue has an uneven and granular appearance due to newly formed capillaries integrated in a weakly laid collagen matrix. Healthy granulation tissue has a granular and irregular structure, is red in colour, and does not cause bleeding easily. The colour and stare of the granulation tissue can be used to predict how the wound will heal in many cases. Poor perfusion, ischemia, or infection can all cause dark granulation tissue. [8]

D. Maturation or remodeling stage.

After the wound has healed, maturation, also known as remodeling occurs. Variations in the structure, quantity, and arrangement of collagen occur throughout the maturation of granulation tissue, leading to an increase in the tissue's strength properties.

This stage involves the remodelling of collagen from type III to type I, which is the main fibrillar collagen in the skin. Collagen which was secreted during first stage, makes the

wound thick and disordered. But during this stage, collagen fibres are aligned in a regular order, due to which collagen fibres are present closer to each other and cross-links. Crosslinking is an important process as this minimizes the scar thickness and improves the skin condition around the injured site. [9]

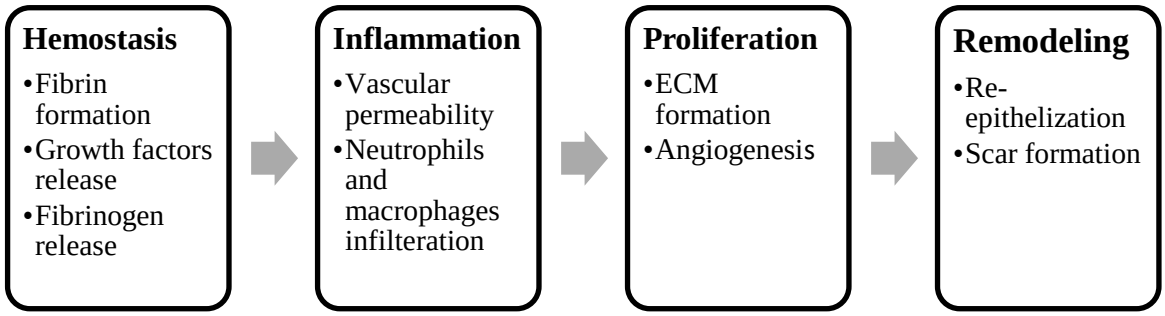


Fig. 1 Stages of wound healing.

1.4 FACTORS AFFECTING WOUND HEALING

A variety of reasons can influence the wound healing process and can contribute to poor wound healing. The factors that can contribute to wound repair is divided into two categories: local and systemic.

1. Local Factors

Oxygenation. Almost all wound-healing processes require oxygen, as does cell metabolism, including energy synthesis via ATP. It prevents contamination, promotes wound contraction, and promotes keratinocyte maturation, migration, and re-epithelialization. It also has an effect on fibroblast proliferation and collagen formation. Wound healing is slowed when oxygen is is less. Although, healing process can be stimulated by acute hypoxia after an injury, but it can be delayed by ongoing or prolonged hypoxia. [10]

Infections. When a wound occurs on the skin, microorganisms that are typically confined to the skin's surface gets internal access of the body and infect the area. Based on the level of contamination and microbe replication, the wound is characterized as contaminated, colonized, limited infection, or spreading invasive infection. Contamination refers to the presence of microorganism which does not undergoes replication at injured site, whereas colonization refers to the presence of microorganism which undergoes replication and does

not cause harm to tissue. In invasive infection, reproducing microorganisms are present at injured site and causes harm to the host. [11]

2. Systemic Factors

Age of the person. Various studies have been conducted to investigate delay in wound healing in people with the increase in their age. It has been seen that, in healthy older adults, ageing can delay the healing process, but not an actual deterioration in healing efficacy. Delay in wound healing in the aged people is associated with a distinct inflammatory response, such as delayed T-cell infiltration into the wound region, alterations in chemokine synthesis, and decreased macrophage phagocytic capability.[12]

Sex Hormones. Sex hormones are also one of the factors influencing the wound healing process in old people. Female estrogen, male androgen hormone, and their steroid precursor dehydroepiandrosterone (DHEA) have been shown to have significant effects on wound healing. [13]

Stress. Stress has been related to a variety of ailments, notably heart related diseases, poor wound healing, high risk of developing cancer and diabetes. According to some studies, stress causes a considerable delay in wound healing process in both humans and animals. [14]

Diabetes. Hundreds of millions of individuals are affected by diabetes around the globe. Several dysregulated cellular process, including decreased T-cell immunity, phagocytosis, and bactericidal activity, as well as fibroblast and epidermal cell dysfunction, are present in diabetic wounds. Diabetic patients experience hypoxia as a result of inadequate circulation and angiogenesis at the wound site. This can increase the initial inflammatory response, causing harm to last longer by raising oxygen radical concentrations**.**[15]

1.5 OBJECTIVE

The objectives of this study are:

i. To evaluate antimicrobial and antioxidant activity of selected plant extracts (Neem and Turmeric)
ii. To evaluate invitro toxicity of plant extracts as well.
iii. To evaluate the in vitro wound healing ability of the selected plants on fibroblast cell line.

CHAPTER 2: REVIEW OF LITERATURE

2. INTRODUCTION

Natural products were the source of all drugs in ancient times, and higher plants supplied the majority of the therapeutic agents. In current times, organic compounds and their derivative products are found to contribute for nearly half of all drugs in medicinal use around the globe and 25% of global drug production. [16] According to the WHO, traditional medicine is used by 80 percent of the population in developing countries for basic health care, and 85 percent of traditional medicine depends only on plant extracts. This indicates that plants are used as a resource of pharmaceuticals by about 3.5 to 4 billion individuals throughout the globe. [17]

2.1 TRADITIONAL MEDICINE

Herbology is the practice of using herbs to heal various illnesses and physiological abnormalities in Ayurveda, Siddha, and Unani. In India's AYUSH systems, around 8,000 herbal remedies have been compiled. In the vast majority of cases, traditional medical systems are still widely used. Massive plants have been used in India for many decades to create a variety of medicinal preparations for both external and internal use.

Ayurveda, the traditional Indian system of medicine, is based on the holistic treatment of diseases that primarily rely on natural herbal drugs. Ayurveda is by far the most primitive of all traditional medicines. It is thought to be older than traditional Chinese medicine and is said to source of systematic medicine.[18]

2.2 SELECTION OF PLANTS FOR THE PRESENT STUDY

Based on a comprehensive analysis of the literature, the following plants were chosen for the current study:

1. *Azadirachta indica* (Neem)
2. *Curcuma longa* (Turmeric)

2.2.1 *Azadirachta indica*

Azadirachta indica is considered as a health-promoting plant because of its high antioxidant content. It has been used in various traditional systems of medicines around the globe such as in Ayurveda and Unani of India. Neem is regarded as safe to use for medicinal purposes as it controls various biological function in body without causing any harm.

2.2.1.2 Taxonomical classification of *Azadirachta indica*

Scientific classification of *Azadirachta indica* (Table 1)

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Sapindales
Family	Meliaceae
Genus	*Azadirachta*
Species	*indica*

Table 1. Classification of neem**.**

2.2.1.3 Botanical Description of Neem

The neem tree is found abundantly in tropical and semitropical regions such as India.The neem tree is about 20–23m tall and stem diameter of neem is about 4-5 feet. The leaves of neem are compound and have 5–15 leaflets each. Fruit of neem are green drupes that ripen to golden yellow color between June and August. [19]

2.2.1.4 Active Compounds of *Azadirachta indica*

From various portions of the neem tree, around 250 natural compounds have been separated. The most important active constituent in neem is Azadirachtin. Additionally, triterpenoid such as nimbin, gedunin, nimbidol, nimbolinin, sodium nimbinate, and quercetin(flavonoid) are present in neem. In 1968, Butterworth and Morgan isolated azadirachtin for the first time. Azadirachtin exerts repellent and toxic effects in insects, therefore act as insecticidal component. [20]

Some of the major phytochemical constituents of neem along with their source and pharmaceutical properties has been shown in table 2. [21]

Active Compounds	Source	Medicinal properties
Nimbidin	Seed Oil	Anti-inflammatory, antifungal, hypoglycaemic, antibacterial
Nimbin	Seed Oil	Anti-inflammatory, antihistamine and antiseptic
Nimbolide	Seed Oil	Anti-malarial, antibacterial, and anti-cancer
Gedunin	Seed Oil	Impaired allergic responses, anticancer
Azadirachtin	Seed Oil	Antimalarial
Quercetin	Leaf	Antioxidant, antibacterial
Polysaccharides	Leaf	Anti-inflammatory
Cyclic Trisulphide	Leaf	Antifungal
Gallic Acid and Catechin	Bark	Antibacterial
Polysaccharides G2A	Bark	Anti-inflammatory

Table 2. Pharmaceutical properties of neem with their active compounds and source.

2.2.1.5 Therapeutic properties of neem

1. Antioxidant Activity

Antioxidant compounds protects the cells from free radicals which are produced as part of the body's regular metabolic function but free radicals can damage the other cells. A study has been conducted to assess the antioxidant activity of *Azadirachta indica* leaf and bark extracts.

The study's findings clearly demonstrated that neem extracts have antioxidant activity.[22] In another study, antioxidant activity of neem leaves in different crude extracts has been evaluated, and it was found that butanol extract shows best activity [23]

2. Anti-Inflammatory and analgesic activities

Carbon tetrachloride extract of neem and its isolated constituent azadiradione has been reported to show significant anti-inflammatory effects, according to the findings. [25] According to one study, neem and its constituent mainly nimbidin interfered and reduced the activity of macrophages and neutrophils. [26]

The seed oil of neem has a significant analgesic effect and it was demonstrated in an experiment which was done on albino rats. Dose dependent analgesic activity was observed in this experiment. [19]

3. Immunostimulant activity

The extract of neem bark shows anticomplement activity in human serum, therefore having a role in activation of complement system. An extract of neem stem has recently been reported to show effective immune response against RBCs of sheep in mice model. [27] In one study, oral administration of neem leaf extracts resulted in increased production of IgM and IgG ab.[28]

4. Antimicrobial Effect

Many microorganisms, including viruses, bacteria, and harmful fungi, are inhibited by neem and its components. Below are the several roles of neem in preventing microbiological development.

Antibacterial Activity

The antibacterial activity of neem extracts against pathogens causing food spoilage and food borne diseases was tested, and the results indicated that neem extracts contain antibacterial components that inhibits the growth of foodborne pathogens. [30]

Antiviral Activity

Neem leaf extract has been demonstrated to exhibit antiviral activity towards many viruses. For instance, coxsackievirus virus. Neem inhibits the growth of viruses by interfering in the early stages of replication cycle. [31]

Antifungal Activity

In one study, antifungal efficacy of different neem extracts on the fungal species of Aspergillus and Rhizopus has been evaluated, and it was seen that both alcoholic and water extracts had inhibited the growth of fungi. Interestingly, alcoholic neem leaf extract outperformed aqueous extract in inhibiting the development of both fungal species. [32]

5. Anticancerous Activity

Flavonoids has been reported to have anti-cancer properties and intaking food with high flavonoid content reduces the cancer risk. [33] Also, oil from neem contains a variety of neem limonoids which reduces the carcinogenic effects of 7,12dimethylbenz(a)anthracene (DBMA). [34]

Other bioactive compounds which show anti-cancerous activity are azadirachtin and nimbolide. These compounds prevents the activation of procarcinogen, DNA damage, and also inhibits angiogenesis. [24]

6. Antidiabetic Activity

The extracts of *A. indica* and *B. spectabilis* has demonstrated effective glucose tolerance and dramatically decreased glucosidase activity.[35] Another study looked into the hypoglycemic action of neem in rats, and it was observed that it has antidiabetic potential as level of glucose were considerably lesser than in the control group. [36]

7. Wound Healing Effect

Excision and incision wound models were used to examine the healing efficacy of *Azadirachta indica* and *Tinospora cordifolia* leaves in rats. Extracts from both species significantly improved wound healing activity in both the wound models. Therefore, showing the potential of plants in wound healing. [37]

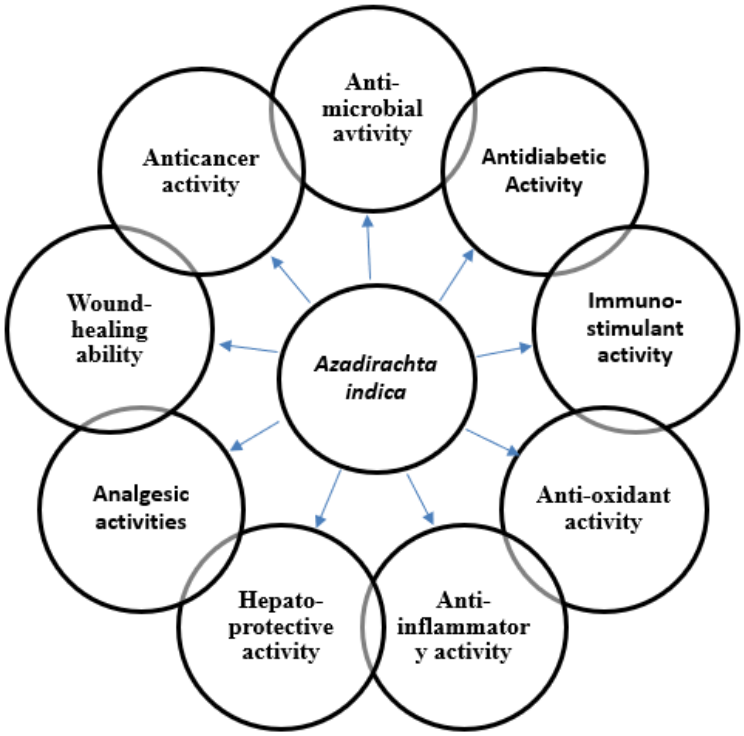


Fig. 2 Some major medicinal properties of neem

2.2.2 *Curcuma longa*

Turmeric is a tropical and subtropical spice that can be found all over the world. Turmeric plant is one of the important medicinal plants and has been used for medicinal purposes for several years in Ayurveda and other Indian traditional systems of medicine. Turmeric has anti-inflammatory, antioxidant, anticancer, antifungal, antibacterial, antiviral, antidiabetic and many more medicinal characterstics. [38]

2.2.2.1 Taxonomical classification of *Curcuma longa*

Scientific classification of *Curcuma longa* (Table 3)

Kingdom	Plantae
Division	Magnoliophyta
Class	Liliopsida
Subclass	Zingiberidae
Order	Zingiberales

Family	Zingiberaceae
Genus	*Curcuma*
Species	*Longa*

Table 3. Classification of *Curcuma longa*

2.2.2.3 Botanical Description of *C. longa*

C. longa is a perennial medicinal spice that can grow up to 1 meter in height. It has oblong shaped leaves, arranged alternately in two rows, with leaf sheaths that divide into false stems, petioles (55–110 cm long), and leaf blades (75–110 cm long). It has dull yellow flowers that are 10–15 cm long and arranged in a spike-like pattern. Turmeric plant have rough segmented rhizomes that are yellow to orange in colour, tubular, and fragrant and measures around 5 cm in length and 2.5 cm wide. [38]

2.2.2.3 Active Compounds of *Curcuma longa*

The flavonoid, curcumin and numerous volatile oils such as turmeron and atlanton are among the major active ingredients in turmeric. Curcumin, a phenolic diketone that gives turmeric its bright yellow colour, is made up of curcumin I, II and III. *Curcuma longa* rhizomes contain a variety of phenolic ketones, including demethoxycurcumin and bis-demethoxycurcumin. [40]

2.2.2.4 *Curcuma longa* as a traditional medicine

C. longa has been utilized in traditional medicine in many regions around the globe for centuries in therapeutic preparations. Many nations in South Asia utilize it as an antibacterial agent on wounds and in Ayurvedic system other therapeutic properties has also been mentioned, such as it helps in removal of kidney stones, aids in proper digestion of food, regulates the menstrual cycles, removing worms from the intestine, and many others.[41]

2.2.2.5 Therapeutic properties of *Curcuma longa*

1. Anti-inflammatory activity

Turmeric appears to have anti-inflammatory properties, according to numerous studies. TNF and prostaglandin E2 production from HL-60 cells was inhibited by crude extracts of

turmeric in one study. [42] In one investigation, a turmeric extract was shown to decrease the activation of dendritic cells against the inflammatory stimuli. [43]

2. Antimicrobial activity

Turmeric has potent antimicrobial properties. At a concentration of 5%, garlic and turmeric extracts inhibited the growth of bacteria involved in production of histamine. [44] *C. longa* extract was reported to have high sensitivity in suppressing the development of the foodborne pathogen. [45] Different strains of *Helicobacter pylori* were also reported to inhibit by a methanolic extract of *C. longa*.[46]

3. Antifungal activity

Turmeric shows strong antifungal properties. In one study, ehanolic extract of *C. longa* inhibited the growth of 29 strains of dermatophytes. The inhibition zone for this extract was 6.1–26.0 mm. [47] Turmeric extracts in chloroform and ether have been found to be antibacterial, antifungal, antiviral, and anti-protozoan. Turmeric showed broad-spectrum antimicrobial activity in an antibiotic screening test. [48]

4. Antiviral Effect

The current antiviral treatments are ineffective and poorly tolerated. Curcumin has been found to have potent antiviral properties. It inhibits various viruses and its potential in inhibiting Epstein-Barr virus activity has been demonstrated. Curcumin also shows anti-HIV activity, they do so by inhibiting the HIV-1 integrase, which is required for virus replication.[39]

5. Anticancer activity

In many developing countries, cancer is the leading cause of death. Numerous studies have revealed that people who consume more vegetables and fruits have a lower risk of cancer. Flavonoids, bioactive composites found in plant foods, are responsible for this result.. [49] Turmeric's anticancer properties include inhibiting cell growth and triggering death of cancer cells.[50]

6. Wound-healing ability

Turmeric's ability to heal wounds is another intriguing feature. In one study, stability of collagen, aldehyde content, and tensile strength were observed to be increased in rat models which were treated with *C. longa*, indicating better collagen maturation and crosslinking. The findings demonstrate that topical administration of *C. longa* boost the wound healing process and has an antioxidant effect. [51]

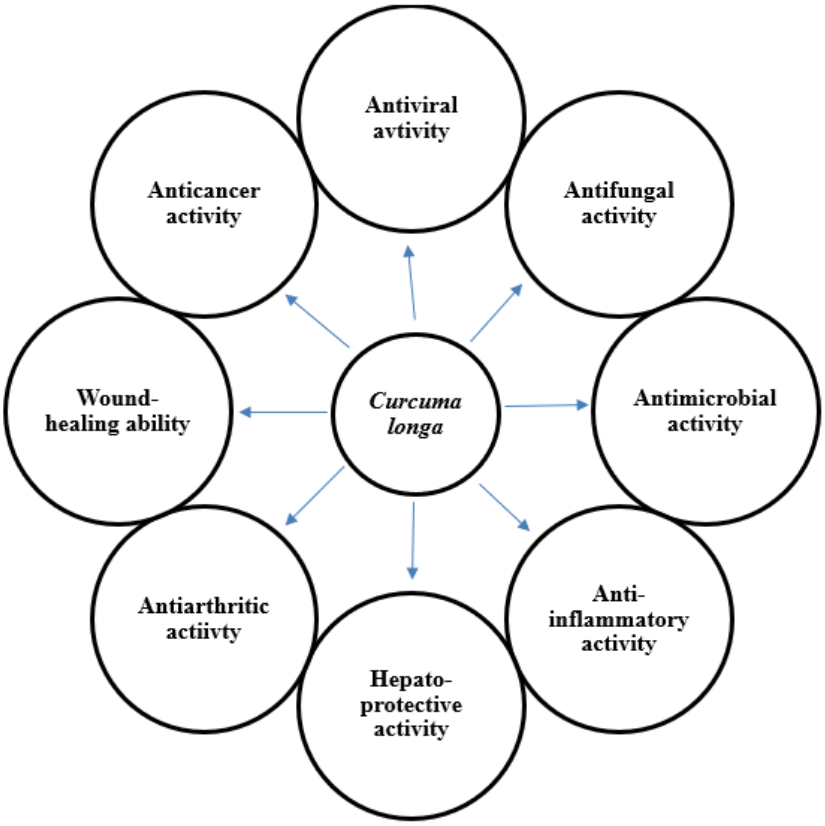


Fig. 3 Some major medicinal properties of turmeric.

CHAPTER 3: MATERIALS AND METHODS

3.1 PREPARATION OF PLANT EXTRACT

3.1.1 Collection of plant material

***Azadirachta indica* (Neem)**

The leaves of neem were collected from the outfield near to village Dumehari, located in Kandaghat, Himachal Pradesh.

***Curcumin longa* (Turmeric)**

The roots of turmeric were collected from the outfield near to village Dumehar, located in Arki, Himachal Pradesh.

The plant leaves and roots were cleaned carefully with water, dried for ten days, grinded in a grinding machine to obtain powdered form, and then it was used for extraction.

3.1.2 Extraction

Soxhlet extraction method was used to extract the powdered plant material from an organic solvent. In this method, ethanol was used as solvent. 250 ml of ethanol was poured in the round bottom flask which was heated using the isomantle. Firstly, the coarsely grounded powder (20 gm) was placed in a porous bag or "thimble" and placed in the Soxhlet apparatus' chamber. The ethanol solvent (250 ml) in the flask was heated at its boiling point (78.37), as a result, solvent begins to evaporate and vapors condensed in the condenser. Then condensate from condenser, drips into the reservoir containing the thimble. The cycle repeats itself once the level of solvent reaches the syphon. The complete process was done for 12 hours for 3 days. The obtained extract was subject to evaporation in a rotary evaporator then extract was refrigerated at 4°C for later use.

Fig. 4 A Soxhlet apparatus.

3.2 CHARACTERIZATION OF PLANT EXTRACT

3.2.1 Qualitative screening of phytochemicals

Different qualitative assays were performed on neem and turmeric extracts to detect the presence of various phytoconstituents.[52] [53]

a) Detection of Carbohydrates

2-3 drops of alcoholic -naphthol solution is added to 3ml of plant extract solution. After that, add few drops of concentrated sulfuric acid to the mixture due to which a violet ring appears at the junction of two liquids in the test tube indicating the presence of carbohydrates.

b) Detection of Alkaloids

Add 2 millilitres of hydrochloric acid in test sample. Then, add few drops of Wagner's reagent into the mixture. The occurrence of reddish-brown precipitates is the indication for the presence of alkaloids.

c) Detection of Flavonoids

Add few drops of NaOH to two ml of the extract producing intense yellow color. After that add few drops of dilute HCl to the mixture. If there are flavanoids present, the solution will turn colourless.

d) Detection of Saponins

Few drops of distilled water were added into the plant extract solution. Then it was shaked vigorously. Emergence of foam indicates the existence of Saponins.

e) Detection of Sterols

2 ml concentrated sulphuric acid is added to 2 ml of the test sample. The appearance of red precipitate indicates the existence of sterols.

f) Detection of Tannins

1 ml of 3% of Ferric chloride is added to 1 millilitre of the plant extract solution. The appearance of a brownish green colour indicates the existence of tannins.

3.2.2 Quantitative screening of phytochemicals

a) Estimation of Total Carbohydrate Content

Prepare different concentration of glucose (0,2,4,6,8 and 10 mg/ml) from plant extract stock of 10mg/ml. Add 1 millilitre of test sample in 4 millilitres of anthrone. Maintain the test tubes in water bath for 8 minutes at 100°C. Using a microplate reader, measure the absorbance of this reaction mixture at 630nm. Calculate carbohydrate content as glucose equivalents (mg/gdw). [54]

b) Estimation of Total Protein Content

Reagents:

Reagent A: 48 millilitres of 2% sodium carbonate in 0.10 N sodium hydroxide + 1ml 0.5% $CuSO_4.5H_20$ in sodium potassium tartrate.

Reagent B: Diluted Folin Reagent (1:1)

Methodology:

Prepare Reagent A by adding 48ml of Na_2CO_3 in 0.1N NaOH and 1 ml of sodium potassium tartrate in 1 ml of copper sulphate. Reagent B was prepared by taking 1:1 ratio of Folin

reagent in distilled water. 1 millilitre of test sample and 4 millilitres of Reagent A should be mixed together, then left to stand at room temperature for 10 minutes. Add 0.5 millilitre of Reagent B to the mixture. After then, set it aside in the dark for 30 minutes. In a microplate reader, measure the absorbance at 750nm. [55]

c) Estimation of Total Flavonoid Content

To make a 10% solution of Quercetin, dissolve 1g of Quercetin in 100 mL methanol. Prepare dilutions of different concentrations (0.1, 0.5, 1.0, 2.5, and 5mg/ml) in methanol from standard quercetin solution to generate a standard gallic acid curve. Allow to stand for 6 minutes after mixing 100 l of each quercetin dilution with 500 ul of distilled water and 100 l of 5% sodium nitrate. After that, add 150 ul of a 10% aluminium chloride solution and let it sit for 5 minutes. After that, gradually add 200 ul of a 1M sodium hydroxide solution. Using a microplate reader, measure the absorbance at 510 nm. Calculate flavonoid content as quercetin equivalents (mgQE/g). [56]

3.3 Evaluating biochemical properties of microorganisms.

3.3.1 Gram staining:

Bacterial suspension was prepared on glass slide and air dried. Crystal violet was added and kept for 30 sec to 1 min and was rinsed with water. Later, iodine stain was added for 1 min and was washed with water. 95 % ethanol was added for 30 sec followed by safranin for 1 min and was washed with water. It was then air dried and observed under microscope.

3.3.2 Catalase test:

By using a sterile loop, small colony of bacteria was picked up from agar plate. It was then transferred to clean glass slide and 3% H_2O_2 was added. Observe the evolution of oxygen bubbles.

3.3.3 KOH test:

Bacterial specimen was kept on the clean slide. 20% KOH was added. It was then observed under microscope.

3.3.4 Methyl Red test:

Prepare MRVP broth in test tubes. Inoculate the broth aseptically with 2 loopful of respective bacterial culture. Label the test tubes with name of organism inoculated. Incubate the test tubes

at 37°C for 48-72 hours. Add few drops of methyl red indicator in the incubated tubes. Observe the results.

3.4 Evaluating biological properties of plant extracts

3.4.1 Evaluation of Antioxidant activity of plant extract.

ABTS assay:

To produce ABTS free radical solution, thoroughly mix ABTS (7 Millimolar) and potassium persulfate (2.45 Millimolar) solutions and incubate 24 hrs in the dark. At 745nm, add methanol to 0.7 to adjust the absorption of this mixture. Incubate for 6 minutes after mixing 300L extract working dilutions with 3.0mL ABTS solutions. Finally, using a microplate reader determine the absorbance at 745nm. Positive control was Gallic acid. The scavenging potential of ABTS in percent was calculated by using the following formula:

$$= \frac{\text{Control absorbance} - \text{sample absorbance}}{\text{Control absorbance}} * 100$$

DPPH scavenging assay:

Make a 0.002% DPPH solution in methanol and measure the absorbance at 515 nm. Mix 50 ul plant extract (1 mg/mL methanol) with 3 ml DPPH solution and set down for 15 minutes in the dark. At 515 nm, record the absorbance once more. Using the formula below, calculate the percentage inhibition of DPPH by plant extracts.

$$\% \text{ Inhibition} = \frac{(\text{A} - \text{B})}{\text{A}} \text{X } 100$$

Here A represents the absorbance of pure DPPH in its oxidised state, and B represents the absorbance of test sample which is measured after 15 minutes of DPPH reaction. Create a percentage inhibition of Galic acid calibration curve for different concentrations to determine the IC50 values, which are the concentration at which 50% of the DPPH solution is inhibited. [57]

3.4.2 Evaluation of Antimicrobial activity of plant extract.

Well Diffusion Method:

To inoculate on Muller-Hinton agar, grow bacteria in Muller-Hinton broth to match the turbidity of 0.5 McFarland standards (106 cells). Dry the plates for 15 minutes after inoculation, then punch the wells with sterile pipette tip. Fill the wells with 50 ul of plant extracts in various concentrations, as well as a positive control (antibiotic) and a blank control (DMSO). Allow plant extracts to diffuse through the agar media and form zones of inhibition by incubating the plates for 24 hours (37°C). After incubation, calculate the diameters of the inhibition zones for various plant extracts when used against various bacteria. [58]

MIC:

Twofold serial dilution method:

It is evaluated in flat-bottomed 96-well micro-titre plates with 100 ul of MH broth in each well. Along the x axis, pour 100 ul extracts of various concentrations into the wells. To each well, add 10 ul of bacterial cells. A number of wells in the plate should be reserved for sterility (no cells), viability (no extract/drug added, cells + drug), and negative controls. At 37°C, incubate the plates for 24 hrs. In each well, add 10 ul of resazurin dye and incubate for 2-4 hours in the dark at 37°C, until the colour of the wells changes from blue to pink, indicating microbial growth. [59]

3.5 Evaluation of proliferation activity of plant extract.

MTT assay:

3T3 fibroblast cell line was purchased from NCCS, Pune. These cells were maintained in high glucose DMEM, 10% FBS and 1% penicillin-streptomycin at 37°C, 95% humidity and 5% CO_2.

Cell proliferation was determined by MTT assay. Using a cell dissociating solution, separate raw cells (trypsin in PBS). For 5-7 minutes, centrifuge the cells. In 96-well culture plates, seed the cells and incubate for 24 hours in CO_2 incubator (at 37°C and 5% CO_2). Fill microtiter plates with 100 ul of different plant extract concentrations (25, 50, 75, 100 mg/ml) and keep it in CO_2 incubator for 24 hours. After incubation, discard the test solutions and fill each well with MTT (0.650 mg/ml MTT in PBS). Incubate the plates at 37°C for 4 hours in a 5% CO2 atmosphere. Remove the supernatant and add 100 ul of DMSO to the plates, gently shaking them to solubilize the formazan that has formed. Measure the absorbance at a

wavelength of 570nm. Calculate the percentage inhibition and 50% (IC50) values. This formula is used to calculate the percent inhibition.

$$\% \text{ Inhibition } = \frac{\text{OD of Control} - \text{OD of sample)}}{\text{OD of control}} \text{X } 100$$

CHAPTER 4: RESULTS

4.1 Percentage yield of plant extract

Dry weight of the neem and turmeric sample was 20gm and after solvent evaporation it came to be 5.7gm for turmeric and 4.9gm for neem.

The percentage yield of plant was calculated by following equation:

$$\frac{\text{Weight of extract after evaporating solvent and drying}}{\text{Dry weight of the sample}} x\ 100$$

Obtained yield of neem was about 24.5% and 28.5% for turmeric.

4.2 Result of qualitative screening of phytochemicals

Qualitative screening was done for both the plant extracts and the result obtained for carbohydrate, amino acid, saponin, glycoside, tannin was positive.

Phytochemical test	Result	Observance
Carbohydrate (Molisch Test)	Positive	Violet ring was observed
Amino Test (Ninhydrin)	Positive	Dark purple colour was observed
Saponin test	Positive	Foam formation was observed
Tannin Test (Ferric chloride test)	Positive	Deep blue colour was not observed

Glycoside Test (Salkowski Test)	Positive	Reddish colour was observed

Table 4. Result of phytochemical screening.

4.3 Result of quantitative screening of phytochemicals

Various quantitative test was done on plant extracts, it was observed that carbohydrate content was more in turmeric and protein content was more in neem extracts. Flavonoid content was also estimated and turmeric was high in flavonoids.

Plants	**Carbohydrates%**	**Proteins%**	**Flavonoids (**mgQE/g)
C. longa	3.64 ± 0.476	2.262 ± 0.49	89 ± 0.153
A. indica	8.946 ± 1.428	2.999 ± 0.43	65.25 ± 0.557

Table 5. Result of quantitative screening of phytochemicals

4.4 Result of biochemical properties of microorganisms

On the different bacterial species, biochemical test was performed and in gram staining *S. aureus* and *B. subtilis* were tested as gram positive and *S.typhi* and E.coli were tested gram negative. All the microbes taken in study were tested positive for catalase test. *S. aureus* and *B. subtilis* were negative for KOH test and positive for *S.typhi* and *E.coli*. In MR test, *S.typhi* and *E.coli* were positive and *S. aureus* and *B. subtilis* were negative.

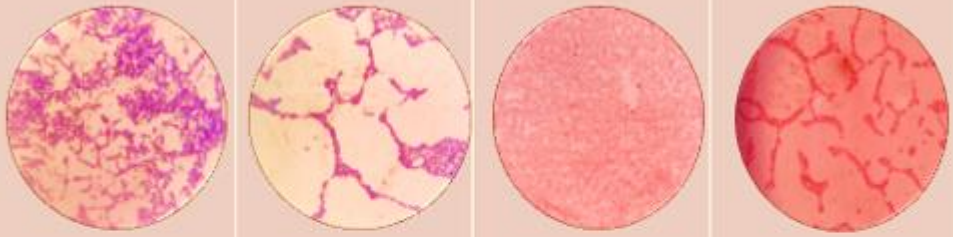
	S. aureus	*B. subtilis*	*S. typhi*	*E. coli*
Gram Staining				
Catalase	+ve	+ve	+ve	+ve
KOH	-ve	-ve	+ve	+ve
MR	-ve	-ve	+ve	+ve

Fig. 5 Result of various biochemical tests.

4.5 Result for minimum inhibitory concentration

Sample	**Concentration (ug/ml)**
A. indica -50mg/ml	6.25
C. longa - 50mg/ml	0.195
Antibiotic (Ampicillin) -50mg/ml	12.25
Cream (Calendula gel) – 50mg/ml	6.25

Table 6: Minimum Inhibitory concentration for *Bacillus subtilis*

Sample	**Concentration (ug/ml)**
A. indica -50mg/ml	6.25
C. longa - 50mg/ml	0.781
Antibiotic (Ampicillin) -50mg/ml	1.56
Cream (Calendula gel) – 50mg/ml	6.25

Table 7: Minimum Inhibitory concentration for *S. aureus*

Sample	**Concentration (ug/ml)**
A. indica -50mg/ml	3.12
C. longa - 50mg/ml	.195
Antibiotic (Ampicillin) -50mg/ml	.390
Cream (Calendula gel) – 50mg/ml	3.12

Table 8. Minimum Inhibitory concentration for *E. coli*

Sample	**Concentration (ug/ml)**
A. indica -50mg/ml	3.12
C. longa - 50mg/ml	.781
Antibiotic (Ampicillin) -50mg/ml	6.25
Cream (Calendula gel) – 50mg/ml	6.25

Table 9. Minimum Inhibitory concentration for *S.typhii*

4.5 Result of Antioxidant activity

4.5.1 For DPPH assay:

Concentration (ug/ml)	**% Inhibition For *C. longa***	**% Inhibition For *A. indica***
0	0	0
250	16.46	15.11
500	32.03	23.65
750	48.80	33.83
1000	62.15	48.05

Table 10 Result of DPPH assay showing percentage inhibition of selected plant extracts.

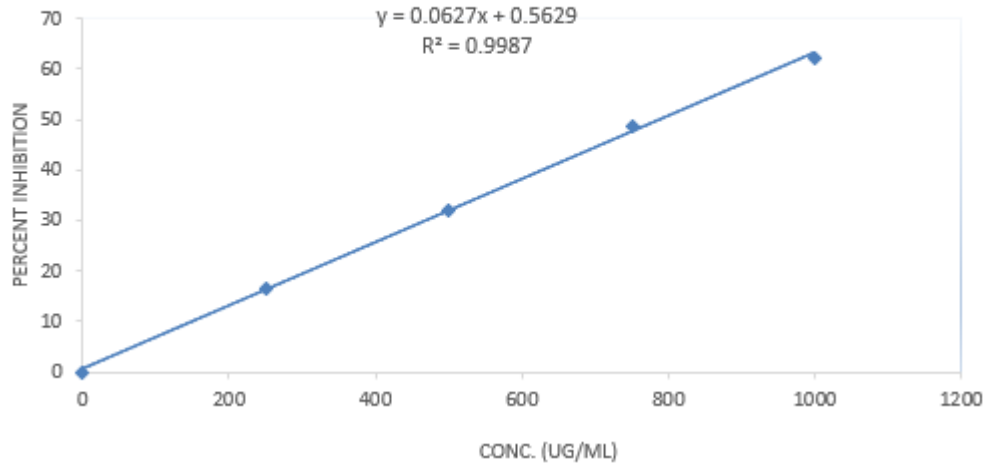


Fig. 6 Graph of percentage inhibition vs. conc. of *C. longa* in DPPH assay.

IC50 Value was calculated by using this equation: y = 0.0627x + 0.5629

IC50 value for *C. longa* was: 0.788mg

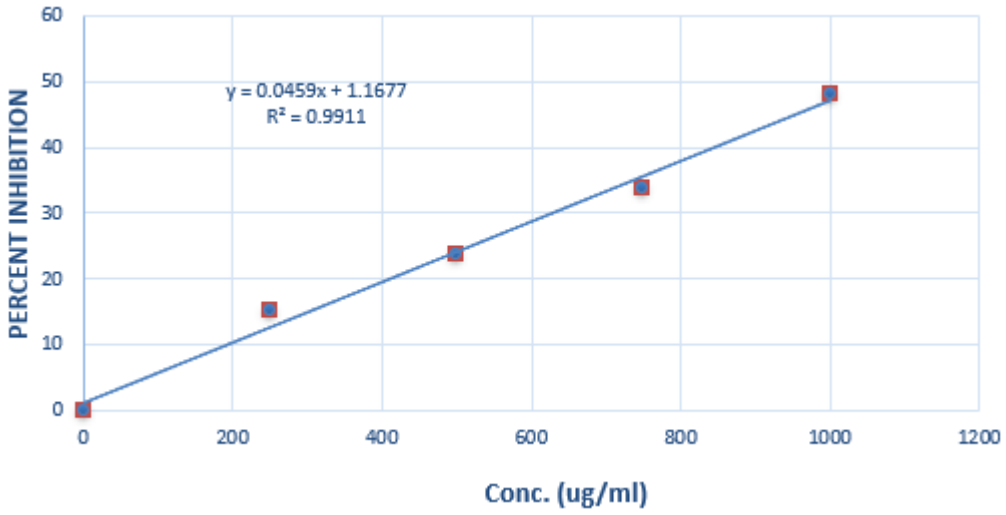


Fig. 7 Graph of percentage inhibition vs. conc. of *A. indica* in DPPH assay.

IC50 Value was calculated by using this equation: y = 0.0459x + 1.1677

IC50 value for *A. indica* was: 1.063mg

4.5.2 Result for ABTS assay:

Concentration (ug/ml)	% Inhibition For *C. longa*	% Inhibition For *A. indica*
0	0	0
250	17.43	15.37
500	29.66	25.22
750	47.77	34.23
1000	60.74	45.81

Table 11 Result of ABTS assay showing percentage inhibition of selected plant extracts.

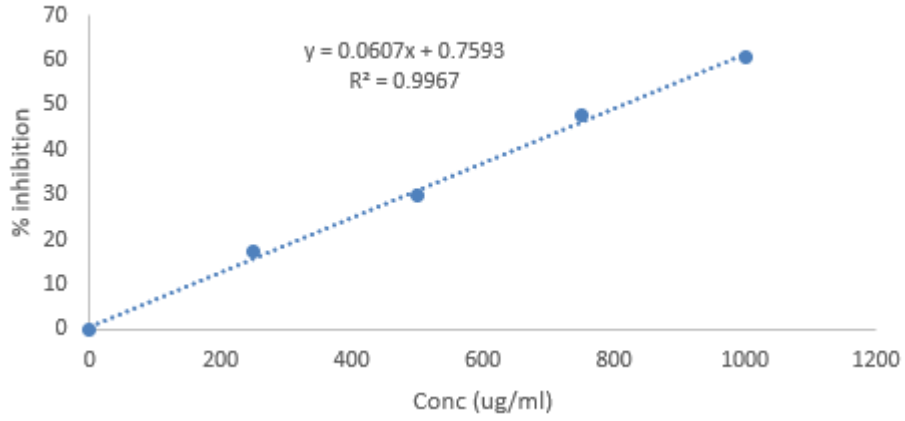


Fig. 8 Graph of percentage inhibition vs. conc. of *C. longa* in ABTS assay.

IC50 Value was calculated by using this equation: = 0.0607x + 0.7593

IC50 value for *C. longa* was: 0.811mg

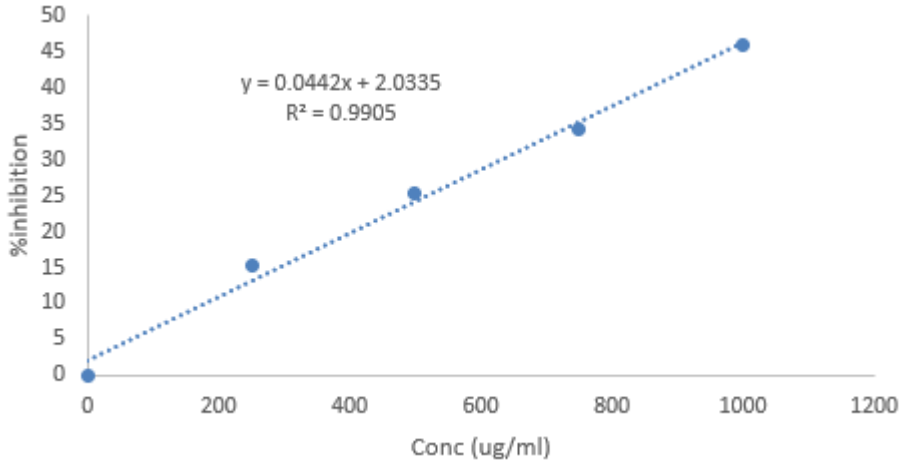


Fig. 9 Graph of percentage inhibition vs. conc. of *A. indica* in ABTS assay

IC50 Value was calculated by using this equation: y = 0.0442x + 2.0335

IC50 value for *A. indica* was: 1.085mg

4.6 Result of proliferation activity

Result from MTT assay revealed that plants extracts are having proliferation effect on 3T3 cells. Both the plants showed 2-fold increase in the cell proliferation. Although, neem plant was more effective in showing proliferation activity than turmeric.

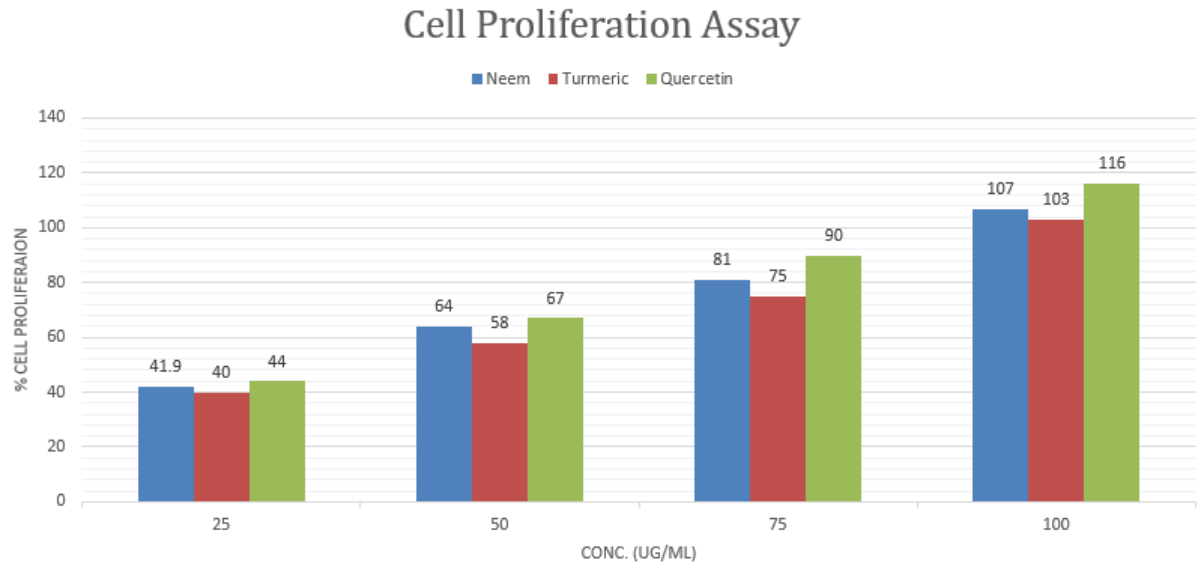


Fig. 10 Graph of percentage cell proliferation activity for *C. longa* and *A. indica*.

CHAPTER 4: CONCLUSION

The purpose of the present study was to evaluate wound healing activity of the selected plants employing the in vitro wound healing assays. The present study endeavours to identify and develop new a therapeutic agent for wound care management. According to the WHO, almost 75% of the world's population has incorporated the use of medicinal products in their life as an alternate of allopathy drugs. Plant based medicines can serve as better alternative therapeutic agents for the people who live in places where conventional drugs cannot be supplied. However, the scientific evidence of their efficacy is limited therefore it is important to introduce a scientific validation for the medicinal effect of plants used in traditional medicine.

In present study, it has been found that selected plants have strong anti-microbial and anti-oxidant activity. An extensive review of literature had revealed that many plants used traditionally in treatment of wound possess anti-microbial, anti-oxidant and wound healing phytochemicals which encourages blood clotting, fight infection and accelerate the healing of wounds. Selected plants also showed cell proliferation effect in cell line assay indicating that they could be a potent candidate for wound healing. So, it is good to assume that these plants can strongly influence the wounds and can further increase the healing process.

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