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IN VITRO ANTIBIOFILM ACTIVITY OF SELECTIVE PLANT EXTRACTS AGAINST ENVIRONMENTAL ISOLATES

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ABSTRACT:

The presence of biofilm producers in environmental settlings is an alarming condition due to the emergence of antibiotic resistant bacteria in such ecological niches. Hence, there is a need to find an alternative to antibiotics and plants may act as a potential source of effective antimicrobial compounds. Novel study was carried out to access the antibiofilm potential of methanolic leaves extracts of *Cinnamomum camphora*, *Cordia dichtoma* and *Citrus maxima* against different environmental isolates. A new study investigated the ability of plant extracts to inhibit biofilm formation in bacteria. Seventeen bacterial isolates from environmental samples were used in the research. The study also determined the minimum inhibitory concentrations (MICs) of the plant extracts to assess their antibiofilm activity. Moreover, phytochemical constituents and antioxidant activity of selective plant extracts were also evaluated. By tube test, 35.29% isolates were strong biofilm producers. Best inhibitory action was observed with methanolic extracts of all selected plants. However, highest inhibitory action against biofilm producers were found with methanolic extracts of *C. camphora* leaves at a concentration of 8mg/ml. Minimum inhibitory concentration (MIC) of methanolic extracts of the *C. camphora*, *C. dichtoma* and *Citrus maxima* were in the range of 2.5mg/ml-8mg/ml. Qualitative phytochemical analysis demonstrated the presence of terpenoids, steroids, reducing sugars, saponins, tannins and carbohydrates in plants tested. Antioxidant activity (85.80%) of methanolic leaves extracts of *C. camphora* was found to be highest at 200g/ml concentration of plant extract. The results obtained in the present investigation regarding antibiofilm potential of plant extracts used against biofilm producers indicates the importance of these plants in drug development.

KEYWORDS:

BIOFILM, AGAR WELL DIFFUSION, MINIMUM INHIBITORY CONCENTRATION (MIC), PHYTOCHEMICAL ANALYSIS.

INTRODUCTION

The environment is a habitat for a wide variety of microorganisms. Microbes can impact their environment in ways that are beneficial, harmful, or undetectable from a human perspective[1]. Pathogenic microbes cause infectious diseases by invading a host and deriving nutrients from it. Pathogenic bacterial strains possess many virulence factors, e.g., endotoxins, inclusion factors, adherence, colonization factors, capsules, biofilm production capacity, or other surface components[2]. Existance of biofilms in different environments can pose significant risks to human health by harboring harmful bacteria and pathogens, which are resistant to antibiotics and disinfectants. In medical settings, they can cause chronic infections, particularly in implanted devices. Additionally, biofilms in water systems can lead to contamination, potentially causing gastrointestinal illnesses. Biofilm is an essential virulence factor in the pathogenesis of various chronic infections. All microorganisms have the capability to form biofilm on any surface. The formation of biofilm depends on many factors such as quorum sensing (QS), iron concentration, and nutritional conditions. Microbial communities communicate with each other during biofilm formation through a process called quorum sensing. Quorum sensing is a process of intercellular signaling or

cell-cell communication for coordinating biofilm formation. The communication mechanism is mediated by the production of self-generated diffusible signal molecules known as autoinducers or pheromones[3]. In the environment, biofilms exist in lakes, rivers, soil, rocks, aquatic plants, and sediments, etc. Presence of biofilm producers in the environment may develop antibiotic resistance to a great extent.

The environment plays a crucial role in the emergence of antibiotic-resistant bacteria among humans and animals. These bacteria have developed due to the overuse of antibiotics in both human and animal populations. Environmental isolates can harbor unique properties that make them more resilient to treatments. These isolates are often used to study the effects of antimicrobial agents, as they can mimic the resistance patterns seen in real-world infections. Furthermore, environmental resistance studies may supplement traditional surveillance by reflecting the clinical resistance situation in the region[4].Therefore it is necessary to develop an alternative method to eradicate these biofilm producers from the environment as they have developed antibiotic resistance. As a result, there has been increasing interest in exploring novel approaches to inhibit biofilm formation and disrupt established biofilms, with natural products, particularly plant extracts, emerging as a promising source of bioactive compounds.

Plants are a rich source of number of secondary metabolites having antibacterial, antifungal as well as antibiofilm potential. Secondary metabolites are phytochemicals present in plants that are not essential for the body's normal functions but contribute to health benefits or aid in disease recovery[5]. The plants are effective in killing biofilm producers as they target the quorum sensing (QS) regulatory system and cause the inhibition of biofilm[6]. Therefore, the present study has been designed to evaluate the antibiofilm activity of native plants from local regions of Himachal Pradesh. The plants used in the present study were *Cinnamomum camphora* (Kapur), *Cordia dichotoma* (Lasura) and *Citrus maxima* (Chakotra). Investigating the antibiofilm activity of selective plant extracts against biofilm producers provides valuable insights into their potential use as effective biocontrol agents. Furthermore, such studies can help to identify the specific plant extracts or secondary metabolites that could be used to prevent biofilm-associated infections, particularly in settings where conventional antibiotics are less effective.

MATERIALS AND METHODS:

ISOLATION AND IDENTIFICATION OF ENVIRONMENT ISOLATES

The samples (n=13) were collected from different environmental sources (soil, water and air) of District Shimla and Solan, Himachal Pradesh. Gram staining and biochemical tests were used to identify the bacterial isolates.Further, these bacterial isolates were preserved by subculturing on nutrient agar and glycerol stocks of pure cultures were stored in a refrigerator at -4°C.

BIOFILM ASSAY

The biofilm formation assay was carried out by a qualitative method, the tube test as mentioned by Christensen *et al*., (1982)[7]. Each test isolate was introduced into a test tube containing 10 ml of trypticase soy broth enriched with 1% glucose and 2% sucrose, and then incubated at 37°C for 24 hours. After incubation, the broth was discarded, and the biofilm was rinsed with phosphate-buffered saline (pH 7.3), dried, and stained with 0.1% crystal violet. Excess stain was removed by washing with deionized water. The tubes were then inverted and allowed to dry completely. Biofilm staining was evaluated by comparing the results with a control strain. A positive result was indicated by the presence of a visible film lining the tube's walls and bottom. The weak biofilm formation was scored as +, moderate ++ and strong as +++.

COLLECTION OF PLANT MATERIAL

Plant material i.e. leaves *Cinnamomum camphora*, *Cordia dichotoma* and *Citrus maxima* were collected from local regions of Kangra, Himachal Pradesh. The plant materials were thoroughly washed, shade-dried, and ground into a coarse powder. The powdered samples were then stored in clean, airtight containers with proper labelling until needed for further analysis.

PREPARATION OF PLANT EXTRACTS

The cold percolation method, as described by Rosenthaler (1930)[8], was employed to prepare plant extracts. The dried powdered sample was immersed in various solvents (petroleum ether, chloroform, acetone, methanol, and distilled water) in a 1:10 ratio and allowed to extract at room temperature for 72 hours with agitation at 150 rpm. The resulting filtrates were concentrated by evaporating the solvents at 40°C until dry. The dried extracts were finely powdered and dissolved in Dimethyl sulfoxide (DMSO) at a concentration of 100 mg/ml. Finally, the extracts were stored at 4°C in a refrigerator for future use.

DETERMINATION OF ANTIBIOFILM POTENTIAL OF PLANT EXTRACTS

The impact of various unrefined plant extracts on biofilm-forming bacteria isolated from the environment was assessed using an agar well diffusion method. Inhibition zones (measured in millimeters) were recorded after a 24-hour incubation period following the method of Nostro (2000)[9]. The Minimum Inhibitory Concentration (MIC) of the plant extract needed to suppress microbial growth was determined using the Resazurin dye assay, with the extract subjected to two-fold serial dilutions[10].

PHYTOCHEMICAL ANALYSIS

The methanolic leaf extracts were subjected to phytochemical screening to detect the presence of carbohydrates, tannins, soluble starch, phenols, alkaloids, steroids, saponins, terpenoids, and flavonoids, following the procedure outlined by Thakur *et al*., 2017[11].

ANTIOXIDANT ACTIVITY

The radical scavenging activity of best inhibitory plant extracts was measured spectrophotometrically by using the 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) as described by Rajendran *et al*., 2014. Plant extract (1 mg/ml) was used as standard. 0.5 ml of 1 mmol/L DPPH solution prepared in methanol was added to 2 ml of plant extract. After 15-minute incubation at room temperature, the absorbance of the test tubes was measured at 517 nm. Radical scavenging activity was then calculated using a specific formula.

DPPH radical scavenging activity (%) = $[(A_0-A_1)/(A_0)]_x100$,

Where,

A_0 was the absorbance of DPPH radical + solvent (blank)

A_1 was the absorbance of DPPH radical + sample or standard.

RESULTS:

CHARACTERIZATION OF ISOLATES

Out of 13 collected samples, a total of seventeen environmental isolates were characterized and selected based on different staining, morphological characteristics analysis and biochemical tests performed. Four isolates were identified as *Staphylococcus spp.,* two as *Streptococcus spp.,* four as *Escherichia spp., one Enterobacter spp., Proteus spp., Bacillus spp., Enterococcus spp., Acinetobacter spp., Shigella spp.,* and *Streptobacillus spp.* Results are shown in Table 1.

BIOFILM FORMATION ASSAY

To know the ability of biofilm formation by environmental isolates, biofilm assay was carried out by the tube test. In the present study, 35.29% (6/17) bacterial isolates were found to be strong biofilm producers, 35.29% (6/17) were moderate and 29.41% (5/17) were weak biofilm producers. Status of biofilm production by isolates is shown in Table 2. However, only strong biofilm producers were further used in the study.

ANTIBIOFILM POTENTIAL OF PLANT EXTRACTS

A higher inhibitory effect was recorded with methanol extract of all selected plant leaves at a concentration of 4 mg/ml. All methanolic extracts were quite effective comparatively when 80 µl of its volume was loaded in the well (Fig.1&2). A higher inhibition zone was observed with methanolic extract of *C. camphora* leaf. However, standard antibiotics used in the study were effective against all biofilm producers.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

The MIC of *C. camphora, C. dichotoma,* and *C. maxima* leaf extracts was in the range of 2.5 mg/ml to 5 mg/ml and is given in the Figure 3.

PHYTOCHEMICAL ANALYSIS

Analysis of the chemical compounds in the crude methanol extract of *C. camphora, C. dichotoma* and *C. maxima* revealed the presence bioactive constituents such as carbohydrates, tannins, terpenoids, steroids, reducing sugars, and saponins, etc. The detail of which is presented in table 3.

ANTIOXIDANT ASSAY OF *C. CAMPHORA* LEAF EXTRACTS

Antioxidant assay of methanol, acetone and chloroform extracts of *C. camphora* was performed for determination of their scavenging activity. Only *C. camphora* plant extracts were selected for evaluation of their antioxidant activity because inhibition of biofilm producers was maximum with extracts of this plant. The results of this assay showed that the DPPH free radicals were scavenged by all extracts of this plant in a concentration dependent manner. The maximum antioxidant activity of *C. camphora* was found for methanol extract i.e., 85.80% and least scavenging activity was recorded for chloroform (39.14%) at 200 µg/ml concentration of plant extract.

DISCUSSION:

Biofilm formation is a phenomenon that plays a crucial role in the pathogenesis of infections. Biofilm producers have developed resistance to traditional antimicrobial agents, making it essential to explore alternative strategies, such as plant-derived compounds, to combat biofilm-associated infections. A total of 35.29% strong biofilm producers were identified among environmental isolates by the tube test. In this regard, several plant extracts have been investigated for their potential antibiofilm activity. The present study reported that the plant extracts from *C. camphora*, *C. dichotoma*, and *C. maxima* have shown promising antibiofilm activity.

Among the three plant extracts tested, *Cinnamomum camphora* (Kapur) has demonstrated the highest effectiveness (ZOI=12mm) in combating biofilm formation. However, Ankita *et al*., 2014[12] reported that the methanol and acetone extracts of the same plant showed maximum antibacterial activity against *E. coli, Pseudomonas spp., and Bacillus cereus* in comparison to other extracts used in their study. Rahman *et al.,* 2016[13] in their study showed higher antimicrobial activity of camphor essential oil against *Streptococcus mutans* as compared to *Enterococcus faecalis.* Other studies have shown that *C. camphora* extracts exhibit potent antimicrobial or antibiofilm properties, whose existence can be ascribed to bioactive compounds such as cinnamaldehyde, camphor, and eugenol[14]. These compounds have been proven to inhibit both planktonic and biofilm forms of bacteria, especially against common pathogens like *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*[15]. The high antimicrobial efficacy of *C. camphora* leaf extracts *in vitro* assays makes it a promising candidate for biofilm inhibition. These findings suggest that *C. camphora* has unique bioactive constituents that significantly enhance its antibiofilm potential. Earlier studies conducted found that *C. camphora* extract was particularly effective in preventing the initial stages of biofilm formation and in

disrupting established biofilms, potentially owing to the existence of bioactive compounds like phenols and flavonoids. Additionally, its high effectiveness is corroborated by its ability to reduce bacterial adhesion to surfaces and its antimicrobial properties, which work synergistically to prevent biofilm maturation [14,16].

On the other hand, *C. dichotoma* and *C. maxima* leaf extracts have demonstrated moderate antibiofilm activity as compared to *C. camphora*. In a different study, Konka *et al*., 2018, reported that the methanol extract of *C. dichotoma* showed good antibacterial activity against *E. coli*, *P. aeruginosa* and Methycilin Resistant *Staphylococcus aureus*[17]. Kanubaddi, 2014reported that fruit pulp of *C. dichotoma* showed considerable activity against MRSA (Methicillin Resistant *Staphylococcus aureus*)[18]. In the same line, Das *et al*., 2013, reported that the ethanolic extract of *C. maxima* leaves had good antibacterial activity against *Pseudomonas aeruginosa* and *E. coli*[19]. The mechanisms through which these plant extracts disrupt biofilms are multifactorial and involve both direct antimicrobial activity and interference with biofilm formation and maturation.

The discrepancies in effectiveness highlight the importance of specific phytochemical profiles in determining the antibiofilm activity of plant extracts. In our study, phytochemical analysis of crude methanol extract of *C. camphora* plant revealed the presence of various bioactive constituents such as terpenoids, steroids, phenols, reducing sugars, saponins, tannins, flavonoids, carbohydrates and methanol extract of *C. dichotoma* leaves revealed the presence of glycosides, reducing sugars, tannins, flavonoids and carbohydrates., etc. Similarly, Ankita *et al*., 2013 reported that *C. camphora* contains flavonoids, tannins, terpenoids and carbohydrates but it was free from resins, anthraquinones, alkaloids and saponins[12] and Rahman *et al.,* 2015 reported the presence of carbohydrates, flavonoids, glycosides in *C. dichotoma* leaves[13]. The high carotenoid content of their leaves contributes to their significant antioxidant activity. Prajapati *et al.,* 2017 revealed that this plant extract included sterols, terpenes, flavonoids, coumarins, pyrrolizidine alkaloids, and saponins[20].

The current research involved an examination of the crude methanol extract's phytochemical composition of *C. maxima* leaves revealed the presence of glycosides, terpenoids, phenols, sterols, tannins, flavonoids, and carbohydrates. Similar phytoconstituents were reported by Khan *et al*., 2018 in the same plant[21]. Alkaloids, terpenoids, flavonoids, and phenolic substances were observed to be present. Sapkota *et al.,* 2022 also reported the presence of saponins, carbohydrates, phenols, alkaloids, flavonoids, glycosides, anthraquninone, carotenoids, terpenoids and amino acids in *C. maxima* plant[22].

The antioxidant activity of *C. camphora* has been studied only and shown to exhibit significant free radical scavenging properties, which align with findings from other studies exploring its phytochemical constituents [23-24]. These results suggest that *C. camphora* could serve as a promising natural antioxidant source. The antioxidant activity of the extracts decreased in the following order: methanol > acetone > chloroform. Therefore, the methanol extract exhibited the strongest free radical scavenging activity.The extract's ability to disrupt biofilm formation and reduce bacterial resistance mechanisms, minimize the side effects and reduce the risk of resistance development.

The exploration of plant extracts as antibiofilm agents presents an exciting avenue for developing novel therapeutic strategies against multi-drug resistant pathogens. Therefore, our study revealed that *C. camphora* plant could be a potential candidate for drug discovery to treat infections caused by biofilm producers or some herbal preparation could also be prepared by using this plant. Such formulations could be applied directly for the destruction of biofilm components in the various ecological niches. This might be a new/better option to explore the new medicines for drug formulation but only after purification and cytotoxicity analysis.

TABLE 1 DETAILS OF NUMBER OF BACTERIA IDENTIFIED IN ENVIRONMENTAL SAMPLES (N=13)

Type of Bacteria	Total No. (n=17)
Escherichia spp.	4/17 (23.52%)
Staphylococcus spp.	4/17 (23.52%)
Enterobacter spp.	1/17 (5.88%)
Streptococcus spp.	2/17 (11.76%)
Enterococcus spp.	1/17 (5.88%)
Acinetobacter spp.	1/17 (5.88%)
Shigella spp.	1/17 (5.88%)
Bacillus spp.	1/17 (5.88%)
Streptobacillus spp.	1/17 (5.88%)
Proteus spp.	1/17 (5.88%)

TABLE 2 STATUS OF BIOFILM PRODUCTION BY ENVIRONMENTAL ISOLATES

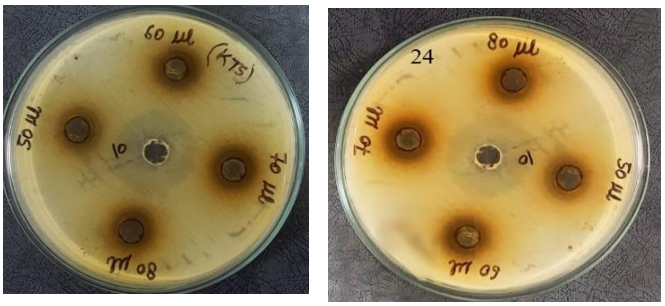
Biofilm Status				
Number of isolates	Non producers	Weak	Moderate	Strong
17	0	05	06	06

TABLE 3 PHYTOCHEMICAL ANALYSIS OF METHANOL EXTRACTS OF PLANTS USED

Compounds	*C. camphora*	*C. dichotoma*	*C. maxima*
Alkaloids	-	-	-
Glycosides	-	+	+
Terpenoids	+	-	+
Sterols	+	-	+

Phenols	+	-	+
Reducing Sugars	+	+	-
Saponins	+	-	-
Tannins	+	+	+
Flavonoids	+	+	+
Starch	-	-	+
Proteins	-	-	-
Carbohydrates	+	+	+

+ indicates presence of compound; - indicates absence of compound



(a) **(b)**

FIG. 1 ANTIBIOFILM ACTIVITY OF METHANOL EXTRACT OF ETALICIZED C. CAMPHORA LEAVES AT HIGHER CONCENTRATIONS AGAINST; (A) ISOLATE NO. S1& (B) ISOLATE NO. F1

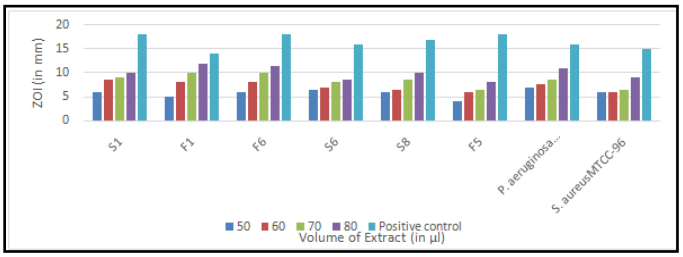


FIG. 2 GRAPHICAL PRESENTATION OF ANTIBIOFILM POTENTIAL OF METHANOLIC EXTRACTS OF *C. CAMPHORA* LEAVES AT DIFFERENT CONCENTRATIONS AGAINST BIOFILM PRODUCERS

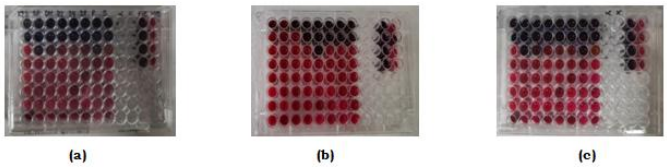


FIG. 3 MINIMUM INHIBITORY CONCENTRATION (MIC) OF METHANOL EXTRACTS OF (A) *C. CAMPHORA*, (B) *C. MAXIMA*,(C) *C. DICHOTOMA* LEAVES AGAINST BIOFILM PRODUCERS

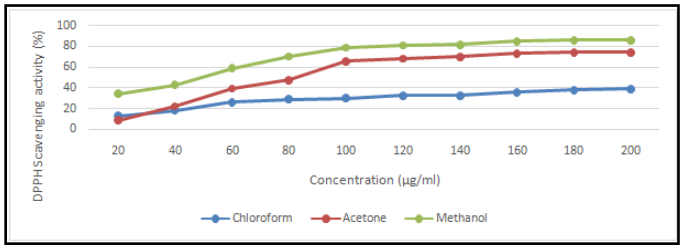


FIG. 4 PERCENTAGE INHIBITION OF DPPH BY *C. CAMPHORA* LEAVES EXTRACTS

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