



SCREENING OF INDIAN MEDICINAL PLANTS AGAINST BIOFILM FORMING BACTERIA

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ABSTRACT

A biofilm is a polymeric conglomeration generally composed of extracellular DNA, proteins and polysaccharides of microorganisms. These biofilms are responsible for microbial diseases and dental carries. Most of plants and herbs are found to be medicinal and have antimicrobial activity to a greater extent. In the present study, we evaluated the antimicrobial activity of petroleum benzene and dicloromethane extracts of leaves of *Azadirachta indica*, *Piper nigrum*, *Cuminum cyminum* and *Ocimum sanctum*. Using the plant extracts, antibacterial assay was performed and the zone of inhibition for each samples were measured. In addition, plant extracts forming the zone of inhibition greater than 15mm was screened to perform biofilm inhibition assay. The biofilm inhibition assay was done with different concentration of the extract against the samples. The sample OD value was recorded and the percentage of inhibition was calculated. All the parameters were found to be effective and within the standards. Altogether, the study suggests a preventive effect of *Azadirachta indica* and *Piper nigrum* in fungal species, but still longer-term randomized clinical trials are necessary to confirm its therapeutic properties.

KEYWORDS: *Azadirachta indica*, *Piper nigrum*, *Cuminum cyminum*, *Ocimum sanctum*, Biofilm, Phytochemicals, Extraction, Antibacterial activity, Evaluation.

INTRODUCTION

A biofilm is a group of microorganisms in which cells stick to each other and often also to a surface. Biofilm is a polymeric conglomeration generally composed of extracellular DNA, proteins and polysaccharides (Stoodley and Lewandowski, 1994).^[1] The microbial cells growing in a biofilm are physiologically distinct from planktonic cells of the same organism, which, by contrast, are single cells that may float or swim in a liquid medium (O'Toole and Kolter, 1998).^[2] These adherent cells are frequently embedded within a self-produced matrix of extracellular substance (EPS) (Stoodley and Lewandowski, 1994). A large proportion of the EPS is more or less strongly hydrated, however, hydrophobic EPS also occur. This matrix encases the cells within it and facilitates communication among them through biochemical signals as well as gene exchange. The EPS matrix is an important key to the evolutionary success of biofilms. One reason is that it traps extracellular enzymes and keeps them in close proximity to the cells. Thus, the matrix represents an external digestion system and allows for stable synergistic micro consortia of different species. Some biofilms have been found to contain water channels that help distribute nutrients and signaling molecules. This matrix is strong

enough that under certain conditions, biofilms can become fossilized (Stromatolites).

Microbes form a biofilm in response to many factors, which may include cellular recognition of specific or non-specific attachment sites on a surface, nutritional cues, or in some cases, by exposure of planktonic cells to sub-inhibitory concentration of antibiotics (Karatan and Watnick, 2009).^[3] The formation of a biofilm begins with the attachment of free-floating microorganisms to a surface (O'Toole and Kolter, 1998). The first colonist bacteria of a biofilm adhere to the surface initially through weak, reversible adhesion via van der Waals forces and hydrophobic effects (Briand et al., 2001).^[4] If the colonies are not immediately separated from the surface, they can anchor themselves more permanently using cell adhesion structures called pili. Hydrophobicity can also with increased hydrophobicity have reduced repulsion between the extracellular matrix and the bacterium (Donlan RM, 2002).^[5] During surface colonization, bacteria cells are able to communicate using quorum sensing (QS) products such as N-acyl homoserine lactone (AHL). Once colonization has begun, the biofilm grows through a combination of cell division and recruitment. The development of a biofilm

may allow for an aggregate cell colony (or colonies) to be increasingly resistant to antibiotics. Cell-cell communication or quorum sensing has been shown to be involved in the formation of biofilm in several bacterial species. Dispersal of cells from the biofilm colony is an essential stage of the biofilm life cycle. Dispersal enables biofilms to spread and colonize new surfaces. Enzymes that degrade the biofilm extracellular matrix, such as dispersin B and deoxy ribonuclease, may play a role in biofilm dispersal (Kaplan *et al.*, 2003).^[6] Biofilm matrix degrading enzymes may be useful as anti-biofilm agents. Recent evidence has shown that a fatty acid messenger, cis-2-decanoic acid, is capable of inducing dispersion and inhibiting growth of biofilm colonies, Secreted by *Pseudomonas aeruginosa*, this compound induces cyclo heteromorphic cells in several species of bacteria and the yeast *Candida albicans* (Davies and Marques, 2009).^[7] Nitric oxide has also been shown to trigger the dispersal of biofilms of several bacteria species at sub-toxic concentrations.

In some cases, antibiotic resistance can be increased a thousand fold. However, biofilms are not always less susceptible to antibiotics. For instance, the biofilm of *Pseudomonas aeruginosa* has no greater resistance to antimicrobials than do stationary-phase planktonic cells, although when the biofilm is compared to logarithmic-phase planktonic cells, the biofilm does have greater resistance to antimicrobials. This resistance to antibiotics in both stationary-phase cells and biofilms may be due to the presence of persistent cells.

Azadirachta indica (commonly known as neem and Indian lilac) is a tree in the mahogany family Meliaceae. Neem oil is also used for healthy hair, to improve liver function, detoxify the blood and balance blood sugar levels. Neem leaves have also been used to treat skin diseases like eczema, psoriasis, etc.

Black pepper is the dried mature berry of *Piper nigrum*, a climbing, perennial shrub mostly found in hot, moist region of Southern India. The major property of Black Pepper is the dried mature berry of *Piper nigrum*, a climbing, perennial shrub mostly found in hot, moist region of Southern India. The major property of *Piper nigrum* is anti-bacterial (Karsha *et al.*, 2010)^[8], anti-colon toxin, antidepressant, antifungal, anti-diarrhoeal, anti-inflammatory, anti-mutagenic, anti-metastatic, anti-tumor, anti-spasmodic, anti-thyroid, hepatoprotective insecticidal activity. Piperine, the pungent principle in pepper oleoresin helps to enhance bioavailability and therefore used in pharmaceuticals.

Cuminum cyminum is a flowering plant in the family Apiaceae, native from the east Mediterranean to South Asia. Cumin has major properties like anti-microbial (Jirovetz *et al.*, 2005)^[9], anti-allergic, anti-oxidant, anti-platelet aggregation and hypoglycemic. In medicine, it is used as a stimulant, carminative, stomachic and astringent.

Ocimum tenuiflorum (synonym *Ocimum sanctum*), commonly known as holy basil, tulsi, is an aromatic plant in the family Lamiaceae which is native to the Indian subcontinent. It has antioxidant, antimicrobial (Joshi *et al.*, 2011)^[10] and antifungal properties.

MATERIAL AND METHODS

All the experiments were done in the period of two months June – July (2017) in Biozone Research Technologies, Saidapet, Chennai. Relevant information on the experimental setup, experimental design, materials used in these investigations and methodology adopted are described in detail.

Microbial sample collection

The dental (plaque) samples were collected from Apollo Dental Hospital, Chennai, Tamilnadu. The samples were named as S1 2a and S1 2b. The samples were isolated by streaking method. Single colonies were isolated by quadrant streaking and the plates were stored for further studies. The samples were characterized using: a) Crystal Violet Assay for calculating the OD value to interpret the biofilm formation at 570nm, b) DNA Isolation and c) PCR (using 16SrRNA).

Plant sample collection

Plant samples of *Azadirachta indica*, *Piper nigrum*, *Cuminum cyminum* and *Ocimum sanctum* were collected and the samples were dried in the shade and were finely powdered.

Preparation of plant extract

150g of finely powdered samples of *Azadirachta indica*, *Piper nigrum*, *Cuminum cyminum* and *Ocimum sanctum* were extracted using petroleum benzene and dichloromethane by Sequential extraction (Soxhlet). The crude extracts were collected and stored in separate vials.

Qualitative analysis of the extracts

Qualitative test for confirming the presence of carbohydrates, alkaloids, flavonoids, tannins, saponins, quinines, glycosides, cardiac glycosides terpenoids, phenols, coumarines, steroids and phytosteroids, phlobatannins and anthraquinones were carried by the method described in (Devendran G *et al.*, 2011).^[11]

Determination of antibacterial activity

Antibacterial activity of the experimental plant was investigated by agar well diffusion method (Bonjar *et al.*, 2005).^[12] Nutrient Agar (NA) plates were inoculated (swabbing method) with test organisms. The plates were evenly spread out. Then wells were prepared in the plates with a cork borer. Each well was loaded with 20µl of corresponding sample and 10 mg of Tetracycline dissolved in 1 ml of 10% DMSO was used as a positive control. The plates were incubated for 24h at 37°C. The development of inhibition zone around the well was measured (diameter) and recorded.

Biofilm inhibition assay

Biofilm inhibition assay was carried out in test tubes adopting modified method of biofilm inhibition spectrophotometric assay.^[13] 3ml of cell suspension of strain S1 2a and strain S1 2b, thus prepared was added into test tubes and different concentration of plant extracts as 200, 400, 600, 800 and 1000 µg/ml was added and incubated at 37° C for 24 hours. After the incubation, the liquid suspension was removed and 3ml of 1% w/v aqueous solution of crystal violet was added. Following staining at room temperature for 30 minutes the dye was removed and the wells were washed thoroughly, 95% ethanol was added and incubated for 15 minutes. The reaction mixture was read spectrophotometrically at 570nm. Inhibition mediated reduction of biofilm formation was calculated by the following formula.

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

Molecular characterization studies

Bacterial strains were analyzed by Agarose gel electrophoresis method. The bacterial DNA was extracted and quantified using UV spectrophotometer. The amplification was done with 16S rRNA primer using the extracted DNA as a template. Sanger's method of sequencing was used to sequence the purified PCR product in a 3730 DNA sequencing analyzer (ABI)36.

Sequencing analysis

A nucleotide BLAST for the strains S1 2a and S1 2b were obtained and found to be congeneric to *Bacillus sp.* with query coverage of 99 and 91 percent. Using Mega 7.0 software the neighbor joining tree was generated which indicated the evolutionary relationship between the various species of *Bacillus* as depicted in result.

RESULTS

Dental bacterial samples isolation

The dental samples were collected from Apollo Dental Hospital, Chennai, Tamilnadu. The samples were named as S1 2a and S1 2b. The samples were isolated by streaking method. The sample S1 2a was found to be *Bacillus mojavensis* (Figure 1) and sample S1 2b was found to be *Bacillus species* (Figure 2).

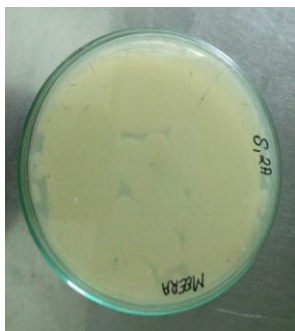


Fig 1: Strain S1 2a



Fig 2: Strain S1 2b

Crystal violet assay

In a micro tetrad pate, NB media was taken and to each well overnight culture grown in NB media was

inoculated (Figure 3). The plates were incubated and the media was discarded without disturbing the biofilm. The cells were stained using of 2% Hacker's Crystal Violet (2% crystal violet and 1% ammonium oxalate). Absorbance was read at 570nm. The OD value was calculated to interpret the biofilm formation.

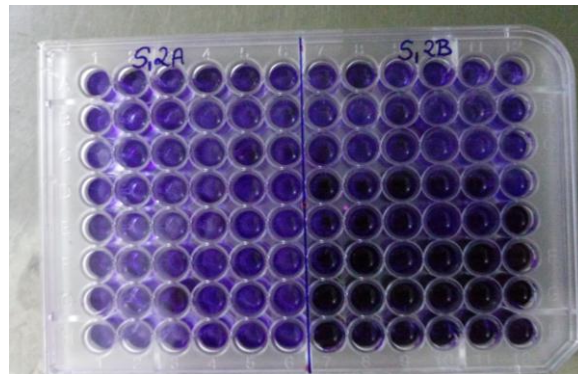


Fig 3: Crystal violet assay in microtetrad plate.

Table 1: The absorbance values of the isolated samples at 570nm.

SAMPLE /READINGS	Strain S1 2a	Strain S1 2b
Reading 1	0.6184	0.7814
Reading 2	0.6324	0.7789
Reading 3	0.6614	0.7905
Reading 4	0.6241	0.7652
Reading 5	0.6421	0.7546

Qualitative assay

The preliminary analysis of petroleum benzene and dichloromethane of *Azadirachta indica*, *Piper nigrum*, *Cuminum cyminum* and *Ocimum sanctum* leaves are listed in Table 2 – Table 5. Most of the phytochemicals were found to be present in the plant extract samples.

Table 2: Presence of phytochemicals in *Azadirachta indica* extract.

S.No.	<i>Azadirachta indica</i>	Petroleum Benzene	Dichloro Methane
1	Carbohydrate	+	+
2	Tannins	+	+
3	Saponins	-	-
4	Flavonoids	+	+
5	Alkaloids	+	+
6	Quinones	-	+
7	Glycosides	-	-
8	Cardiac Glycosides	+	+
9	Terpenoids	+	+
10	Phenols	+	+
11	Coumarins	+	+
12	Steroids	+	+
13	Phytosteroids	-	-
14	Phlobatannins	-	-
15	Anthroquinones	-	-

Table 3: Presence of phytochemicals in *Piper nigrum* extract.

S.No.	<i>Piper nigrum</i>	Petroleum Benzene	Dichloro Methane
1	Carbohydrate	+	+
2	Tannins	+	+
3	Saponins	-	-
4	Flavonoids	+	+
5	Alkaloids	+	+
6	Quinones	-	+
7	Glycosides	-	-
8	Cardiac Glycosides	+	+
9	Terpenoids	+	+
10	Phenols	+	+
11	Coumarins	+	+
12	Steroids	+	+
13	Phytosteroids	-	-
14	Phlobatannins	-	-
15	Anthroquinones	-	-

Table 4: Presence of phytochemicals in *Cuminum cyminum* extract.

S.No.	<i>Cuminum cyminum</i>	Petroleum Benzene	Dichloro Methane
1	Carbohydrate	+	+
2	Tannins	+	+
3	Saponins	-	-
4	Flavonoids	+	+
5	Alkaloids	+	-
6	Quinones	+	+
7	Glycosides	-	-
8	Cardiac Glycosides	+	-
9	Terpenoids	+	+
10	Phenols	+	+
11	Coumarins	+	+
12	Steroids	+	+
13	Phytosteroids	-	-
14	Phlobatannins	-	-
15	Anthroquinones	-	-

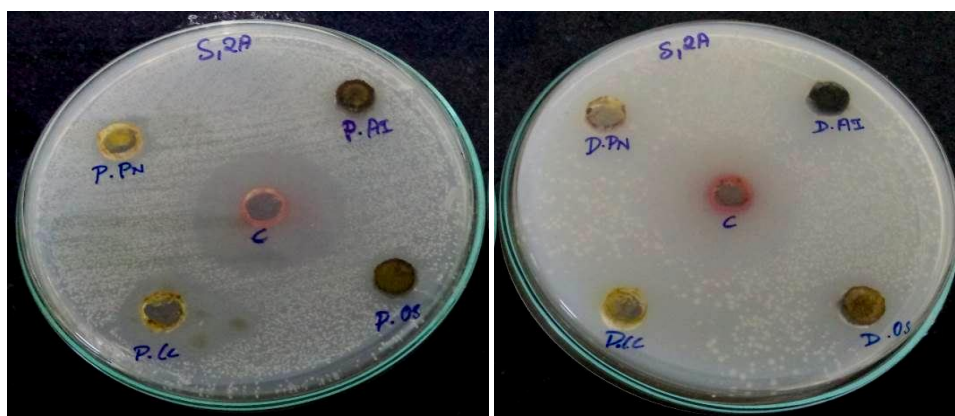
Table 5: Presence of phytochemicals in the *Ocimum sanctum* extract.

S.No.	<i>Ocimum sanctum</i>	Petroleum Benzene	Dichloro Methane
1	Carbohydrate	+	+
2	Tannins	+	+
3	Saponins	-	-
4	Flavonoids	+	+
5	Alkaloids	+	-
6	Quinones	-	+
7	Glycosides	-	-
8	Cardiac Glycosides	+	-
9	Terpenoids	+	+
10	Phenols	+	+
11	Coumarins	+	+
12	Steroids	-	+
13	Phytosteroids	+	-
14	Phlobatannins	-	-
15	Anthroquinones	-	-

Antibacterial activity

The antibacterial activity of petroleum benzene and dichloromethane of *Azadirachta indica*, *Piper nigrum*, *Cuminum cyminum* and *Ocimum sanctum* leaves were analysed by diffusion method by using tetracycline as the positive control. For strain S1 2a, petroleum benzene and dichloromethane extract of *Cuminum cyminum* and

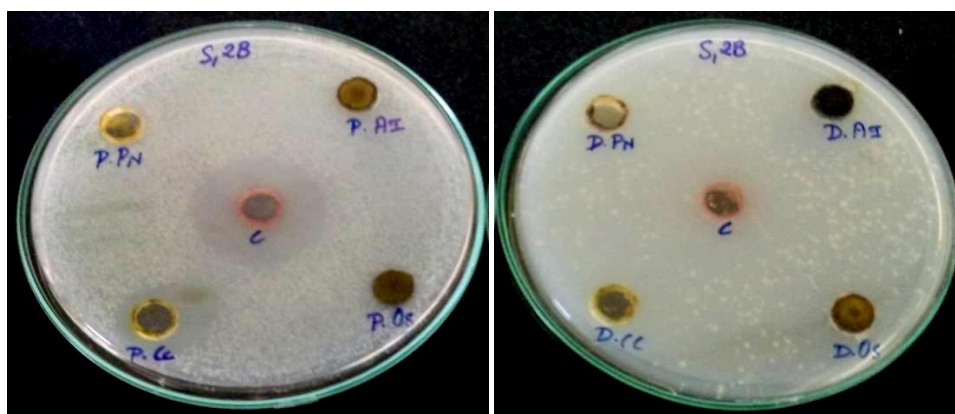
dichloromethane extract of *Azadirachta indica* shows significant zone of inhibition (Figure 6). For strain S1 2b, petroleum benzene and dichloromethane extract of *Cuminum cyminum* and dichloromethane extract of *Azadirachta indica* and *Piper nigrum* shows significant zone of inhibition (Figure 7).



Petroleum benzene extract

Dichloromethane extract

Fig 4: Zone of inhibition for strain S1 2a.



Petroleum benzene extract

Dichloromethane extract

Fig 4: Zone of inhibition for strain S1 2b.

Biofilm inhibition assay

Biofilm inhibition assay was carried out in test tubes adopting modified method of biofilm inhibition spectrophotometric assay for Petroleum benzene and Dichloro methane extracts of *Azadirachta indica*, *Piper nigrum*, *Cuminum cyminum* and *Ocimum sanctum*. For strain S1 2a, petroleum benzene extract of *Azadirachata indica* and dichloromethane extract of *Piper nigrum* showed a high range of inhibition (Table 6 and Table 7) and for strain S1 2b, petroleum benzene extract of *Azadirachata indica* and dichloromethane extract of *Piper nigrum* showed a high range of inhibition (Table 8

and Table 9). The inhibition was found to be directly proportionate to increasing concentration of the extract. However dichloromethane extract of *Cuminum cyminum* against strain S1 2a was also found to be satisfactory. The graphical representation of the inhibition assay of plant extracts in petroleum benzene and dichloromethane are shown in Figure 8 and Figure 9.

Table 6: The absorbance value of biofilm inhibition assay for strain S1 2a.

Concentration(μ g)	P.Cc	D.Os	D.Cc	D.Pn	D.Ai
200	0.6194	0.6018	0.5974	0.5773	0.6418
400	0.5928	0.5869	0.5862	0.5738	0.6
600	0.5887	0.5825	0.5537	0.5709	0.5828
800	0.5842	0.5784	0.5449	0.5635	0.5598
1000	0.5790	0.5464	0.5402	0.5513	0.5328

Note: P.Cc-Petroleum benzene extract of *Cuminum cyminum*, D.Os-Dichloro methane extract of *Ocimum sanctum*, D.Cc-Dichloro methane extract of *Cuminum cyminum*, D.Pn-Dichloro methane extract of *Piper nigrum*, D.Ai-Dichloro methane extract of *Azadirachta indica*.

Table 7: Percentage of inhibition for strain S1 2a

Sample – Strain S1 2a

Control=0.6781

Concentration(μ g)	P.Cc	D.Os	D.Cc	D.Pn	D.Ai
200	8.65654	11.25203	11.9009	14.86506	5.353193
400	12.57927	13.44934	13.55257	15.38121	11.51748
600	13.1839	14.09822	18.34538	15.80888	14.05397
800	13.84752	14.70285	19.64312	16.90016	17.4458
1000	14.61436	19.42191	20.33623	18.69931	21.42752

Note: P.Cc-Petroleum benzene extract of *Cuminum cyminum*, D.Os-Dichloro methane extract of *Ocimum sanctum*, D.Cc-Dichloro methane extract of *Cuminum cyminum*, D.Pn-Dichloro methane extract of *Piper nigrum*, D.Ai-Dichloro methane extract of *Azadirachta indica*.

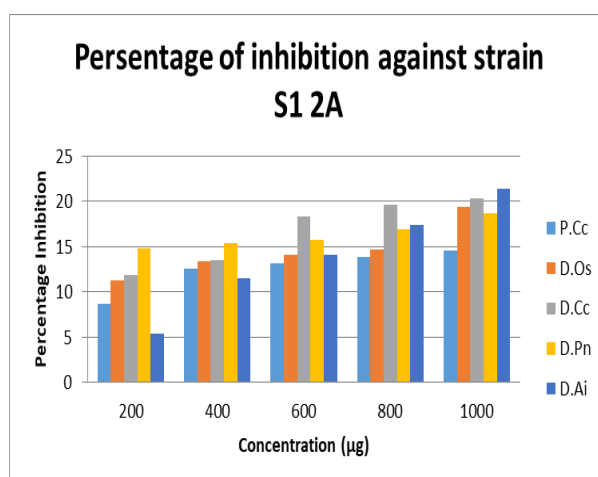
**Fig 5: Percentage of Inhibition against strain S1 2a.**

Table 8: The absorbance value of biofilm inhibition assay for strain S1 2b.

Concentration(μ g)	P.Cc	D.Os	D.Cc	D.Pn	D.Ai
200	0.7604	0.7435	0.7496	0.7541	0.7668
400	0.7390	0.7389	0.7409	0.7209	0.7568
600	0.7319	0.7183	0.7244	0.6877	0.7501
800	0.7290	0.7077	0.7196	0.6642	0.7471
1000	0.7189	0.6941	0.7011	0.6412	0.7369

Note: *P.Cc*-Petroleum benzene extract of *Cuminum cyminum*, *D.Os*-Dichloro methane extract of *Ocimum sanctum*, *D.Cc*-Dichloro methane extract of *Cuminum cyminum*, *D.Pn*-Dichloro methane extract of *Piper nigrum*, *D.Ai*-Dichloro methane extract of *Azadirachta indica*.

Table 9: Percentage of inhibition for strain S1 2b

Sample – Strain S1 2b

Control=0.7728

Concentration(μ g)	P.Cc	D.Os	D.Cc	D.Pn	D.Ai
200	1.604555	3.791408	3.00207	2.419772	0.776398
400	4.373706	4.386646	4.127847	6.715839	2.070393
600	5.292443	7.052277	6.26294	11.0119	2.937371
800	5.667702	8.423913	6.884058	14.0528	3.325569
1000	6.974638	10.18375	9.27795	17.02899	4.645445

Note: *P.Cc*-Petroleum benzene extract of *Cuminum cyminum*, *D.Os*-Dichloro methane extract of *Ocimum sanctum*, *D.Cc*-Dichloro methane extract of *Cuminum cyminum*, *D.Pn*-Dichloro methane extract of *Piper nigrum*, *D.Ai*-Dichloro methane extract of *Azadirachta indica*.

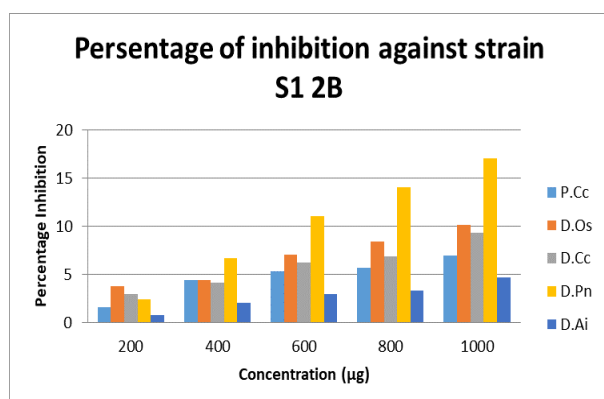


Fig 6: Percentage of Inhibition against strain S1 2b.

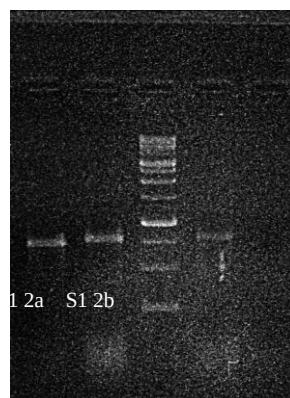


Fig 7: DNA isolation

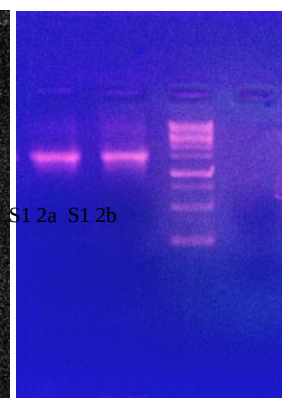


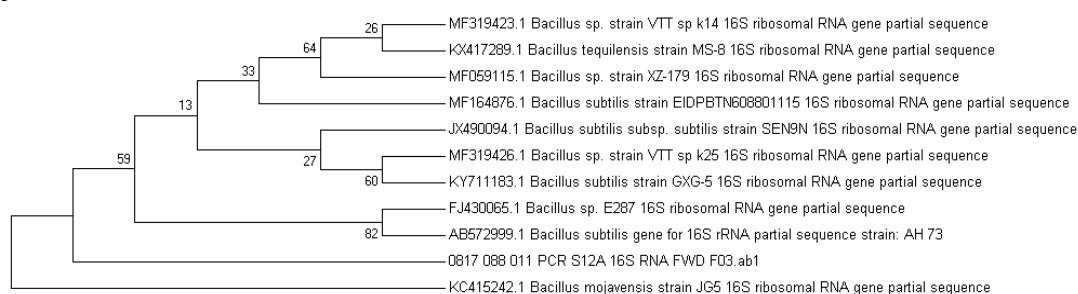
Fig 8: PCR sample in agarose gel

DNA isolation and PCR technique

DNA was isolated from both the cultures using agarose gel electrophoresis (Figure 4). Plasmid was isolated from the transformed colony and used for PCR study (Figure 5).

Phylogeny

Bootstrap Consensus tree

Fig 9: Evolutionary relationship of S1 2a between various species of *Bacillus* by MEGA 7.0.

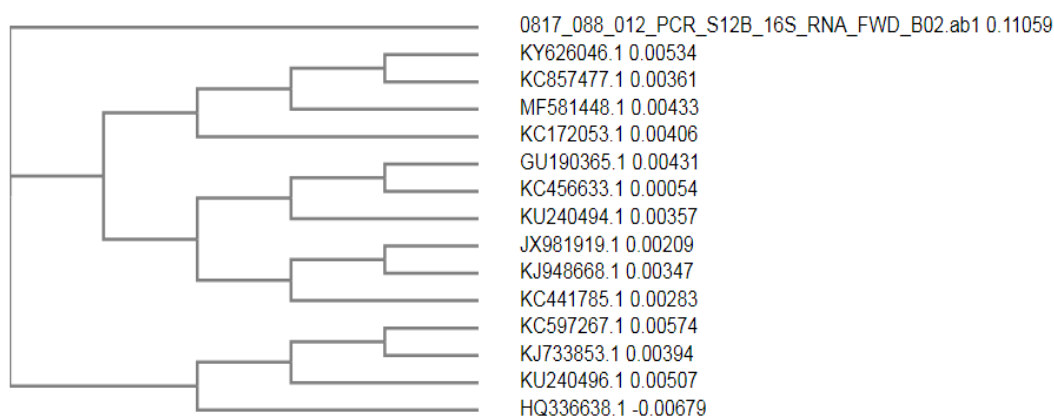


Fig 10: Evolutionary relationship of S1 2b between various species of *Bacillus* by MEGA 7.0.

DISCUSSION

In the previous study conducted, antibacterial activity of Neem and Peppermint extracts were observed. The pure, ethanol, acetone and methanol extracts of Neem were screened against bacterial strains i.e. *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhi*, *Pseudomonas*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Staphylococcus epidermitis* by using agar disc-diffusion assay and fractions obtained from gel filtration chromatography. Similarly, zones of inhibition of Peppermint by agar disc diffusion assay and fractions by gel filtration chromatography were measured. The fractions of Neem were screened against seven bacterial strains as mentioned above. The antibiotic Ampicillin drug was loaded as a control to check the comparison of antibacterial activity with different crude extracts of medicinal plants. The maximum antibacterial activity observed by ampicillin against *E.coli* was 22 mm.

In the above mentioned paper they used pure ethanol, acetone and methanol extract of Neem (*Azadirachta indica*) against two different organisms, but I used Petroleum Benzene and Dichloro Methane extract of Neem (*Azadirachta indica*). The control used in agar disc-diffusion assay they used Ampicillin drug but I used Tetracycline and that drug to showed a maximum antibacterial activity of 23mm.

A study conducted explained a total of 8 methanolic plant extracts against 5 clinical bacterial isolates. Methanol was selected as extraction solvent, because it is one of the best solvents used for the extraction of antimicrobial substances. Results of preliminary antimicrobial tests, performed by the well diffusion method, were quite variable between each plant extract ranging from 0 to 2.8 cm. *P.laevigata* extract was active against all the clinical isolates, while *N. bivalve* bulb did not show activity against any microorganism. The highest diameter of inhibition was obtained with *P.laevigata* extract, against *S.aureus* strain, followed by *G.microcephala* and *O.ficus-indica* also against *S.aureus*. Meanwhile *E.coli* was less susceptible to these extracts, respectively *K.pneumoniae* and *E.faecalis* were more resistant to the extracts, only inhibited

by *P.laevigata* and *O.ficus-indica* with inhibition zones ranging high; on the other hand, *S.maltophilia* was the only microorganism that was not inhibited by the extracts.

In the above paper they used 8 methanolic plant extracts against biofilm forming bacteria and they concluded the zone of inhibition of different organisms. Here I used 4 Petroleum Benzene extract and 4 Dichloro Methane extract of 4 different plants (*Azadirachta indica*, *Cuminum cymunim*, *Ocimum scatum*, *Piper nigrum*) against 2 different organisms. I conclude that Petroleum Benzene extract of *Azadirachta indica* and Dichloro Methane attract of *Piper nigrum* showed a high range of inhibition against *Bacillus mojavensis* (S1 2a) and S1 2b (Uncultured *Bacillus* species) respectively.

The composition of the matrix varies according to the nature of the organism and reduction in the biochemical composition of the biofilm matrix leads to weakening of the biofilm thus facilitating the entry of the drugs. Purification and identification of the phytochemicals responsible for the anti-biofilm effect and the coating of the bioactive compound with chitosan will lead to development of effective anti-microbial against harmful pathogenic microorganism.

The plant extracts evaluated in present research had potential antimicrobial and antibiofilm activities against isolated nosocomial bacteria, which can be an alternative to control the formation of microbial biofilms or can be used as model to the search for new drugs.

CONCLUSION

The medicinal plants find application in pharmaceutical, cosmetic, agricultural and food industry. The use of the medicinal herbs like *Azadirachta indica*, *Piper nigrum*, *Cuminum cymunim* and *Ocimum scantum* are used for curing disease caused by *Bacillus mojavensis* and *Bacillus species* has been documented in history of all civilizations. Before onset of synthetic era, man was completely dependent on medicinal herbs for prevention and treatment of diseases. With introduction of scientific procedures the researchers have concluded that the plants

contain active principles responsible for curative action of the herbs. After reviewing, there is considerable evidence that plants extracts, and purified phytochemicals have potential to be developed into agents which can be used as a preventive or treatment therapies for oral diseases such as dental caries.

This study paves way for further studies of plant extracts for treating microbial infections and analyzing the chemical composition of the organism and the gene responsible for the formation of biofilm which helps to study the methods of inhibition better.

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