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Determination of the Antibacterial and Anti-biofilm Capability of Selected Leaf Extracts

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Abstract

Antibiotics are epochal examples of medical development in human history. The naturally occurring molecules may, however, develop into new sources of antibacterial and anti-biofilm drugs for clinical use as a result of an impending antibiotic crisis brought on by the emergence and widespread spread of antimicrobial resistance among bacterial agents, as well as the rising number of patients with chronic and recalcitrant bacterial bio-film-associated infections. There is a need for alternate treatment regimens, especially those derived from natural resources like plants because they are efficient and have less side effects, especially with the rising incidence rate of periodontitis and resistance among oral bacteria to antibiotics. The major goal of the current work is to determine the antibacterial and anti-biofilm capability of leaf extracts from *Bryophyllum pinnata*, *Coriandrum sativum*, *Eucalyptus globulus*, and *Mentha piperita*. Plant leaves were collected, and maceration method was used to make extractions. *E. coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* that produce biofilms, were used as test organisms for the obtained extracts. It was calculated how much biofilm inhibition there was. Potential antibacterial and anti-biofilm action was demonstrated by leaf extracts. *Bryophyllum pinnata* and *Eucalyptus globulus* demonstrated good results among four plant leaf extracts. A phytochemical screening was done on the extracted materials.-

Keywords: antibiofilm, antimicrobial, *Bryophyllum pinnata*, *Coriandrum sativum*, *Eucalyptus globulus*, *Mentha piperita,* and maceration.

Introduction

The establishment and spread of resistant bacterial strains are evolving into a significant global problem, with the potential for the return of the pre-antibiotic period. *E. coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* are some bacteria responsible for nosocomial infections that are most frequently seen and are showing signs of increasing drug resistance. These biofilm-forming bacteria have emerged as a significant global cause of both hospital- and community-acquired illnesses. Staphylococcal infections are challenging to treat because of their innate propensity to produce powerful biofilms and to become resistant to the used medicines. Additionally, management of such infections has become extremely difficult due to the emergence of resistant strains, such as Methicillin-Resistant *S. aureus* (MRSA), Glycopeptide Intermediate *S. aureus* (GISA), and Vancomycin Intermediate *S. aureus* (VISA) (1)

Pseudomonas aeruginosa started acting like a deadly infection in the past few years. It has long been a challenging contributor to secondary infections of wounds, particularly burns, and it has also been linked to eye infections that lead to blindness. Being biochemically adaptable and able to employ a variety of disinfectants as food sources, *Pseudomonas aeruginosa* is resistant to numerous antibacterial substances.

Similar to this, *E. coli* is an opportunistic pathogen that causes the majority of nosocomial infections. When it grows in immunocompromised individuals' biofilms, *E. coli* causes significant infection. Infections connected to intravenous catheters (2), joint prosthesis, and Foley urinary catheters are all brought on by *E. coli* biofilms, which also cause pyelitis, pyelonephritis, and cystitis. It results in diarrhoea and deadly dehydration in babies.

The investigated bacteria are known to form biofilms, and it has been demonstrated that bacteria living in biofilms are extremely resilient to the effects of antibiotics. The biofilm matrix that serves as a barrier prevents most antibiotics from penetrating (3, 4), which causes the phenotypic resistance of bacteria living there. Additionally, bacteria that form biofilms grow slowly and act as persister cells, evading (5) the majority of medications that act to kill or affect them. Biofilm-associated cells are 100–1,000 times more resilient than free-swimming planktonic cells. Therefore, despite the prolonged duration of many treatments, treatment failures (6) are common under clinical conditions where biofilms play a significant role in pathogenesis (7), such as wounds in diabetic patients, catheter-associated urinary tract infections, and endocarditis (8)

In nature, bacteria frequently live in biofilms that are very well hydrated, which provides a favorable environment for cell adhesion to one another and to other surfaces. This community's microorganisms form a cement-like matrix that can serve as "biological superglue" to adhere to or trap on various biotic or abiotic surfaces. In particular, biofilm infections (9, 10) on implants or indwelling devices are challenging to treat because of their superior resistance to macrophages and antibiotics. As a result, they frequently result in fatal clinical consequences. Due to its formation on medical implants, presence in human tissue, and involvement in numerous severe chronic infections, it is a serious issue in the medical field.

Antibiotic resistance rates are increasing globally; hence it is urgent to look into safe and effective alternatives. Although the emergence of resistance is inevitable given that it is a crucial aspect of microbial evolution, it is equally important and essentially prudent to develop novel antibacterial products. Herbal remedies have long been utilized in conjunction with conventional therapy and have been shown to have few negative effects (11). The scientific community has, however, recently revived its interest in looking into this as an alternative kind of treatment to combat the problem of drug resistance.

The well-known medicinal herb *Eucalyptus globulus* has pharmacological and biological characteristics. It is the primary source of essential oils, which have demonstrated antibacterial, anti-inflammatory, anti-oxidant, anti-diabetic, and even cancer-fighting properties (12).

In general, earaches, coughs, diarrhoea, dysentery, abscesses (13, 14), ulcers, insect bites, heart problems, epilepsy, arthritis, dysorrhea (15), and whitlow are treated with *Bryophyllum pinnatum* in ethno-medicine.

The seeds of coriander (*Coriandrum sativum* L.) are used as a flavour agent or spice in seafood, curries, bread, meat, and meat confections. Coriander seeds contain up to 1% essential oil. The primary ingredient with antioxidant, antimicrobial, hypolipidemic, and anti-diabetic activities is linalool. Additionally, it has stimulat-

ing and appetising effects throughout the digestion process (16)

Mentha piperita, a member of the Lamiaceae family, is found growing in North America, Europe, and India. Numerous beneficial properties, including antiviral, antimicrobial, antioxidant, mildly anaesthetic, anti-inflammatory, antispasmodic, antiulcer, and hepatoprotective, are said to exist in it (17).

The present study carried out on assessing the anti-biofilm potential of methanolic extracts taken from leaves of *Bryophyllum pinnata*, *Coriandrum sativum*, *Eucalyptus globulus*, and *Mentha piperita* against clinically significant pathogens like *E. coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* capable of forming potent biofilms and associated with life threatening infections and issues of drug resistance. This research helps us further understanding of the usage of traditional herbal remedies as a powerful and secure substitute for the widely used antibiotics in both the prevention and treatment of biofilm infections.

Materials and Methods

Collection of leaves

Fresh plant leaves were collected and sent right away to the lab for processing in order to be tested for antibiofilm activity. The leaves were cleaned with tap water to eliminate dust, air dried to a constant dry weight, ground into a coarse powder, and then maintained at room temperature for subsequent studies in airtight containers.

Making extracts from plant leaves

Using a mortar and pestle, four different solvents (diethyl ether, ethyl acetate, methanol, and ethanol) were separately extracted from fresh leaves. The pulverized leaves were then kept submerged in the appropriate solvents for two days to allow for complete extraction. The resulting extracts were filtered, concentrated in a hot air oven at 40° C until nearly dry, weighed, and stored at 4° C until the antibiofilm assay (18).

Bacterial Cultures

E. coli (MTCC739) was procured from the Microbial Type Culture Collection Center, Chandigarh, India. *Pseudomonas aeruginosa* (MCC2048) and *Staphylococcus aureus* (MCC2408) were obtained from the National Centre for Microbial Resource, Pune, India, and were employed as bacterial cultures in the

Table 1: Methods for phytochemical screening (19, 20)

Phyto-chemical	Test	Observation
Alkaloid	Wagner's reagent (I2/KI) for alkaloids was applied. The extracts were filtered after being dissolved in weak HCl. Two milliliters of the filtrate were added to a few drops of Wagner's reagent (I2/KI).	Alkaloid presence was determined by the appearance of a brownish/red precipitate.
Flavo-noids	A portion of the extract was subjected to a concentrated H_2SO_4 test to determine the presence or absence of flavonoids	Formation of orange colour confirms the presence of flavonoids.
Steroids	It employed the Liebermann-Burchard test. 0.5 mL of acetic anhydride and 0.5 mL of acetic acid were used to treat four milligrams of the extracts. H_2SO_4 concentration was gradually added.	Steroids were present if a reddish-brown colour develops.

Terpenoids	Liebermann Burchard test was used. Four mg of the extracts was treated with 0.5 ml of acetic anhydride and 0.5 mL of acetic acid. Concentrated H_2SO_4 was slowly added	development of a blue-green color indicated the presence of terpenoids
Saponins	Exactly 0.5 g of the plant extract was dissolved in 2.5 mL of distilled water. The mixture was shaken vigourously.	presence of foam indicated the presence of saponins
Tannins	Ferric chloride test was used to test for the presence of tannins. An exact amount of 0.5 g of the extract was boiled in 20 mL of distilled water and filtered afterwards. Few drops of 0.1% of $FeCl_3$ were added	presence of brownish-green, brownish-black, or blue-black color was used to detect the presence of tannins
Glycosides	Benedict s test was used for the detection of glycosides. Precisely, 0.5 g of plant extract was dissolved in 5 ml of distilled water. Exactly 2 mL of Benedict s solution was heated and 8 drops of the dissolved sample were added and allowed to boil for 5 minutes	Formation of brick-red precipitate indicated the presence of glycosides

study.

Phytochemical analysis

The obtained effective extracts underwent qualitative phytochemical screening to check for phytochemicals as tabulated in Table 1.

Methodology

Detection of biofilm

Microtiter plate assay was used to quantify biofilm development. Mueller-Hinton Broth (MHB) is used to prepare bacterial suspension. The concentration of this bacterial suspension is changed to 5.10^6 cfu/ml. Then, in accordance with Stepanovi et al.'s 2007 description, 180 ml of MHB supplemented with 1% glucose and 20 ml of bacterial suspensions are inoculated into 96-well flat-bottomed sterile polystyrene microplates to produce a final concentration of 5.10^5 cfu/ml (21). At 37°C, microplates are incubated for 24 hours. Planktonic cells are removed from micro-titre plate wells by treating them with PBS (pH 7.2), and then the wells are dried at 60°C for roughly 30 minutes. By flooding for 20 minutes with 150 µl of methanol, the biofilm is fixed. Only 150 µl of 0.1% crystal violet is used to stain the sessile isolates of biofilms that have formed on the walls of microplate wells for 15 minutes (22). Microplate wells that have been dyed with crystal violet are then rinsed twice with PBS to remove the stain. Following the air drying of the microplate's wells, 150 µl of 95% ethanol is used to resolubilize the dye from the biofilms that lined the plate's walls. A microplate reader then uses spectrophotometry to measure the microplate at 570 nm. The uninoculated wells containing sterile MHB are taken as blanks and are regarded as the negative controls.

Anti-bacterial activity assessment

The agar well diffusion bioassay was employed to assess antibacterial activity. On Muller Hinton agar (MHA) plates, 100 µl of new culture was evenly distributed using a sterile L-shaped glass rod. The inoculated plates were then left to dry at room temperature for 20 minutes. Then, 100 µl of each extract being studied was poured into the wells that had been made in the agar using a sterilized cup borer. The control was set using methanol. At 37°C, plates were then incubated the next day. The development of an obvious inhibition zone surrounding the well provided proof that the area was antibacterial. The diameter of this zone was determined and noted (23, 24).

Evaluation of minimum inhibitory concentration

MIC was carried out using a micro-dilution of extracts in MHB. Various extract concentrations, ranging from 0.1 to 1 mg/ml, were prepared and put into tubes. Fresh culture is injected in 100 µl at the proper concentration, then left to incubate for 24 hours at 37 °C. The lowest concentration of extract that prevented discernible growth was known as the minimum inhibitory concentration (MIC).

To evaluate MBC, 100 µl from each tube was reinoculated in Muller Hinton Agar. At 37°C, agar plates were incubated for 24 hours. The MBC of an extract can be calculated using the lowest concentration of extract that inhibited growth (24)

Biofilm formation inhibition assay:

By using a microtiter plate assay percentage of biofilm inhibition was measured. Mueller-Hinton Broth (MHB) is used to prepare bacterial suspension. The concentration of this bacterial suspension is changed to 5.10^6 cfu/ml. Then, as described by Stepanovi et al. in 2007, 20 µl of bacterial suspensions and 100 µl of MHB supplemented with 1% glucose are inoculated into 96-well flat-bottomed sterile polystyrene microplates to achieve the desired final concentration. At 37°C, microplates are incubated for 24 hours. Planktonic cells are removed from microtitre plate wells by treating them with PBS (pH 7.2), and then the wells are dried at 60°C for roughly 30 minutes. The plant extracts were utilized in a method of serial dilution ranging from 10µg to 300 µg. By flooding for 20 minutes with 150 µl of methanol, the biofilm is fixed. Only 150 µl of 0.1% crystal violet is used to stain the sessile isolates of biofilms that have formed on the walls of microplate wells for 15 minutes. Microplate wells stained with crystal violet are then rinsed twice with PBS to remove extra stain. Following the air drying of the microplate's wells, 150 µl of 95% ethanol is used to resolubilize the dye from the biofilms that lined the plate's walls. A microplate reader then uses spectrophotometry to measure the microplate at 570 nm. Uninoculated wells containing sterile MHB are used as blanks and are regarded as the negative controls. The percentage biofilm inhibition was calculated using the formula below (25, 26).

Percentage of biofilm inhibition = [1-(A570 of the test/A 570 of non-treated control)]x 100

Results and Discussion:

The obtained effective extracts underwent qualitative phytochemical screening to check for phytochemicals. The results are tabulated in the table 1.

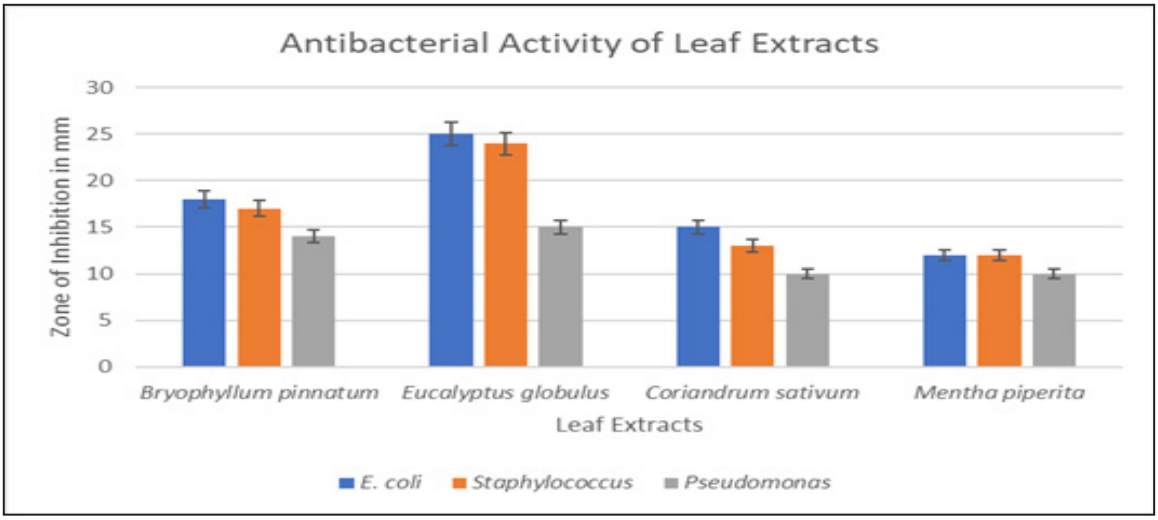
Table 1**:** Phytochemical screening of leaf extracts

S. No.	Name of the test	*Mentha piperita*	*Coriandrum sativum*	*Bryophyllum pinnatum*	*Eucalyptus globulus*
1	Bayer□s	+	+	+	+
2	Triterpenes	+	+	+	-
3	Coumarins	+	+	+	+
4	Test for quonins	+	+	+	+
5	Carboxl group	+	+	+	+

6	Tanins	-	+	+	+
7	Steroids	-	+	+	-
8	Saponins	+	-	-	+
9	Carbohydrates	+	+	+	+

Table 2: Antimicrobial activity

Bacterial cultures	*Bryophyllum pinnatum*	*Eucalyptus globulus*	*Coriandrum sativum*	*Mentha piperita*
E. coli	18±0.2 mm	25±0.1 mm	15±0.2 mm	12±0.2 mm
Staphylococcus	17±0.1 mm	24±0.3 mm	13±0.2 mm	12±0.1 mm
Pseudomonas	14±0.2 mm	15±0.1 mm	10±0.1 mm	10±0.2 mm

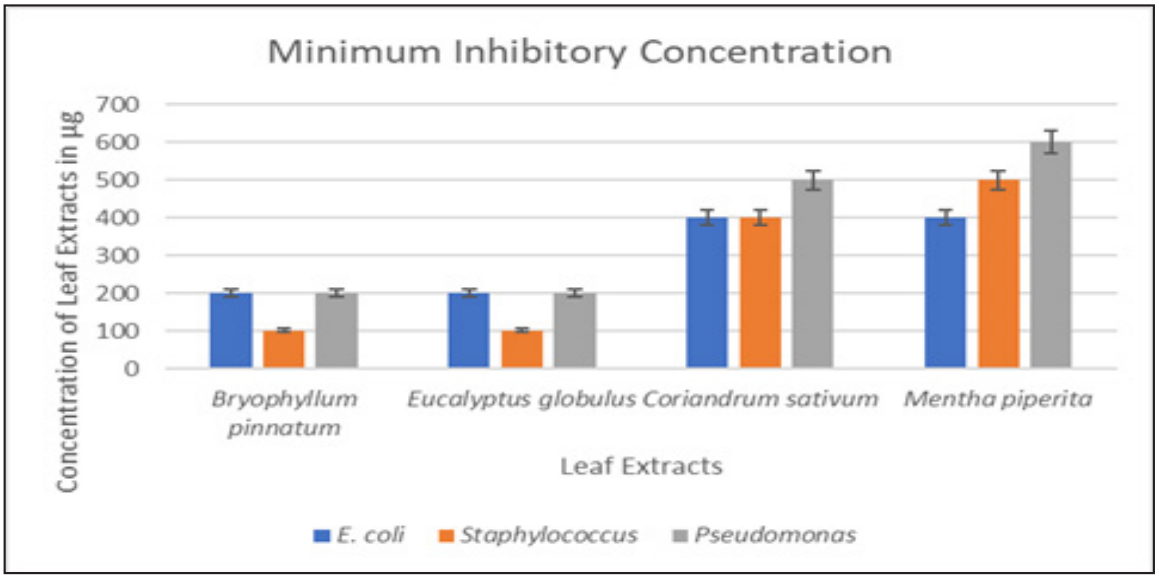


Graph 1: Representing the antibacterial activity of the leaf-extracts on *E. coli, Staphylococcus aureus, Pseudmonas aerogenosa*.

Table 3: Minimum Inhibitory Concentration

Bacterial cultures	*Bryophyllum pinnatum*	*Eucalyptus globulus*	*Coriandrum sativum*	*Mentha piperita*
E. coli	200 µg	200 µg	400 µg	400 µg
Staphylococcus	100 µg	100 µg	400 µg	500 µg
Pseudomonas	200 µg	200 µg	500 µg	600 µg

Determination of the antibacterial and anti-biofilm capability of selected leaf extracts



Graph 2: Representing the minimum inhibitory concentration.

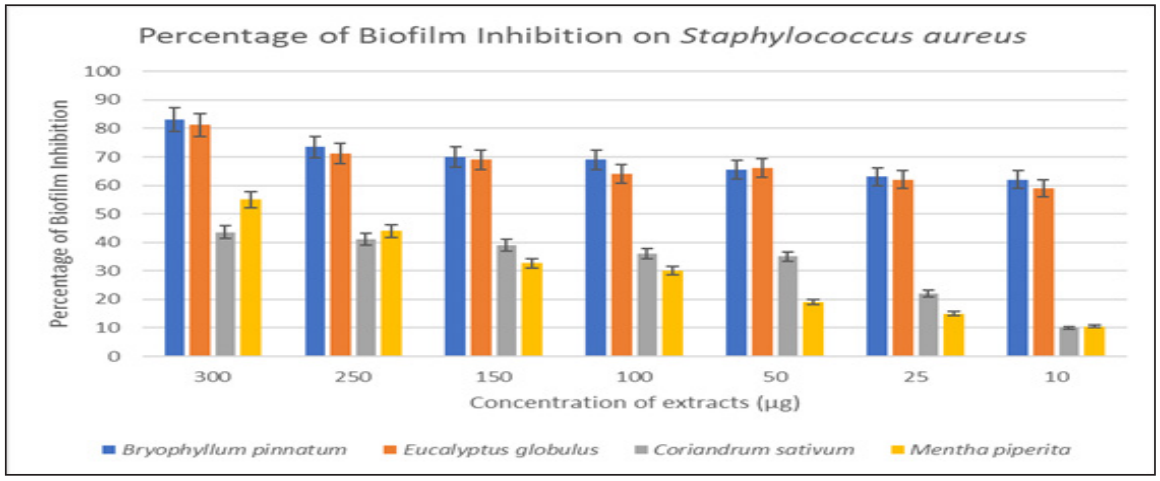
Table 4: Percentage of biofilm inhibition on *Staphylococcus aureus*

S. No.	Concentration of extracts (µg)	*Bryophyllum pinnatum*	*Eucalyptus globulus*	*Coriandrum sativum*	*Mentha piperita*
1	300	83.2	81.25	43.6	55.3
2	250	73.5	71.25	41.2	44.1
3	150	70.1	69.2	39.1	32.7.1
4	100	69.1	64.1	36.01	30.1
5	50	65.5	66.2	35.1	19.1
6	25	63.2	62.1	22.2	15.2
7	10	62.1	59.1	10.1	10.5

phylococcus aureus by the various leaf-extracts.

Table 5: Percentage of biofilm inhibition on *E. coli*

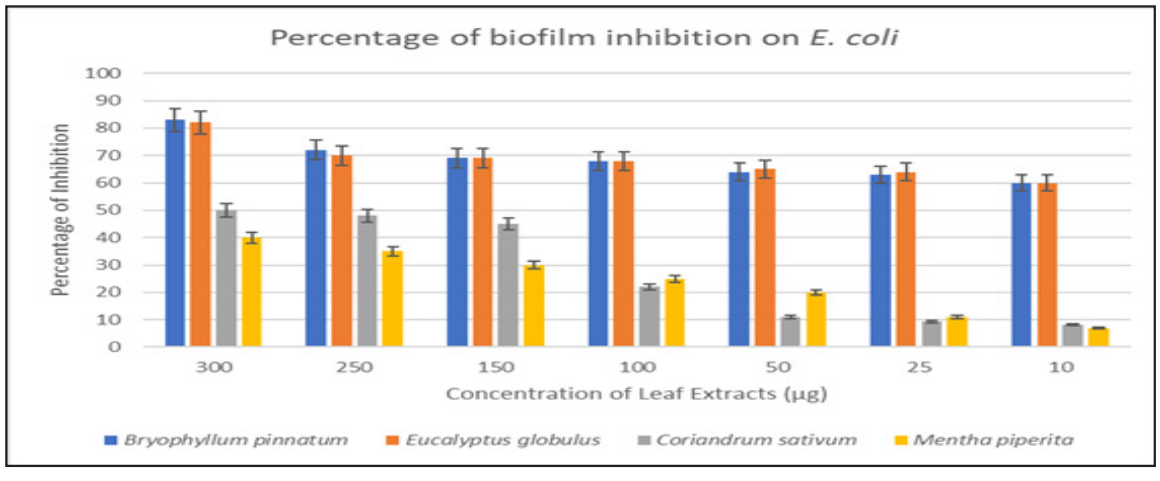
S. No.	Concentration of extracts(µg)	*Bryophyllum pinnatum*	*Eucalyptus globulus*	*Coriandrum sativum*	*Mentha piperita*
1	300	83.01	82.1	50.1	40.1
2	250	72.02	70.02	48.02	35.02
3	150	69.1	69.01	45.01	30.01
4	100	68.02	68.03	22.1	25.02
5	50	64.01	65.02	11.01	20.2
6	25	63.03	64.2	9.4	11.05
7	10	60.02	60.1	8.2	7.2



Graph 4: Representing the Biofilm inhibition percentage of *E. coli* by the various leaf extracts.

Table 6: Percentage of biofilm inhibition on *Pseudomonas aeruginosa*

S. No.	Concentration of extracts	*Bryophyllum pinnatum*	*Eucalyptus globules*	*Coriandrum sativum*	*Mentha piperita*
1	300	82.5	80.2	40.6	56.2
2	250	72.02	74.25	34.3	46.8
3	150	70.2	69.01	31.2	37.5
4	100	69.05	68.2	21.8	31.2
5	50	68.5	65.1	9.3	21.8
6	25	64.01	64.02	6.2	18.7
7	10	62.2	62.01	3.2	12.5



Graph 5: Representing the Biofilm inhibition percentage of *Pseudomonas aeruginosa* by the various leaf extracts.

Determination of the antibacterial and anti-biofilm capability of selected leaf extracts

Three bacterial cultures were evaluated with a total of four methanolic extracts. Methanol is used as the solvent in the extraction process because of its suitability for polar and moderately polar active chemicals from plants, such as terpenoids, tannins, flavones, and polyphenols. These leaf extracts obtained from other solvents like di ethyl ether and ethanol are less effective compared to methanolic extracts.

Results of exploratory antibacterial tests using the well diffusion method on three bacterial species varied amongst extracts. From the table 2, it can be confirmed that all the four-leaf extracts exhibited antimicrobial activity on the three bacterial cultures. However, *Eucalyptus globulus* and *Bryophyllum pinnatum* were found to be more effective against these three bacterial cultures.

The Minimum inhibitory concentration (MIC) values of *Bryophyllum pinnata, Eucalyptus globulus, Coriandrum sativum*, and *Mentha piperita* on *Pseudomonas* sps. is 200 µg, 200 µg, 500 µg and 600 µg respectively.

The Minimum inhibitory concentration (MIC) values of *Bryophyllum pinnata, Eucalyptus globulus, Coriandrum sativum*, and *Mentha piperita* on *E. coli* are 200µg, 200µg, 400µg and 400µg respectively. The MIC values of *Bryophyllum pinnata, Eucalyptus globulus, Coriandrum sativum*, and *Mentha piperita* extracts on *Staphylococcus aureus* is 100 µg, 100 µg, 400 µg and 500µg respectively. The results of MIC are mentioned in the Table: 3 and Graph 2.

All four plant leaf extracts underwent phytochemical screening, and the findings are shown in Table 1. This table illustrates the presence of several functional groups. Triterpenes, Coumarins, Quonins, Carboxyl groups, Tanins, Saponins, and Carbohydrates were discovered. By adding various quantities of plant leaf extracts, *Pseudomonas* sp., *E. coli*, and *Staphylococcus aureus* biofilm formation is inhibited. Higher extract concentrations were shown to have the highest biofilm formation suppression (results are listed in Tables 4 ,5and 6). The best activity was shown in leaf extracts of *Eucalyptus globules* and *Bryopyllum pinnatum* on all three bacteria when the percentage inhibition of biofilm formation was determined. The other leaf extracts, including those from *Mentha piperita* and *Coriandrum sativum*, displayed the least or no activity of biofilm formation inhibition.

A promising option to be investigated in the fight against biofilm bacteria is eucalyptus extract. One of the components of the eucalyptus oil (EO) found in the leaves, eucalyptol (also known as 1, 8-cineole), is primarily responsible for the eucalyptus' therapeutic effects (27). Eucalyptus has been used as an antiseptic, to treat respiratory tract infections, wound healing, diabetes, and fungal infections, as well as to relieve symptoms of cough, cold, and sore throat. Several researches have looked into the therapeutic properties of this plant. Additionally, polysaccharides and essential oil extracted from eucalyptus appear to possess a variety of antimicrobial properties, according to in vitro and in vivo studies. Studying the anti-biofilm potential of Eucalyptus leaf extract against nosocomial infections has, however, received little attention. The current study uses in vitro experiments to examine the effect of a eucalyptus leaf methnolic extract on the capacity of *P. aureus*, *E. coli*, and *S. aureus* to produce biofilms.

According to the findings, *Eucalyptus* methnolic extract from leaves is a viable option that has strong antibiofilm properties against *E. coli*, *P. aeruginosa*, and *S. aureus*.

Bryopyllum pinnatum methonolic leaf extracts have high levels of alkaloids a phytochemical compound which could be majorly responsible for antibacterial and antibiofilm activity (28).

The *Bryophyllum pinnatum* plant's phytochemical content as well as the presence of imminent mineral elements may be some of the

contributing aspects that point to potential medicinal uses for the plant. This plant is an excellent source of human nutrition, so it should be consumed as a dietary supplement, according to this evidence as well (29).

This study indicates that an ethnobotanical approach should be taken into account when examining the antibacterial properties of plants, in addition to demonstrating the scientific basis for some of the therapeutic uses of this plant in conventional medicine. It also explains why, despite their differences, these herbs provide similar therapeutic effects when taken interchangeably (30). If the active principle can be separated, the broad-spectrum effect of several of these extracts suggests that they may be useful in chemotherapy as well as antiseptic and disinfection formulations (31). It is possible to investigate the anti-pseudomonal and anti-staphylococcal properties of certain of these plants' potent extracts further.

Fewer studies have been done on the antibiofilm effects of plant extracts, despite the fact that there are many reports available on the antimicrobial properties of plant extracts. Therefore, the objective of the current study was to evaluate the antimicrobial and antibiofilm properties of medicinal plant leaves extracts from *Mentha piperita*, *Coriandrum sativum*, *Eucalyptus globules*, and *Bryophyllum pinnatum* against multidrug resistant *P. aeruginosa*, *S. aureus*, and *E. coli* strains.

Conclusions

The medicinal and pharmacological effects of natural compounds with plant origin are the current areas of study. The results of this study demonstrate the antibiofilm and antibacterial efficacy of various plant leaves extracts against *P. aeruginosa*, *E. coli*, and *S. aureus* strains that are multidrug resistant. For medical professionals and scientists, complete eradication of microbial biofilms still represents a significant problem. Natural antibiofilm agents are an attractive target in the fight against infectious disease since they are safer for the environment and less dangerous than synthetic substances. According to our research, *Bryophyllum pinnatum* and *Eucalyptus globules* both possess potent antibacterial and antibiofilm properties. The specific bioactive components from the extracts of these plants' leaves should be further analyzed, and toxicity tests of the bioactive compounds should also be conducted to calculate the extracts' safety indices.

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