

ANTIBIOFILM ACTIVITY OF METHANOLIC AND AQUEOUS EXTRACTS OF PLANTS *VITEX NEGUNDO* AND *ECLIPTA PROSTRATA*

Submitted in partial fulfillment of the requirements for the award of Bachelor of
Technology degree in Biotechnology

by

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**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING**

SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

**Accredited with "A" grade by NAAC | 12B Status by UGC | Approved by AICTE
JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI - 600 119**

April - 2021



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BONAFIDE CERTIFICATE

This is to certify that this project report is the bonafide work of Badugu Reddy Baveen (37230010) and Dubbaka Sai Teja (37230015) who carried out the project entitled “ANTIBIOFILM ACTIVITY OF METHANOLIC AND AQUEOUS EXTRACTS OF PLANTS *VITEX NEGUNDO* AND *ECLIPTA PROSTRATA*” under our supervision from December 2020 to March 2021.

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Certification of Project Work

This is to certify that Badugu Reddy Baveen (Reg: 37230010), B.tech Biotechnology, Sathyabama Institute of Science and Technology, Chennai, has been with us for working on a project work under the title " Antibiofilm activity of methanolic and aqueous extract of plants Vitex negundo and Eclipta prostrata" from December 7th 2020 to March 8th 2021. The work embodied in this dissertation is strictly confidential and is the sole property of the institute and should not be published / used in part of full or patented without the prior permission of the supervisor.

Thanks and regards,

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DECLARATION

We Badugu Reddy Baveen (37230010) and Dubbaka Sai Teja(37230010) hereby declare that the Project Report entitled “Antibiofilm activity of methanolic and aqueous extracts of plants *Vitex negundo* and *Ecliptaprostrata*” done by us under the guidance of Dr. Thyagarajan.R, Assistant Professor (Internal) and Dr. Sabitha.Y (External),Managing director at CienciaLife Sciences,Hyderabad is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

DATE:

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ABSTRACT

Biofilm is a complex microbial community highly resistant to antimicrobials. The formation of biofilms in biotic and abiotic surfaces is related to high rates of morbidity and mortality in hospitalized patients. Plants are the oldest source of pharmacologically active compounds and have provided humankind with many therapeutic properties for hundreds of years. In this study aqueous and methanol extracts of leaves of *Vitex negundo* and *Eclipta prostrata* were tested for phytochemical analysis, minimum inhibitory concentration, antibacterial, antifungal and biofilm inhibition. Phytochemical analyses reveal the presence of phenols, steroids, saponins. The minimum inhibitory concentration (MIC) was determined ranging from 0.019 µg/mL to 1.25 µg/mL against *E.coli*. The diameter of inhibition zone was obtained for antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Bacillus cereus* at different concentrations. Antifungal activity against *Aspergillus Niger* were tested at 50µL and 100µL concentrations. In this study biofilm formation was actively shown in *E.coli* and *S. aureus*. The ability of plant extracts to inhibit preformed bacterial biofilms was also determined. Among the 4 plant extracts, methanol extract of *E.prostrata* significantly inhibited the growth of both bacteria by over 71.5% and over 84.7% at 200µL concentration. The present result demonstrates that the medicinal plants *V.negundo* and *E.prostrata* are potentially useful as antibiofilm agents in preventing biofilm infections.

Keywords:

Anti-biofilm; *Vitex negundo*; *Eclipta prostrata*; methanol; aqueous; antimicrobial; MIC; phytochemicals; therapeutics.

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ABBREVIATIONS

AAM	American Academy of Microbiology
AMR	Antimicrobial resistance
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming units
CuSO ₄	Copper sulphate
CV	Crystal violet
CVCs	Central venous catheters
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EMJH	Ellinghausen-McCullough-Johnson-Harris
EP	<i>Eclipta prostrate</i>
EPS	Exopolysaccharide
EPS	Extracellular polymeric substances
FeCl ₃	Ferric chloride
GAA	Glacial acetic acid
GIT	Gastrointestinal tract
GTase	Glucosyltransferase
H ₂ SO ₄	Sulfuric acid
HCL	Hydrochloric acid
HIV	Human immunodeficiency virus

LD	Lethal dose
MBC	Minimum bactericidal concentration
MH	Muller-Hinton agar
MIC	Minimum inhibitory concentration
NaCl	Sodium chloride
NaOH	Sodium hydroxide
OD	Optical density
PDA	Potato dextrose agar
QS	Quorum sensing
SBF	Specific biofilm formation
UTI	Urinary tract infection
VN	Vitex negundo
°C	Celsius
μL	Microliter
μg	Microgram
mL	Millilitre
Mg	Milligram
Gms	Grams
M	Molarity
H	Hours

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CHAPTER-1

INTRODUCTION

Biofilms are formations that occur when a group of microorganisms like bacteria, fungi, parasites and viruses attached to the living or inert surface and is embedded within the extracellular polymeric substances (EPS) and create a colony. These biofilms attach to the epithelia layer of multicellular organisms, abiotic surfaces and interfaces between air and water. There are two basic forms of microbial existence: free-floating or free-swimming(planktonic) and anchored-sessile forms (biofilms and spores) (Moldenhauer,2018).

In 1970s,the study on biofilm has been extensive, with significant evidence showing that bacteria exist primarily as a biofilm phenotype in medical, natural and industrial ecosystem. The biofilm forms itself into a form of 'shield'that features a glue-like consistency, often mentioned as 'slime'. These biofilms act as a barrier and makes the microbes stronger and more resistant to attack, e.g., antimicrobials, disinfectants, and host immune defenses.

Biofilms are often a part of the rationale that some wounds could also be difficult to heal, and protracted infections keep recurring. They adhere to surfaces such as lining of the gastrointestinal tract (GIT), urinary tract, respiratory system, heart, mouth (including teeth), sex organs, eyes, middle ear and skin. However, biofilms are not always undesirable, and can have useful effects on food chains, sewage treatment of plants, to eliminate petroleum oil or hydrocarbon spillage from the oceans and human chronic bacterial infections (Georgiou,2017).

1.1 HOW DO BIOFILMS FORM:

The formation of bacterial biofilms must, necessarily, begin with the adhesion of a small number of bacterial cells to a surface. It seems ironic that trained microbiologists often have considerable difficulties in producing biofilms for their first experiments in this area, even while their children's aquariums are filthy and opaque because of the sessile bacterial growth. The most issue emanates from the tendency of the microbiologists to use laboratory-adapted microorganism

strains, whose behaviour they'll anticipate, in distinction with 'wild' strains contemporary from their natural habitats.

Many laboratory-adapted strains have virtually lost their ability to adhere to surfaces because, whenever they are transferred into contemporary medium, the inoculum for this operation is taken from the bulk fluid and any bacterial cells that are adherent to the walls of the vessel are residual. After a few hundreds of transfers of this kind, most laboratory-adapted bacterial strains have been heavily selected for rapid growth and against adhesion to surfaces. Bacterial strains that have been selected in fast moving streams or in the lungs of cystic fibrosis patients, actually grow as 'slime balls' during their initial few transfers in laboratory culture.

Wild bacterial strains should survive in nature, among a host of antibacterial factors, and that they would like their protecting outer layers even a lot of desperately than they have to stick to surfaces. Wild bacterial strains, in real ecosystems, have a thick layer of exopolysaccharide (EPS) surrounding each cell, through which long fimbriae protrude to form an effective part of the cell surface. These wild strains of bacteria would rely on their fimbriae for specific adhesion to well defined ligands on tissue surfaces, and on their EPS for nonspecific adhesion to inert surfaces. It is important to note that the specific adhesions mediated by fimbriae are not strong, being based on the interaction of protein structures, which are not, in themselves, mechanically strong (Gayathri and Mani, 2017).

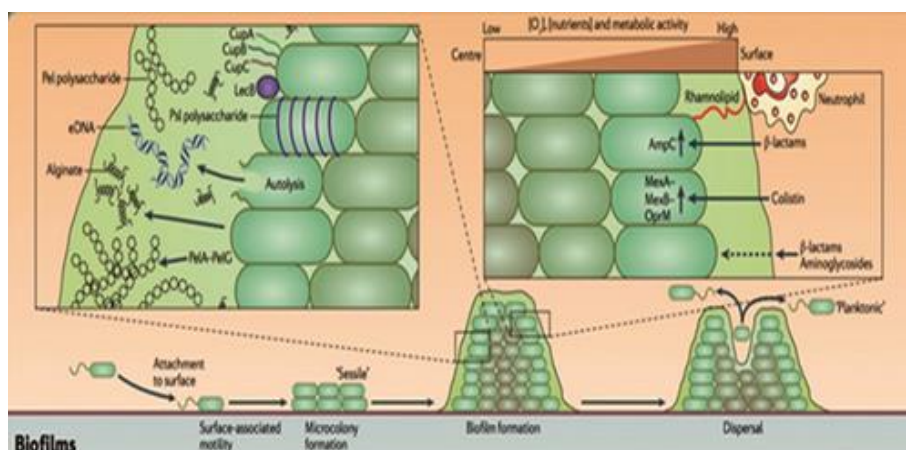


Fig: 1.1: Formation of bacterial biofilm.

(source : Gayathri and Mani, 2017)

Fimbriae can be removed from bacterial cells by simple sonication, and it is clear that these fragile protein structures serve primarily to align the bacteria in close juxtaposition to the specific receptors of the tissues concerned. Firm adhesion to surfaces requires the interaction of the remarkably elastic polymers of the EPS layer, even when the location of the eventual adhesion is dictated by the interaction of fimbriae with specific receptors. It is the peculiar ability of these EPS polymers to conform very closely to the surface to be colonized that makes them so very effective as nonspecific ligands in aquatic ecosystems.

1.2 LIFE CYCLE OF BIOFILMS

The life cycle of biofilm contains 4 stages: First the cells are adhered to a substrate such as polysaccharides or glycoprotein and form microcolonies (Hauser,2011). As a lot of cell aggregates, the concentration of chemical signals reaches a point that activates genetic changes within the cells that cause them to attach firmly to the surface and neighboring cells. These microcolonies produce a thick extracellular matrix composed of exopolysaccharides (EPS), proteins, extracellular DNA and other polymer that forms a protective physical barrier around the bacteria, allowing them to grow into a mature biofilm of complex communities that are capable of chemical communication, a process called upon quorum sensing (QS), these molecules have been shown to be essential for biofilm formation (Chen et al.,2004) (Hornby and Nickerson,2004).

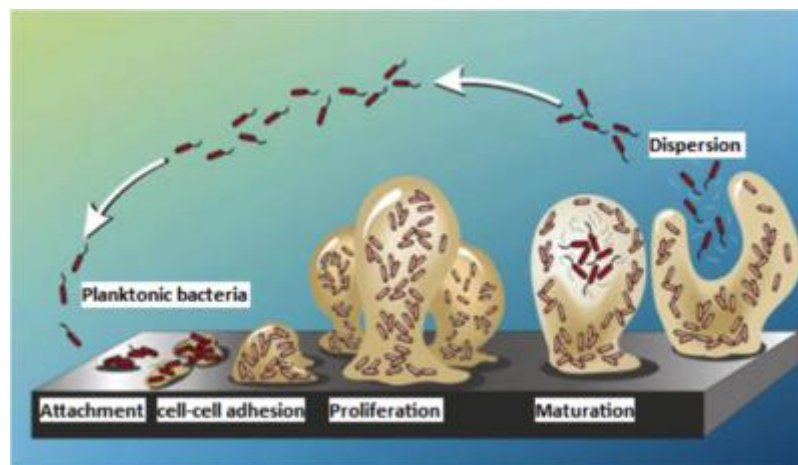


Fig: 1.2: Life cycle of biofilm.

Once the biofilm reaches particular cell density, point of saturation, biofilm turns off the expression of gene producing EPS products and reactivates flagella motility genes to disperse new planktonic cells from disseminative biofilm in search of recent surroundings. The relationship between the host and its microbial communities is fastidiously balanced, however under certain conditions, it will break down and end in infectious diseases. Microbes of biofilm secrete specific toxins, generate a hypoxemic microenvironment, and therefore the host immune system, from all of which can contribute to delayed wound healing. According to a recent publication announcement from the National Institutes of Health, quite 60% of all microbial infections are caused by biofilms (Zhao et al., 2012).

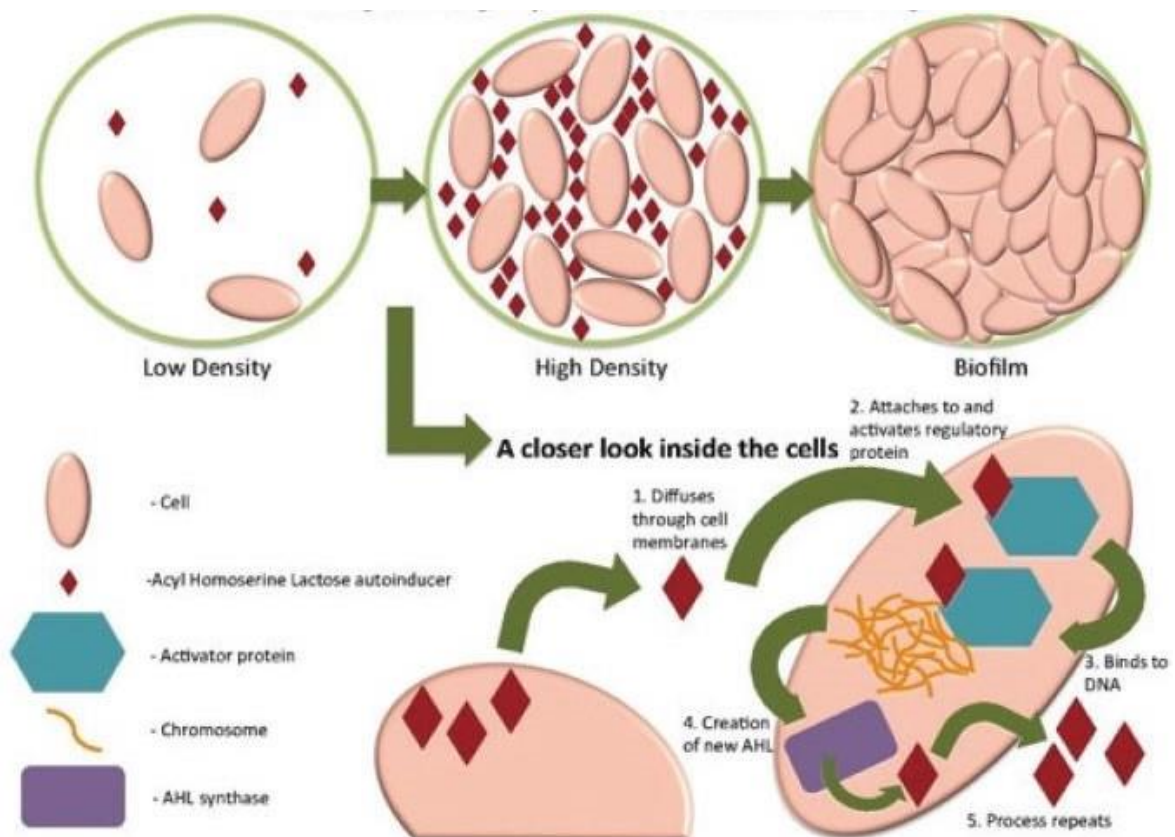


Fig: 1.3: Gram-negative single species bacteria Quorum Sensing.

(source: <https://www.google.co.in/search?q=Gramnegative+single+species+bacteria+Quorum+Sensing.>)

The studies conducted by Cho's team (Cho et al., 2007) provided new potential mechanisms for the formation of complicated biofilms. Interactions between the cells and therefore the corresponding organization through the propagation and attachment phases are vital. Formation of biofilms are affected by the cell morphologies and the physical boundaries confining them that this understanding may aid in the control, growth and treatment of biofilms.

Till now, 80% of human bacterial infections are formed by biofilm-associated, mostly infections are frequently being caused by *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Gayathri and Mani, 2017).

1.3 VITEX NEGUNDO

1.3.1 Plant description:

Vitex negundo also known as the Chinese chaste tree, five-leaved chaste tree, or horseshoe vitex, is an aromatic deciduous shrub with quadrangular, densely whitish, tomentose branchlets. It is an erect large shrub or sometimes a small slender tree growing from 2 to 8 m in height. Its bark is thin, grey or reddish brown; branchlets quadrangular, whitish with a white tomentum. Leaves are digitate 3-5 foliolate; leaflets lanceolate, acute, the terminal leaflet 5-10 by 1.6-3.2 cm. with a petiole 1-1.3 cm long, the lateral leaflets smaller with a very short petiole, all nearly glabrous above, covered with a fine white tomentum beneath, base acute; common petioles 2.5-3.8 cm long. The leaf edges are toothed or serrated and the bottom surface is covered in hair.

The numerous flowers are borne in panicles 10 to 20 cm in length. Each flower is around 6 to 7 cm long and is white to blue in colour. The petals are of different lengths, with the middle lower lobe being the longest. Both the corolla and calyx are covered in dense hairs. Calyx 3 mm long, white-tomentose; teeth triangular, 0.8-1 mm long. Corolla 1 cm long, bluish purple,

tomentose outside, hairy inside at the insertion of the stamens. Filaments hairy at the very base. Ovary glabrous; stigma forked. Drupe less than 6 mm. diameter, black when ripe. The fruit is a succulent drupe, 4 mm (0.16 in) in diameter, rounded to egg-shaped. It is black or purple when ripe.

1.3.1.1 Morphology :

VN has trifoliate, occasionally Penta foliate leaves with petiole of 2-4 cm. Trifoliate leaves are lanceolate with 5-10 cm long and 2-3.5 cm wide middle leaf. The remaining leaves on either side are sub-sessile. Leaves have a glabrous surface, tomentose bottom, and leathery texture.



Fig:1.4:Five-leaved chaste tree.

1.3.1.2 Range:

Widespread in the tropics and subtropics from east Africa, through Asia to Indonesia, the Philippines and Mariana Islands.

1.3.1.3 Habitat:

Wasteland up to 2,000 metres in the Himalayas. Mixed thickets on mountain slopes at elevations of 200 – 1,400 metres in China.

1.3.1.4 Parts Used:

Leaves, Seeds, Roots and essential oil of fresh leaves, flowers and dried fruits and bark.



Fig:1.5: Inflorescence of VN.

(source : nhp.gov.in)

1.3.1.5 Common Names:

Bengali:	Nirgundi, Nishinda, Nisinda, Samalu
Marathi:	Lingur, Nirguda, Nirgunda, Nirgundi, Nirgur
Sanskrit:	Indrani, Nilapushpa, Nilanirgundi, Nirgundi, Shephali,
Tamil:	Nirkkundi, Nochi, Sinduvaram, Vennochi
Telugu:	Nallavavili, Sinduvaramu, Vavili, Vayila

1.3.1.6 Scientific Classification:

Kingdom:	Plantae
Subkingdom:	Tracheobionta
Division:	Magnoliophyta
Class:	Magnoliopsida

Subclass:	Asteridae
Order:	Lamiales
Family:	Lamiaceae
Genus:	<i>Vitex</i>
Species:	<i>negundo</i>

1.3.2 Therapeutic applications:

Seeds soaked in vinegar are used as hot fomentation in case of flatulence. It cures cough and boils. It is beneficial in leprosy, asthma and rectal prolapse. Oral use of powdered seeds helpful in regularising the menstrual cycle. Seeds are used orally along with sikanjabeen to resolve hard swellings of spleen. Oral use of powdered seeds helpful in reducing pain during menses. Powdered seeds are used orally in case of poor lactation. Oral uses of powdered seeds act as emmenagogue. Oral use of powdered fruit in the form of tablet reduces arthralgia. Oral use of pounded leaves is helpful in relapsing fever. Powdered dry fruit is used orally to reduce back pain and headache.

Local application of pounded leaves is helpful in fissure in ano. Pounded leaves cooked in oil is applied locally to treat putrefied ulcers and also to resolve hard swellings. Local application of warm leaves is beneficial in resolving warmebarid (cold swelling) and flatulence. Juice of green leaves dropped into eyes to improve the vision. Gargling with decoction of leaves is helpful to treat mouth ulcers and throat pain. Sitz bath in its decoction is helpful in warmemaqaad (proctitis). Leaves soaked in cold water whole night and are used as gargle to treat toothache, gingivitis, bleeding gums, halitosis and excessive salivation. Oral use of decoction of leaves helpful in cold, coryza and headache.

Pillow stuffed with its leaves is used to reduce headache. Juice of leaves is used locally to treat skin diseases and swellings. Tablet formed with pounded leaves in the dose of 3gm and cooked in ghee is used for one week in puerperium with benefit. Dried leaves are used in hukka which is beneficial in cold and headache. Its root and leaves act as a diaphoretic and diaphoresis. Grind together its leaves

along with garlic, rice and jaggery and used orally to get wormicidal effect. Fumigation with its leaves and fruit along with pudinasehrai act as emmenagogue. Sitz bath with decoction of leaves of *Vitex negundo* Linn. Or boiled leaves can be applied locally to reduce inflammation of uterus. Sitz bath with decoction of leaves of *Vitex negundo*. Linn is beneficial in Inflammatory condition of ovaries. Application of pounded root over umbilical region or over the vulvae causes easy delivery of the baby.

1.3.3 Medicinal properties:

Whole plant: astringent, cephalic and stomachic; Fruits: cephalic, emmenagogue and nervine tonic; Seeds: cooling; Flowers: astringent, cardiogenic and cooling; leaves: alterative, antiparasitic, discutient, anti-inflammatory and antirheumatic; root-bark: tincture; root: anodyne, diuretic, expectorant, febrifuge and anthelmintic.

1.3.4 Medicinal uses:

This species is widely used in Chinese herbal medicine; it is the second most important treatment for chronic bronchitis (The sub-species *V. negundo cannabifolia* is used). The aromatic leaves are astringent, febrifuge, sedative, tonic and vermifuge. They are useful in dispersing swellings of the joints from acute rheumatism, and of the testes from suppressed gonorrhoea. The juice of the leaves is used for removing foetid discharges and worms from ulcers, whilst an oil prepared with the leaf juice is applied to sinuses and scrofulous sores. The leaves are stuffed into pillows, which are then used to relieve headache. The leaves are harvested in early summer and used fresh or dried.

A decoction of the stems is used in the treatment of burns and scalds. The dried fruit is vermifuge. The fruit is also used in the treatment of angina, colds, coughs, rheumatic difficulties etc. The fresh berries are pounded to a pulp and used in the form of a tincture for the relief of paralysis, pains in the limbs, weakness etc. The root is expectorant, febrifuge and tonic. It is used in the treatment of colds and rheumatic ailments. The root is harvested in late summer

and autumn, and dried for later use. The plant is said to be a malarial preventative and is also used in the treatment of bacterial dysentery - extracts of the leaves have shown bactericidal and anti-tumoractivity.

1.3.5 Edible uses:

SEEDS - occasionally used as a condiment. It is a pepper substitute. When washed to remove the bitterness it can be ground into a powder and used as a flour, though it is very much a famine food used only when all else fails. Tea is made from the roots and leaves.

1.3.6 Agroforestry uses:

The plant is traditionally grown in living fences in the north-western Himalayas, where it helps to exclude livestock and other animals; mark out land boundaries; whilst also providing a range of medicinal and other uses. The roots of hang ping are strong and deep, suckering profusely. The plant can be used as a contour hedge in sandy arid areas for soil retention and moisture conservation. The plant has been found suitable for shelterbelts and windbreaks. It is also used as a live hedge. It can be used for reforestation, especially for the reclamation of forestlands which are affected by floods, and in arid areas.

1.3.7 Other uses:

The young stems are used in basket making and for making wattles. The leaves yield an essential oil. Extract of the leaves have insecticidal properties and are used to repel insects in grain stores. The fresh leaves are burnt with grass as a fumigant against mosquitoes. The stems are used as firewood.

1.4 ECLIPTA PROSTRATA

1.4.1 PLANT DESCRIPTION:

Eclipta prostrata commonly known as false daisy, yerba detago, Gunta kalagaraku/Gunta galagaraku, Karisalankanni, and bhringraj, is a species of plant in the helianthus family. It is widespread across much of the world.

This plant has cylindrical, greyish roots. The solitary flower heads are 6–8 mm (0.24–0.31 in) in diameter, with white florets. The achenes are condensed and narrowly winged. A much-branched, variable, prostrate, ascending or erect, rough-hairy annual herb, up to 90 cm tall with slim, reddish stems roofed with short, stiff hairs, rooting at the lower nodes. Flowers throughout the year; a quantitative short-day plant . A profuse seeder.

1.4.1.1 Leaves:

Opposite, simple, rough, dull green, ovate to oblong-lanceolate, 2-10 cm long, 1-3 cm wide, apex acute or blunt, base attenuate, margin entire or slightly serrate, juvenile, mostly sessile, the lower leaves generally short-petioled, basally swollen hairs on each surface, veins prominent.

1.4.1.2 Inflorescence:

Flower heads up to 1 cm in diameter, a cluster of sessile white flowers, in superior axils or terminal, solitary or two heads along. Peduncle, thickened at the upper,



variable in length, 0.5-7 cm long, hairy. Involucral bracts 5-6, green, ovate, in two rows, outer ones 4-6 mm long, inner ones usually shorter, prominent, hairy. Ray flowers marginal, pistillate, fertile, corolla white, ligulate, 2-3 mm long. Disk flowers various, central, perfect, fertile, corolla whitish, tubular, minute, 1.5-2 mm long.

Stamens five, separated filaments, anthers blended to form a tube around the style.

Fig:1.6: Inflorescence of EP

1.4.1.3 *Seedling:*

Stems are pale green, seldom purple, with fine, small hairs. Primary or cotyledonary leaves show spongy and are opposite, elliptical to egg shaped, with a smooth margin and often with short, pointed, randomly dispersed hairs on the lower side. True leaves are up to 6 mm long, 3 mm wide and opposite. Later leaves appear as if the primary true leaves and have fine, clear marginal hairs. They are up to 12 mm long and 5 mm wide, sessile, lanceolate or linear lanceolate and whole or shallowly toothed, and each surface are rough with scattered, stiff hairs .

1.4.1.4 *Range:*

It is widely distributed in the tropical, subtropical and warm temperate regions mostly in India, China, Japan.

1.4.1.5 *Habitat:*

E. prostrata occurs under both upland and lowland conditions. It is widespread in damp places (heavy soils with a constant and abundant water supply are preferred - Kranz et al., 1977), in ditches, and close to rivers and swamps. It is a common weed of rice. However, its prevalence in lowland rice fields could also be determined entirely by water management .

1.4.1.6 *Parts Used:*

Leaves, Seeds, Roots, flowers and fruits.

1.4.1.7 *Scientific Classification:*

Kingdom:	Plantae
Subkingdom:	Tracheobionta
Division:	Magnoliophyta
Class:	Magnoliopsida
Subclass:	Asteridae
Order:	Asterales
Family:	Asteraceae
Genus:	<i>Eclipta</i>
Species:	<i>prostrata</i>

1.4.2 Therapeutic applications:

Eclipta prostrata belongs to a family of medicinal plants (Asteraceae) and plays a role in the treatment of many diseases, as well as hepatitis, snake venom poisoning, gastritis, and respiratory diseases like a cough and asthma. Variety of compounds, as well as thiophene derivatives, steroids, triterpenes, flavonoids, polyacetylenes, polypeptides, and coumestans, are isolated from *E. prostrata*. The plant functional compounds will act as reducing agent within the field of nanoparticle synthesis. The extracts of *E. prostrata* are widely used for green biosynthesis of numerous metal and metal oxide nanoparticles, nanoparticles, that showed a potential for pharmaceutical, biotechnological, and biomedical applications. Establishment of an efficient in vitro regeneration and genetic transformation method of *E. prostrata* could be an important necessity for application of biotechnology in order to enhance secondary metabolite yields. So that the pharmaceutical potential of the plant may be higher evaluated.

1.4.3 Medicinal properties:

Antiseptic, Astringent, Anticytotoxic, Antihemorrhagic, Antiproliferative, Antioxidant, Antitumor, Antihyperglycemic, Antidementia, Antimicrobial, Antihyperlipidemic, Antivenom, Anti-HIV, Depurative, Styptic, Larvicidal activities.

1.4.4 Medicinal uses:

Eclipta Alba (False Daisy) is an herb that has traditionally been utilized in Ayurvedic medicine for being a liver tonic (for which it is one among the simpler herbs apparently) and having beneficial effects on diabetes, eye health, and hair growth.

The plant *Eclipta prostrata* is used in the traditional medical practices of India to treat hepatic diseases and hyperlipidaemia. The total alcoholic extract of the plant when tested for antihyperlipidemic potential, exhibited a dose-dependent activity in albino rats when compared to standard drugs. The activity was assessed by studying the lipid profiles of serum, liver and heart of the control and drug-treated animals. The results lend support to the traditional use of *Eclipta prostrata* within the treatment of hyperlipidaemia. It has traditional external uses, like athlete's foot, eczema and dermatitis.

Bhringraj powder has various health benefits. Powdered leaves of Bhringraj are found to be effective in the management of high cholesterol and liver toxicity due to its hepatoprotective activity. It also prevents seizures and improves cardiovascular health by reducing arterial pressure. The leaves have been used in the treatment of scorpion stings. It is used as anti-venom against snake bite in China and Brazil.

1.4.5 Edible uses:

Bhringraj powder can be consumed twice or thrice in a day. Consume it before meals along with lukewarm water, Cow's ghee, Honey in respiratory diseases. For chronic fever, you can consume it along with milk. For liver diseases or jaundice, it is recommended to consume Bhringraj powder with mishri or sugar.

1.4.6 Other uses:

Bhringraj darkens the shade of your strands, so if your hair is blonde, wash it off after only 15 minutes. The powder of this plant works perfectly as a hair dye. It darkens your wisps; however, the effects are not as stable as the effects of amla.

CHAPTER-2

REVIEW OF LITERATURE

2.1 BIOFILM CONCEPT:

The idea that bacteria grow preferentially on surfaces has come to the fore at regular intervals, for quite 150 years, whenever microbiologists have used direct methods to look at natural populations of these organisms growing in real ecosystems. Antoine Philips van Leeuwenhoek a Dutch scientist also known as “Father of Microbiology” and one of the first microscopists and microbiologists, used his primitive but effective microscope to define aggregates of ‘animalcules’ that he had scraped from human tooth surfaces. The term biofilm was introduced into medicine in 1985 by Costerton.

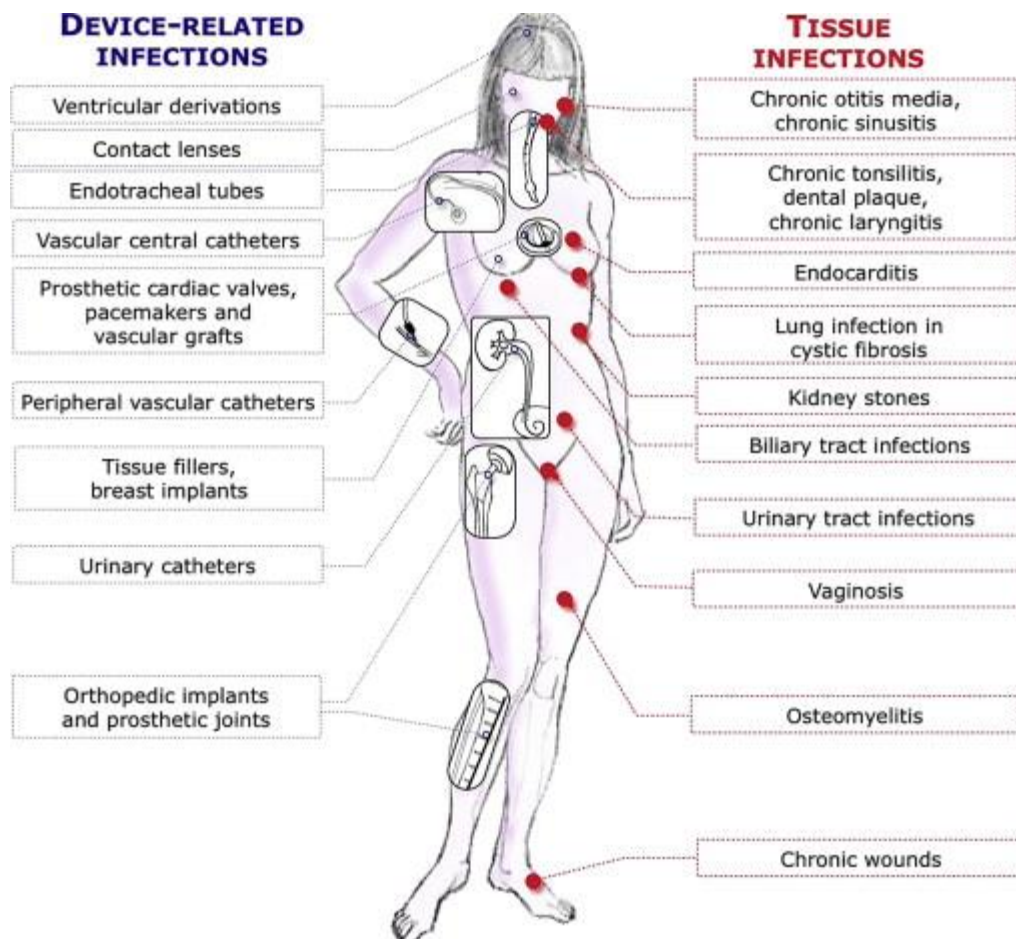
Almost 200 years later, in 1934, Claude E. ZoBell examined natural marine populations by direct microscopy, and concluded that these bacteria are interested in the surfaces to which they often adhere, to form sessile populations. Steadily, throughout the history of microbiology, a very small proportion of microbiologists have chosen to look at their subjects directly, by microscopy, and have found that bacteria grow differently after they adhere to a surface and initiate biofilm formation. Between 1935 and 1978, microbiologists at the Forsyth Dental Center, particularly Ron Gibbons and van Houte, examined the microbic biofilms that represent dental plaque and form macroscopical growths on tooth surfaces.

In 1964, Ralph Mitchell and Kevin Marshall examined the primary stages of biofilm formation by bacteria in pure cultures, and distinguished between the reversible adsorption of bacteria to surfaces and also the future irreversible attachment that constitutes the primary stage of biofilm formation. All of those bellwether studies of the association of bacterial cells with surfaces meet the standards of the recent report of the taskforce of the American Academy of Microbiology (AAM). This AAM taskforce has taken the position that the discipline of microbiology has been seriously misled by its tendency to conclude from studies of planktonic cells in monospecies cultures, and these early studies that discovered biofilms represent one amongst its most convincing examples.

2.2 BIOFILM INFECTIONS IN HUMAN BODY:

Biofilm is implicated in numerous bacterial infections including those associated with the urinary tract, ear, sinuses, cystic fibrosis, indwelling catheters, chronic wounds, and periodontal disease (Hoiby, 2014).

Many different bacteria form biofilms, including gram-positive e.g., *Bacillus species*, *Listeria monocytogenes*, *Staphylococcus species*, and *Lactic acid bacteria*, including *Lactobacillus plantarum* and *Lactococcus lactis* and gram-negative species e.g., *Escherichia coli*, or *Pseudomonas aeruginosa*, *Klebsiella*



pneumoniae.

Fig: 2.1: Typical biofilm infections

(Source: Hoiby et al., ESCMID guideline for diagnosis and treatment of biofilm infections, 2014).

2.2.1 Surgical Implants :

Biofilms form on implanted medical devices such as prosthetic joints, mechanical heart valves, arteriovenous shunts and Central Venous Catheters (CVCs). Device-associated infections are often caused by a single species of bacterium or by a mixture of bacterial species or fungi. These microorganisms are human commensals such as *Staphylococcus epidermidis* and *Escherichia coli* or are present in the environment like *Pseudomonas aeruginosa*. CVCs are of great clinical importance. They are widely used in surgical and critically ill patients for hemodynamic monitoring and administration of total parenteral nutrition. In cancer patients they permit administration of chemotherapy and other therapeutic drugs. CVC-related sepsis, however, is common and has been reported to occur in up to 19% of cases (Pemberton et al., 1986). Microorganisms gain access to the catheter via various routes. Most frequently commensal skin bacteria such as *Staphylococcus epidermidis* contaminate the insertion site or contaminate the catheter hub, leading to biofilm formation and catheter-related infection. Biofilm formation around breast implants may play a crucial role in the establishment and recurrence of fibrous capsular contracture following breast augmentation. *Staphylococcus epidermidis*, which is endogenous to the breast, is assumed to realize access and infect the mammary implant. In a prospective blinded study, we investigated the role of biofilm in capsular contracture. We demonstrated a significant correlation between *Staphylococcus epidermidis* positivity and contracture. Scanning electron microscopy confirmed the presence of extensive biofilm on implants and within capsules (Linares et al., 1985).

2.2.2 Urogenital Infections :

Persistent infections in the urinary tract are widely associated with biofilm formation, in particular with catheter-associated infection, prostatitis and struvite (infected stone) calculogenesis. Urinary tract infections may be caused by ascending or creeping colonisation by enteric bacteria that may first colonise the vagina and introitus, the periurethral area, the bladder and occasionally the

kidneys when bacterial virulence overcomes the numerous natural defence mechanisms. Cells shed from biofilms provide an unbroken source of measurable infection. Foley catheters are essential in urology for draining the bladder, monitoring urine output in patients and to assist repair of the urethra and surrounding tissues. Bacterial biofilms forming on urinary Foley catheters have been shown to be the source of biomaterial-related sepsis. Planktonic bacteria are continually shed from the biofilm of colonised catheters into residual urine around the tip and balloon of the catheter. While indwelling Foley catheters are essential in-patient care, the presence of this foreign body breaches the normal host defence by disruption of the bladder mucus, by mechanical or chemical irritation, exposing surfaces that promote bacterial adhesion and biofilm formation. Once bacteria adhere to the bladder wall, symptomatic catheter-associated cystitis develops (Nickel et al., 1994).

In the case of bacterial prostatitis, bacteria enter the prostate from the urethra and multiply rapidly within the prostatic ducts activating host defences. As a result of the inflammatory response, the ducts become engorged with infiltrates composed of dead or living bacteria, inflammatory cells and cellular debris. At this point organisms can still be eradicated with antibiotic therapy. If bacteria persist, they can adhere to the epithelium of the ducts forming microcolonies and eventually form biofilms that cannot be eliminated with standard antibiotic therapy. Treatment regimens that deliver much higher concentration of antibiotics to the biofilm within prostatic ducts are likely to show improved treatment success rate (Nickel and McLean, 1998).

Infection stones can cause significant health problems as they cannot be eliminated by antibiotics alone and often recur after surgical removal. The initial step in struvite calculogenesis is adherence of urea lytic bacteria such as *Proteus mirabilis* to Foley catheters, mucosal scars in the bladder or to calcium stones. Infection stones are associated with urinary tract infection (UTI). Urease-producing bacteria split urea into ammonia, generating an alkaline pH that subsequently causes precipitation of Mg^{2+} and Ca^{2+} as struvite and apatite. Crystals formed within the biofilm microenvironment adhere to the mucosa and coalesce to form the initial stone which then continues to grow with the addition of more bacteria,

matrix production and crystal deposition. Hence, the biofilm bacteria permeated throughout the infection stone are protected from antibiotics used for the treatment of UTI. Other indwelling prosthetic devices can also be the nidus for infection or encrustation causing blockage and include ureteric stents, penile prostheses, peritoneal dialysis catheters, femoral and subclavian central venous catheters(Nickel and McLean, 1998).

2.2.3 Cystic Fibrosis :

Cystic fibrosis (CF) is a genetic disease caused by the loss of CF transmembrane conductance regulator (CFTR) protein. This loss leads to secretion of thicker alveolar mucus and blocked ducts, altering the natural immunity of the airways and promoting colonisation of the epithelium by bacteria. Biofilms of *Pseudomonas aeruginosa* are now recognised as leading contributors to morbidity and mortality in CF patients since they suffer recurrent chronic respiratory infections. As with other biofilm infections, antibody reaction is elicited in response to the infection yet the antibodies are ineffective against bacteria sheltered within the biofilm. Progressive loss of lung function occurs as the pulmonary tissue is damaged by continual inflammation. Antibiotic therapies developed against planktonic cells are also inadequate in eradicating biofilm bacteria, allowing the biofilm to act as a nidus for spread of infection(Hoiby ,1995).

2.2.4 Dental Practice :

The soft bacterially deposited films on teeth have long been recognised as plaque. Plaque may form on any part of a tooth surface, particularly within the gingival margin, causing gingivitis and other mouth infections. Oral treatment to reduce plaque formation is a continuing subject of research. Biofilm formation in dental evacuation devices continually presents problems in maintaining adequate hygiene. An important recent finding was the existence of biofilms within the cannula of reusable dental syringes. Blood borne virus resisted normal cleaning, high level chemical disinfection and autoclaving for 3 minutes at 134°C when shielded by biofilm(Vickery et al.,2000).

2.2.5 Removal of Biofilms from Endoscopes :

Conventional practices for cleaning endoscopes use neutral and near neutral detergents enclosing a variety of enzymes that are claimed because the important ingredient for removing surgical soils. As is widely appreciated, pre-cleaning of endoscopes and other medical equipment before applying high-level disinfectants is essential for assuring the performance of chemical biocides. High-level chemical disinfectants based on peracetic acid and glutaraldehyde; all fail to effectively disinfect endoscopes if pre-cleaning is not performed correctly. The study of biofilms generated in endoscopic and surgical tubing has shown conclusively that while present methods of cleaning remove the bulk of the biofilms, there still remains on surfaces a very thin, tenacious, strongly adherent chemical resistant basal film. The retained basal film can contain pathogens encountered during routine surgery (unpublished results). Routine pre-cleaning with either neutral detergent or any of the three-common enzyme-based medical detergents tested failed to dislodge laboratory-generated biofilms of *E. coli* and *P. aeruginosa*. The scanning electron microscope views of a *P. aeruginosa* biofilm before and after cleaning with a widely used endoscope reprocessing detergent(Deva et al.,1998).

It is clear that the viscous glycocalyx or EPS, the bulk of typical biofilms, is readily removable with currently accepted detergents. The research is directed at deciphering the chemical behaviour of this unique basal layer generated on medical equipment surfaces by typical medical pathogens. What is not clear at thatpoint is whether or not biofilms manufactured under laboratory conditions will reliably reproduce the conditions encountered in routine endoscopic practice. Different laboratory models were used to generate and test biofilms may well yield different solutions to this serious problem. Those findings must be interpreted against epidemiological considerations, in particular the very low incidence of post-operative infections following endoscopic surgical procedures. Clearly, although present endoscope decontamination procedures may have demonstrable weaknesses, there is no doubt that if carried out meticulously, a satisfactory result is obtained.

2.3 Prevention of Biofilms :

In conclusion, biofilm infection is difficult to treat and determination of infection around implants is usually only achieved by removal of the implant, adding to patient morbidity and increasing health costs. As antibiotic concentrations required to eradicate biofilm bacteria far exceed the maximum therapeutic dose, researchers have attempted to prevent biofilm formation by incorporating biocides into the biomaterial during polymerisation or by silver coating of catheters. Such attempts have been unsuccessful due to leaching of the biocide or silver coating. Functional monomers with structures almost like the active sites of common antibiotics have also been grafted onto the polymer surface without success (Carpentier and Cerf, 1993).

Other medical conditions primarily associated with persistent bacterial infection due to the formation of biofilms will undoubtedly be diagnosed in the future. At this point, it is clear that the importance of biofilms in infection processes is merely now being understood. In recent studies, many methods and techniques were identified by using Nanoparticles and crude extract of plants to eradicate the biofilms formation. However, the means of overcoming the difficulties posed by antibiotic and biocide resistance of bacteria and other cells located within biofilms remains a matter of serious research within the medical community.

From past few years, several researches were performed on eradication of biofilm through medicinal plants. These are the list of plants which contain very high antibacterial, antifungal and antibiofilm properties.

Table 2.1:List of medicinal plants which have anti-biofilm activity

Name	Parts used	Types of plant extracts	Medicinal uses of plants	Pathogens used for biofilm	Activities	References
<i>Azadirachta indica</i>	Leaves	Methanol	Various ailments, act as contraceptive and sedative agent.	<i>Escherichia coli</i> , <i>Pseudomonas aeruginosa</i>	antibiofilm , antibacterial activities.	Kusum et al, 2013
<i>Vitex negundo</i>	Leaves	Methanol, ethyl acetate, petroleum ether, and hexane	Wound, ophthalmic, Verminosis, ulcer, bronchial smooth muscles relaxant	<i>Escherichia coli</i>	Antinociceptive, antimicrobial, anti-inflammatory, antipyretic activities.	Namsivayam and Roy, 2013
<i>Tridax procumbens</i>	Leaves	Methanol, ethyl acetate	Bronchial, dysentery hair growth promotes, and insect repellent	<i>Escherichia coli</i>	Wound healing, Hepatoprotective, antimicrobial and anti-inflammatory activities	Mundada and Shivhare, 2010
<i>Ocimum tenuiflorum</i>	Leaves	Methanol	Coughs, colds, vomiting, flatulence, heal peptic ulcers.	<i>Escherichia coli</i>	Wound healing effect, Radio protective, anti-carcinogenic properties and anti-genotoxic	Namsivayam and Roy, 2013
<i>Piper lonum</i> <i>Piper nigrum</i>	Grains	Methanol, petroleum ether,	Malaria, asthma, cough, diabetes and	<i>Streptococcus pyogens</i>	Anti-biofilm,anti-bacterial, antioxidant	Darsini et al, 2015

		chloroform and ethyl acetate	heart problems		activities.	
<i>Pittosporum tetraspermum</i>	Leaves	ethyl acetate	Chronic bronchitis, rheumatism, skin diseases, and leprosy.	<i>Escherichia coli</i> , <i>Salmonella typhi</i> , <i>pseudomonas aueroginasa</i>	Antibacterial, antifungal, antibiofilm, Antioxidant and anticancer activities.	Abdullah Al-Dahabi et al, 2015
<i>Couroupitaguianensis</i>	Fruits	Chloroform	Tumours, pain and hypertension	<i>Pseudomonas aueroginasa</i>	Antimicrobial, antimycobacterial, antifungal, antibiofilm activities.	Al-Dahabi et al, 2012
<i>Hakea sericea</i>	Aerial parts	Methanol	No medicinal uses	<i>Staphylococcus aureus</i> MRSA	Antioxidant, antibacterial, anti-biofilm and cytotoxicity activities	Luis et al, 2014
<i>Chromolaena odorata</i>	Leaves	Chloroform, ethanol	Cough, malaria	<i>Pseudomonas aeruginosa</i>	Antibacterial, antibiofilm, antioxidant, wound healing	Yahya et al, 2014
<i>Terminalia fagifolia</i>	Stem bark	Ethanol	Gastrointestinal disturbances, such as ulcer, gastritis and diarrhoea	<i>Staphylococcus aureus</i> , <i>Staphylococcus epidermidis</i>	Antibiofilm, antioxidant, gastric antiulcerogenic, and hypokinetics activities. In vitro and in vivo cytotoxicity	De Araujo et al, 2015

					studies.	
<i>Pityrocarpam o moniliformis</i>	Leaves	Aqueous	Healing process	<i>Staphylococcus epidermidis, Pseudomonas aeruginosa</i>	Anti-biofilm, anti- bacterial activities, Antinociceptive,a nti-inflammatory agent	De Queiroz et al, 2010 and Silva et al, 2013

(Source : Gayathri and Mani, 2017).

CHAPTER-3

AIM AND SCOPE OF THE PROJECT

The overall aim of the present study was to eradicate the biofilm forming microorganisms by analysing how plants are an excellent source to provide abundant natural compounds for the development of preventative and therapeutic agents of biofilm-based infections.

Objectives of the present investigation are:

- Preparation of methanolic and aqueous extracts of *Vitex negundo* and *Eclipta prostrata*.
- Phytochemical analysis in *Vitex negundo* and *Eclipta prostrata* plants.
- Assessment of Minimum Inhibitory Concentration in plant extracts.
- Evaluation of Antibacterial and Antifungal activities in *Vitex negundo* and *Eclipta prostrata* extracts.
- To examine the Antibiofilm activity in *Vitex negundo* and *Eclipta prostrata* extracts.

CHAPTER-4

MATERIALS AND METHODS

4.1 GLASSWARES

Petri plates, conical flask, beakers, test tubes, falcon tubes, funnels, 12 well plates, Eppendorf tubes, micro-Pipettes, micro-tips, Whatman filter paper, absorbent and non-absorbent cotton, lighter, inoculation loop, spirit lamp, marker, mortar and pestle, vortex mixer, horizontal laminar flow, micro-oven, autoclave, spectrophotometer, cotton swabs, weighing scale, corkborer, gloves, spatula.

4.2 CHEMICALS

95% Methanol, sodium chloride (NaCl), gelatin, sodium hydroxide (NaOH), chloroform, conc. H₂SO₄, dilute HCL, 5% ferric chloride (FeCl₃), Copper sulphate (CuSO₄), Glacial acetic acid, Molisch reagent, dimethyl sulfoxide (DMSO), Nutrient broth media, Muller-Hinton agar (MH), Potato Dextrose Agar (PDA), ethanol, acetone, sodium acetate, crystal violet, distilled water.

4.3 SAMPLING OF PLANTS:

The leaves of *V. negundo* were collected from the mature plant in Birding site (17°18'52.9"N 77°51'58.3"E), Anantagiri, Telangana, India during December 1st, 2020. The leaves of *E. prostrata* were collected from the mature plant in Anantagiri hills (17°18'45.7"N 77°51'19.0"E), Vikarabad, Telangana, India during December 2nd, 2020.

These leaves are dried in an oven dryer at 40°C for 48 hours. The dried leaves were then pulverized by mortar and pestle and passed through sieve to get fine powder, stored in dark glass bottles and kept at 20°C until further analysis.

4.4 PREPARATION OF EXTRACTS:

Extracts were prepared following the methodology proposed by Sanchez et al. with minor modifications.

4.4.1 Aqueous extract:

10 gms of leaves powder is added in the conical flask containing 100 ml of distilled water and heated in heating mantle for 5 mins at 50°C and kept aside for 72 hours with occasional shaking . Then it is filtered by using Whatman No 1 filter paper to obtain pure aqueous extraction.



Fig: 4.1: Filtration of crude extracts.

4.4.2 Methanol extract:

10 gms of leaves powder is added in the conical flask containing 100 ml of 70% Methanol and kept aside for 72 hours with occasional shaking at room temperature. Then it is filtered by using Whatman No 1 filter paper to obtain pure methanol extraction.

These two extracts were poured into Glass tray and allowed it to air dry for 48 h. Then it was macerated in mortar and pestle until the fine powder was obtained for further analysis.



Fig: 4.2: Final product of plant extracts

4.5 BACTERIAL STRAINS AND CULTURE CONDITIONS:

A collection of 4 organisms, 2 Gram-negative (*Klebsiella pneumoniae* -2451 and *Escherichia coli* -2412) and 2 Gram-positive (*Staphylococcus aureus* -2044 and *Bacillus cereus* -2128) were used for this study of clinical isolates and tested for purity by standard microbiological methods. The bacterial stock cultures were maintained on Mueller-Hinton (MH) agar slants and stored at 4°C. Active cultures were obtained by inoculation of a loopful of each strain into separated 5 ml MH broth and incubated for 18 h at 37°C.

4.6 PHYTOCHEMICAL STUDIES (QUALITATIVE ANALYSIS):

The phytochemical screening of methanol and aqueous extracts for the presence of these secondary metabolites: Tannins, flavonoids, saponins, steroids, phenols, terpenoids, anthocyanins, proteins, glycosides, carbohydrates were evaluated according to the methods as described by Edeoga et al., 2005. In general, tests for the presence of phytochemical compounds involved the addition of appropriate reagents.

4.6.1 Test for Tannins :

- Dissolve 50 mg extract in 5 ml distilled water and add 2 ml of 1% solution of gelatin containing 10% NaCl is added to it (0.5 gms NaCl, 0.1 gm gelatin in 2 ml distilled water). White precipitate indicates the presences of tannins.

4.6.2 Test for Saponins :

- Dilute the extract 50 mg with distilled water and make up to 20 ml.
- Shake the suspension in a graduating cylinder for 15 mins. A two layer of foam indicates the presence of saponins.

4.6.3 Test for Flavonoids :

- On treating the extract 50 mg with few drops of sodium hydroxide solution forms intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

4.6.4 Test for Steroids :

- Dissolve 2 ml of organic extract in 1 ml of chloroform and add 1 ml of conc. H_2SO_4 . A red colour is produced in the lower chloroform layer indicates the presence of steroids.

4.6.5 Test for Terpenoids :

- Dissolve 2 ml of organic in 1 ml of chloroform and evaporate till dry.
- Add 1 ml of conc. H_2SO_4 and heat for about 2 mins at 80°C . Development of reddish-brown colour indicates the presence of terpenoids.

4.6.6 Test for Anthocyanins :

- Weigh about 0.1 gm of plant extract in a separate test tube add 1 ml of 2 M sodium hydroxide (NaOH) and heat for 5 mins at 100°C and observe for the

formation of bluish green colour which indicates the presence of anthocyanin.

4.6.7 Test for Phenols :

- Weigh about 0.1 g of plant extract and treat with 5% FeCl_3 and observe for the formation of deep blue colour which indicates the presence of phenols.

4.6.8 Test for Proteins :

- Biuret Test – Mix 0.1 g of extract with 2 ml of 10% NaOH and 2 – 3 drops of 1% CuSO_4 solution. Appearance of violet or purple colour indicates presence of protein.

4.6.9 Test for Glycosides :

- Keller – Killani Test – To 0.1 g of extract add glacial acetic acid and few drops of FeCl_3 and conc. H_2SO_4 . If reddish brown colour is formed at the junction of two layers and upper layer turns bluish green confirms the presence of glycosides.

4.6.10 Test for Carbohydrates :

- Add 0.1 g plant extract into 2 ml of distilled water and add 200 μL of Molisch reagent . Formation of violet colour ring indicates the presence of carbohydrates.

4.7 ASSESSMENT OF MINIMUM INHIBITORY CONCENTRATION (MIC):

The Minimum Inhibitory Concentration (MIC) was determined on plant extracts that showed antimicrobial activity, by a broth microdilution method proposed by Novy et al. with minor modifications. Briefly, 100 μL of each of the aqueous and methanol plant extracts initially dissolved in 1% DMSO to make 10 mg/mL was added into

the first well was serially diluted in 12-well (4) microtiter plate with 2ml of Mueller-Hinton Broth was performed by transferring 1ml of the suspension to the subsequent wells up to the 10th well to obtain a concentration ranging from 1.25µg/mL to 0.0195 µg/mL. Then , 5 µL of standardized E.coli inoculum size of 10⁶ CFU/ml in each well. Then, 25 µL of Azophloxin a standard antibiotic is added in all 4 plates for comparative analysis with the effectiveness of various extracts against tested clinical isolate and drug resistant bacteria . Microplates were incubated at 37°C for 24 h. Following incubation, the MIC was calculated as the lowest concentration of the extract that restricted the visible growth of microorganism tested. Two replications were maintained (Arokiyaraj et al, 2009).

4.8 EVALUATION OF ANTIBACTERIAL ACTIVITY :

An agar-well diffusion method was employed for evaluation of antibacterial activity. The bacterial strains were reactivated from the stock culture by transferring into Mueller-Hinton Broth (MH) and incubating at 37°C for 18 h. A final inoculum containing 10⁶ colony forming units (1 × 10⁶ CFU/ml) was added aseptically to MH agar medium and poured into sterile petri plates. After that, wells of 6 mm in diameter were made in the agar using sterilized cup-borer and concentrations of 25 µL, 50 µL, 75 µL, 100 µL of plant extracts were added to wells. Plates were incubated at 37°C for 72 h . Experiments were performed in duplicates. Antibiotic such as ciprofloxacin (10µL, 20µL) in two wells are used as positive reference to determine sensitivity of microorganisms tested. DMSO (50µL, 100µL) in other two wells are used as negative control. Antibacterial activity was evidenced by the presence of clear inhibition zone around each well. The diameter of this zone was measured and recorded (Sanchez et al., 2010).

4.9 EVALUATION OF ANTIFUNGAL ACTIVITY :

Antifungal activity for screening antifungal agent in the material was used in agar dilution method. Clotrimazole is used as standard drug and Potato dextrose agar

was employed as medium. In vitro screening of antifungal activity was carried out against *Aspergillus Niger* fungal strain. The culture of organisms was maintained on Potato dextrose agar (PDA). The broth was incubated at 37° C for 24 hours. Inoculum was prepared by dilution of 24 hours old culture in saline. A dilution of 1:100 was used in the test, prepared in distilled water. The Petri plates of PDA were prepared and 0.1 ml of diluted culture was poured on each plate. Size of well was 6mm in diameter and concentrations at 50µl, 100µl of plant extracts were added to wells. The plates were incubated for 72 hours at 37° C. The zone of inhibition of fungal growth was measured and compared with standard drug. Each experiment was performed in triplicate; their mean of linear growth of zone of inhibition was recorded and calculated (Alves et al., 2002).

4.10 MICROTITRE PLATE BIOFILM ASSAY(QUANTITATIVE METHOD):

Biofilm assay is conducted in triplicate in 12 wells microtiter plates (tissue culture treated, flat-bottom wells-TPP). The EMJH broth is used as the negative control. Two reference strains *Escherichia coli* and *Staphylococcus aureus* , selected because of their strong biofilm -forming ability study , were used in the assay of plant extracts.. Ciprofloxacin is used as an antibiotic in the positive control as reference to determine the antibiofilm activity. Firstly, 2ml of media is added all well plates. Then 30µl of inoculated culture were mixed with different strains to concentrations at 50µl, 100µl, 150µl, 200µl and incubated for 48 h at 37°C. The procedure to conduct microtiter plate biofilm assay is as follows. Briefly remove liquid culture every day for 10 days with time intervals of 24 hours. Rinse the wells gently with distilled water to remove non-adherent planktonic cell. Allow it to air dry for 15 minutes before fixation using 2% sodium acetate. Remove the sodium acetate solution and allow to air dry again. Stain the cells using 500µl of 1% crystal violet solution and remove. Rinse the cells three times with distilled water. Finally, the crystal violet solution remaining in the wells is dissolved in 750µl of ethanol/ acetone(80/20 ,v/v solution). Prior to official density at 600nm(OD 600). To correct the background staining of crystal violet, the mean official density at 600nm of the

negative control is subtracted from the mean optical density at 600nm of the biofilm formation of pathogen.

The percentage of biofilm inhibition was calculated by the following formula (Namasivayam et al., 2012).

$$\% \text{ of inhibition} = \frac{\text{OD in control} - \text{OD in treatment}}{\text{OD in control}} \times 100$$

CHAPTER-5

RESULTS AND DISCUSSION

5.1 QUALITATIVE PHYTOCHEMICAL SCREENING :

Crude phytochemicals were determined quantitatively in *V.negundo* and *E.prostrata* using the literature methods and results of extracts are summarized in table 5.1 and shown the presence of different functional groups. These leaves showed the presence of phytochemicals like saponins, steroids, phenols, terpenoids, anthocyanins, glycosidic compounds. Among these compounds' phenols, steroids, and saponins shows high presence in both plant extracts.



Fig 5.1: Phytochemical tests of VN and EP

The methanol extract of *Eclipta prostrata* shown maximum phytochemical compounds like tannins, saponins, steroids, terpenoids, phenols, glycosides which help us to understand that methanol extracts is better than aqueous extracts. Moreover , methanol polarity ensured the extraction of polar compounds from plants against microorganisms likesaponins, steroids, and phenols(Cowan, M.M.,1999).

Table 5.1:Phytochemical screening of aqueous, methanol extracts of

s.no	secondary metabolites	VN methanol	VN aqueous	EP methanol	EP aqueous
1	Tannins	-	-	+	-
2	Saponins	+	-	+	+
3	Flavonoids	-	-	-	-
4	Steroids	+	+	+	-
5	Terpenoids	-	+	+	-
6	Anthocyanins	+	+	-	-
7	Phenols	+	+	+	+
8	Proteins	-	-	-	-
9	Glycosides	-	-	+	+
10	Carbohydrates	-	-	-	-

Vitex negundo* and *Eclipta prostrata

‘+’= present; ‘-’ = absent

The results of present study are helpful for the discovery of potent remedies on various diseases. Medicinal plants are the main source for potent bioactive compounds(Sunil and Vanita , 2017). The present study also useful for preparing phenol-based drugs which have been most important in antioxidant, antimicrobial properties.

5.2 MINIMUM INHIBITORY CONCENTRATION STUDIES :

Minimum inhibitory concentration (MIC) results are comparable to those obtained in the agar-well diffusion technique, because the lowest MIC were obtained using the extracts showing the best antimicrobial activity (Sanchez E, 2016).

The MIC figure of the plant extracts were in agreement with its zone of inhibition of antibacterial activity in the case of *E. coli* with the preliminary zone of inhibition mean diameter which means having higher inhibition and lower concentration.

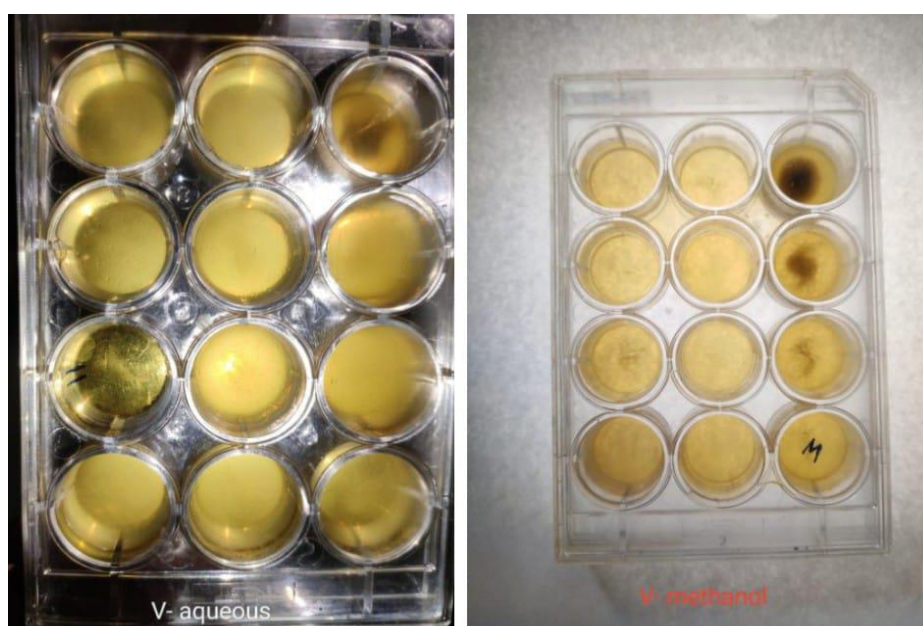


Fig: 5.2: MIC in methanol and aqueous extract ofVN

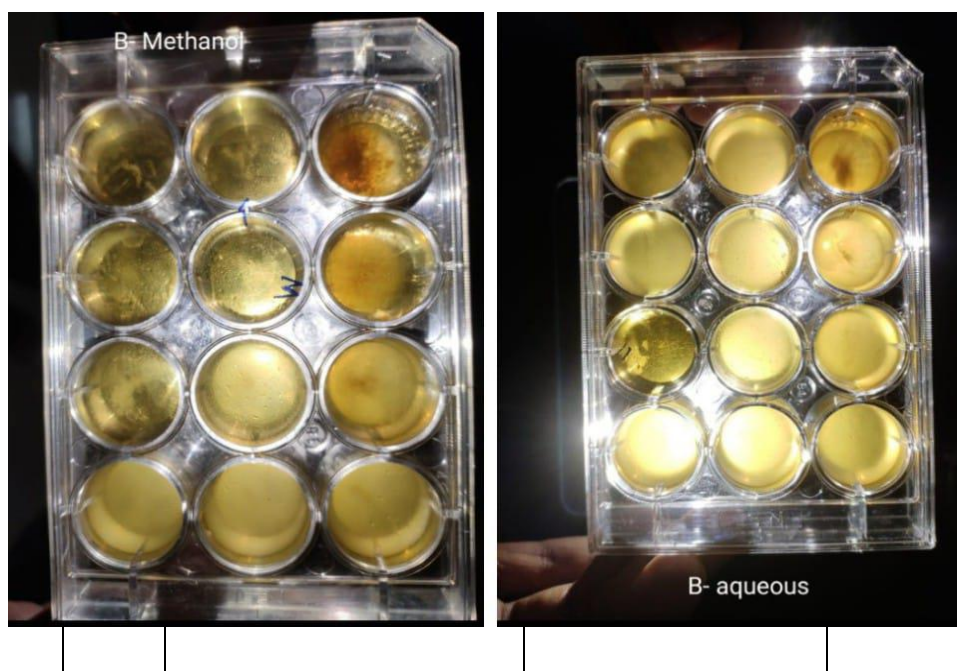


Fig: 5.3: MIC in methanol and aqueous extracts of EP

As indicated in Table 5.2, the methanol extract of plant *Vitex negundo* had the maximum MIC of 1.25 μ g/mL and the minimum MIC value was 0.019 μ g/mL against *E. coli*. The MIC values order for both methanol and aqueous extracts starting from the higher to the lower were 1.25 μ g/mL , 0.156 μ g/mL, 0.039 μ g/mL, and 0.019 μ g/mL with the respective extracts of VN-methanol, EP-methanol, EP-aqueous and VN-aqueous respectively.

Table : 5.2: MIC of plant extracts against *E.coli*

VN= *Vitex negundo*; EP= *Eclipta prostrata*

The results presented in this paper demonstrate that both of the plants with aqueous extracts shows inhibited their growth with the higher serial dilution or with lower concentration of plant extract even with 0.019µg/mL .

5.3 ANTIBACTERIAL ACTIVITY TEST :

The zones inhibition of mean diameter of the plant extracts against the tested bacteria is tabulated in table 5.3, 5.4, 5.5,5.6.

Plant extract	Conc. (µL)	<i>S. aureus</i>	<i>E. coli</i>	<i>B. cereus</i>	<i>k. pneumoniae</i>
	100	16	14	16	NI
VN-methanol	75	12	12	12	NI
	50	10	12	10	NI
	25	NI	10	10	NI

Table: 5.3: Zone of inhibition(in mm) of VN- methanol

NI : no inhibition

Table: 5.4: Zone of inhibition(in mm) of VN- aqueous

Plant extract	Conc. (µL)	<i>S. aureus</i>	<i>E. coli</i>	<i>B. cereus</i>	<i>k. pneumoniae</i>
	100	12	14	14	NI
VN-aqueous	75	12	12	14	NI
	50	10	10	12	NI

	25	NI	6	10	NI
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NI : no inhibition

Table: 5.5: Zone of inhibition(in mm) of EP- methanol

Plant extract	Conc. (μL)	<i>S. aureus</i>	<i>E. coli</i>	<i>B. cereus</i>	<i>k. pneumoniae</i>
	100	12	14	12	14
EP-methanol	75	10	14	10	12
	50	10	12	8	10
	25	8	10	NI	6

NI : no inhibition

Table : 5.6: Zone of inhibition(in mm) of EP- aqueous

Plant extract	Conc. (μL)	<i>S. aureus</i>	<i>E. coli</i>	<i>B. cereus</i>	<i>k. pneumoniae</i>
	100	14	14	16	16
EP-aqueous	75	12	12	10	12
	50	12	10	6	10
	25	10	NI	NI	NI

NI : no inhibition

We selected the target strains with the aim of studying the suppression or inhibition of pathogenic growth in the body by the extracts of medicinal plants. Among the tested bacteria , *B. cereus* (16 mm) was moderately susceptible compared to the other tested bacteria within the concentration of 100μL of plant extract of aqueous, methanol of *Eclipta prostrata* and *Vitex negundo* leaves part in this study. As depicted in tables, the moderate susceptible bacterium at 100μL

was *B. cereus* (16mm) followed by *S. aureus*, *K. pneumoniae* and *E. coli* with a zone of inhibition diameters (16mm, 16mm and 14mm), respectively. From the tested bacteria, *S. aureus* is slightly resistant to the plant extracts at the concentration of 100µl with 6mm of inhibition zone diameter; *E. coli* in the lower plant extract, concentrations showed lower inhibition zone of diameter. At the concentration of 75µL of the plant extract *V. negundo* methanol, the antibacterial inhibition activities were nearly similar for the tested bacteria.

Based on the zone of inhibition, the plant extracts' antibacterial activity ability was depending on the concentrations of the plant extracts used. For the *Vitex negundo* plant extract, *K. pneumoniae* had no inhibition zone, whereas other plant shows little zone of inhibition diameter in the same concentrations. The Zone of inhibition of the plant extracts at the concentration of 25µL was significantly different when compared to 100µL of the plant extract for all tested bacteria. All the tested bacteria in all concentrations of plant extract were significantly different from that of positive control (Ciprofloxacin, 30µg/disc). Generally zone of inhibition of the antibacterial activity of the plant extracts was depending on its concentrations.

However, the well diffusion assay is considered a qualitative technique and is mainly used for selecting extracts with antimicrobial activity, mostly when diameters zones of inhibition are $\geq 10\text{mm}$ (Usman and Abdulrahman, 2009). It is important to recognize that the size of inhibition zones of different extracts could be due to the compound's polarity obtained, since a more diffusible but less active extract could give a bigger diameter of inhibition than a nondiffusible but more active extract (Savaroglu et al., 2011).

5.4 ANTIUNGAL ACTIVITY TEST :

The antifungal activity of methanol and aqueous extracts of *Vitex negundo* and *Eclipta prostrata* has been investigated against clinically isolated fungal strain. The disc was prepared in 100µL concentrations and therefore the disc was placed on MH agar plates with standard disc containing reference antibiotic Clotrimazole were used as a positive control.

Table : 5.7: Zone of inhibition (in mm) of *Aspergillus Niger*

Plant extracts	Zone of inhibition (<i>A. Niger</i>)	
	100 μ L	50 μ L
V- methanol	8	NI
V- aqueous	16	NI
E- methanol	14	10
E- aqueous	16	12

NI : no inhibition

The antifungal activity of the standard antibiotic Clotrimazole disc at 100 μ L concentration used, the zone of inhibition obtained is 18mm. In aqueous extract of *Eclipta prostrata* and *Vitex negundo* at 100 μ L concentration the inhibition zone was high (16mm) against methanol plant extracts. For *Vitex negundo* plant extracts there is no zone of inhibition at 50 μ L concentration. The results showed that the antifungal activity increases with the increase in concentration of the extract (table 5.7).

The result of this study showed that the leaf extracts of *Vitex negundo* and *Eclipta prostrata* exhibit antifungal properties justifies its traditional use as medicinal plant. The inhibition of the growth of the pathogenic fungi is due to the active ingredients predominantly found in the plant (Shetty et al., 1989). The present investigations are in line with the investigations carried out by other workers (Sarvamangala et al., 1993), which infers that leaf extracts in general have great potential in the control of fungal diseases in commercially important crop plants.

5.5 EFFECT OF PLANT EXTRACTS ON INHIBITION OF BIOFILM

FORMATION :

Two of the selected plant extracts have shown the maximum inhibition activity against the biofilm of *E. coli* and *S. aureus*. The methanol extracts of *V. negundo* and *E. prostrata* efficiently inhibit biofilm formation where 70.99%, 64.71% and 71.55%, 84.71% of both the organisms tested. The best biofilm reduction is observed in higher concentrations of the plant extracts effectively in methanol extract.

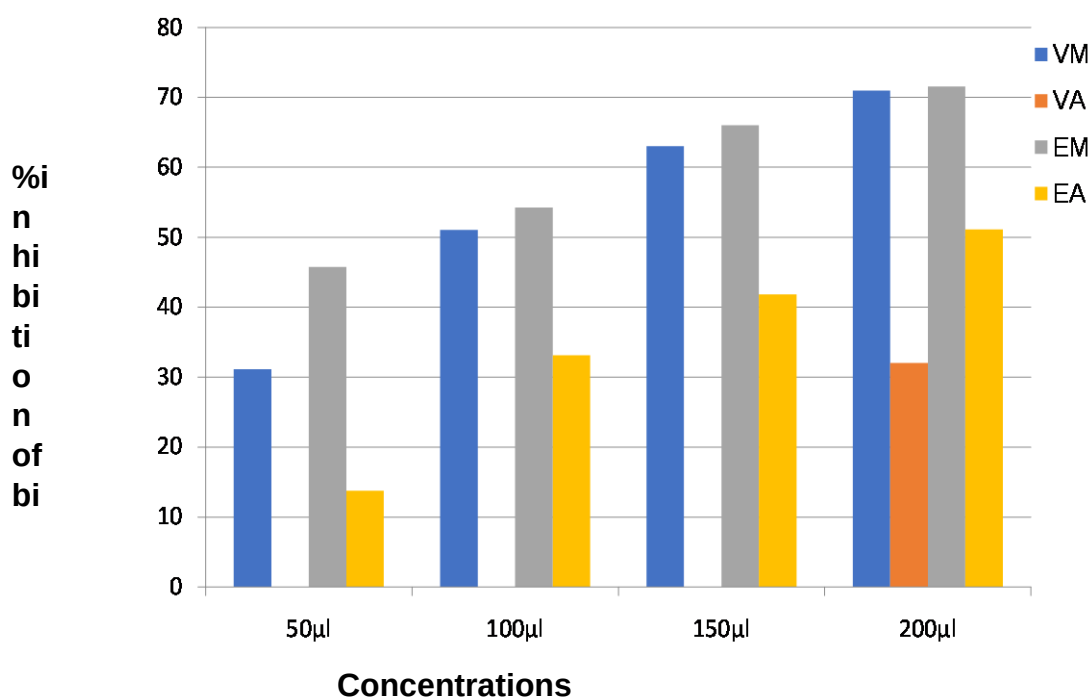


Fig: 5.4 : Biofilm inhibition of plant extracts against *E. coli*

VM= *Vitex negundo* methanol VA= *Vitex negundo* aqueous

EM= *Eclipta prostrata* methanol EA= *Eclipta prostrata* aqueous

Additionally, the least activity was observed in aqueous extracted plant sample of *V. negundo*. However, aqueous extract of *E. prostrata* also shows the less activity of biofilm inhibition between 13% to 54% in both tested bacteria. These %inhibition values are shown in table 5.8 as below .

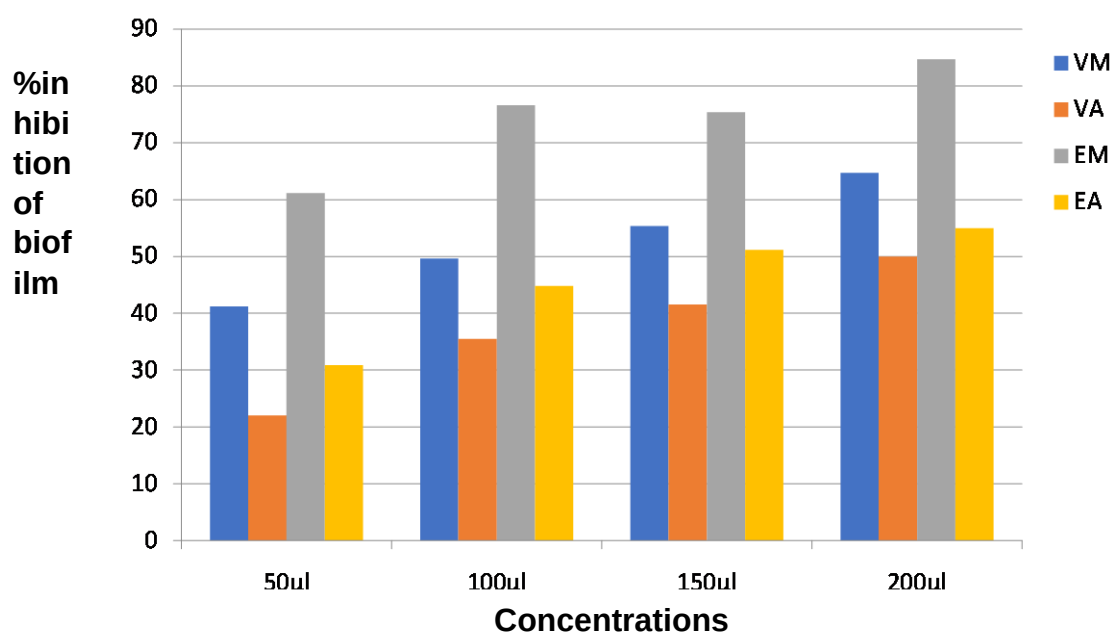


Fig : 5.5: Biofilm inhibition of plant extracts against *S. aureus*

Table :5.8 : %Biofilm activity of plant extracts at different concentrations

Plant extracts	E. coli (μL)				S. aureus (μL)			
	50	100	150	200	50	100	150	200
VN- aqueous	-	-	-	32	22	35.5	41.5	50
VN – methanol	31.1	51	62.9	70.9	41.1	49.6	55.3	64.7
EP – aqueous	13.7	33.1	41.8	51.1	30.8	44.8	51.1	54.9
EP – methanol	45.7	54.2	66	71.5	61.1	76.6	75.3	84.7

From the tested bacteria *S. aureus* shows the best biofilm inhibition by comparing with the antibiotic in positive control having 79.8% and 87.5% with bacterial values 71.5% and 84.7% at higher concentrations only. From the table we also seen that *E. prostrata* of methanol extract at 100μL concentration shows high percentage biofilm inhibition of 76.6% than 150μL concentration of 75.3% in *S. aureus*

bacteria. Previous studies revealed a strong evidence that the medicinal plants have promising attitude to combat various types of infectious diseases caused by numerous microbial species (Manandhar et al., 2019). From these results it can be concluded that E. prostratamethanol extract may find a use as broad spectrum antibiofilm agents after further extensive investigations.

CHAPTER – 6

SUMMARY AND CONCLUSIONS

The aim of the present study was to eradicate biofilm forming microorganisms and to evaluate antibiofilm effect of both methanolic and aqueous coated extracts of *Vitex negundo* and *Eclipta prostrata*. These biofilms have caused serious damages to the human body by causing diseases like cystic fibrosis, urinary tract infections, dental plague etc. In recent studies, inhibition of biofilm formation through several medicinal plant extracts on bacterial survival and virulence factors involved in tissue colonization and biofilm formation was evaluated.

In the present study, phytochemical investigations have shown that *V.negundo* and *E. prostrata* extracts contain mainly saponins, steroids and phenol derivatives. According to the results of this study , Minimum inhibitory concentration shows that aqueous extracts of both plants inhibit the growth of *E. coli* at lower concentrations 0.039µg/mL and 0.019µg/mL.

The present study revealed that the methanol-aqueous extracts of *V.negundo* and *E . prostrata* has antibacterial activities against the growth of the selected pathogenic bacteria with varying antibacterial spectrum ability even if its inhibition zone of diameters was lower when compared to the other studies on the same plant species with different extraction protocol, bacterial strains , and geographical location of plant collection sites. The antibacterial activities of the plant might be linked with the presence of saponins, phenols, alkaloids and other bioactive phytochemical constitutes in its leaves. Unfortunately , only a few research groups described the antibiofilm activity of *V. negundo* and *E. prostrata* extracts. For this reason, the purpose of our study was to determine the eradication and inhibition of tissue colonization and biofilm formation.

The findings of the present study clearly indicate that the tested methanolic plant extracts exhibit 70% to 80% of biofilm inhibition at higher concentrations for both *E. coli* and *S. aureus* organisms.

In conclusion, the plants extracts evaluated in the present research had potential antimicrobial and antibiofilm activities against isolated *E. coli* and *S. aureus* bacteria , which can be alternative to control the formation of microbial biofilms or can be used as model to the search for new drugs.

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