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RESEARCH ARTICLE

Investigation of Bactericidal Effects of Medicinal Plant Extracts on Clinical Isolates and Monitoring Their Biofilm Forming Potential

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ABSTRACT

This study aims at checking the inhibitory effects of different plant extracts on biofilm forming microorganisms isolated from clinical setting. A total of 60 samples including 30 from oral sites and 30 from urine and wounds were collected and 50 morphologically different strains were isolated. Six highly resistant strains were characterized morphologically, physiologically, biochemically and genetically. Isolated strains were tested for biofilm formation using test tube assay, Congo red assay and liquid-interface coverslip assay. Antibacterial activity of aqueous and methanolic extracts of 5 different plants including *Camellia sinensis* (green tea), *Syzygium aromaticum* (clove), *Musa sepientum* (banana), *Mentha piperita* (peppermint) and *Allium sativum* (Garlic) was determined both individually and in combination against selected strains in both planktonic and biofilm mode. 16srRNA sequencing identified strains as *Providencia stuartii*, *Shigella sonnei*, *Escherichia coli*, *Bacillus cereus*, *Enterobacter aerogenes* and *Macrococcus caseolyticus*. Significant biofilm formation was observed by each of the three methods for all strains except for *E. coli* and *P. stuartii*. Aqueous extract of *A. sativum* showed highest antibacterial activity against all strains with MIC ranging from 75-735 mg ml^{-1} and MBC from 255-740 mg ml^{-1}. Aqueous extracts of *M. sepientum* exhibited maximum biofilm reduction in *B. cereus*. Reported knowledge of medicinal plants as antibacterial and antibiofilm agents against both highly contagious and antibiotic resistant gram positive and the gram negative isolates provide novel information necessary to control their formation in clinical setting. Hence, there is an increasing need to research new substances with the ability to inhibit these strains.

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INTRODUCTION

A balanced and healthy relationship between people and their environment must exist, to survive on earth. Humans have been dependent on plants, not only for oxygen and food but also for their medicinal effect and remedies. Books and many other informational sources are available to tell the medicinal effectiveness of various plants (York *et al*., 2011; Memon *et al*., 2015).

Microbes are everywhere on planet earth constituting varieties of habitats. These microbes may live singly or in colonies performing various functions of life. The work of various scientists has revealed that more than 99% bacteria exist as biofilms in natural environments (Kirti *et al.*, 2013; Ali *et al*., 2015; Nasir *et al*., 2015).

Bacterial biofilm is an aggregate or a structured community of bacterial cells in which cells adhere to non-living or living surface, and are embedded in a self-produced matrix of extracellular polymeric substance (EPS). Development of these sessile biofilms and their remarkable resistance against host immune system and a variety of antibiotics is the major cause of many infectious bacterial diseases (Hoiby *et al.*, 2010).

Plants have immense therapeutic potential with respect to their antimicrobial activity. The development of increasing resistance in a wide variety of infectious pathogens against commonly used antibiotics and therapeutic agents, has promoted great interest in developing new natural anti-microbial agents (Palombo, 2011; Hameed and Ahmed, 2014).

Syzygium aromaticum is commonly known as Clove. Eugenol is its basic antimicrobial agent. In folk medicine, buds of *S. aromaticum* were used for odontalgic, tonicardic, diuretic, stomachic, aromatic condiment properties (Ayoola *et al*., 2008). *Camellia sinensis* is known as Green tea. It has significant antibacterial activity especially against *E. coli* and *Salmonella* (Amit *et al.,* 2012). *Allium sativum*, commonly known as Garlic has been used for its medicinal activity for centuries. It is also well known for its inhibitory effects against *Shigella, S. aureus and Salmonella*. Because of its bacteriocidal as well as bacteriostatic activities, garlic can be used as a sterilizer or disinfectant (Karuppiah and Rajaram, 2012).

Mentha piperita (peppermint) extracts are able to cease the growth of various pathogens like *Streptococcus pyrogens, S. aureus, E. coli* and *Mycobacterium avium*. It is reported that peppermint oil is strongly effective against *Bacillus subtilis, Klebsiella pneumoniae, Staphylococcus aureus, B. cereus* and *E. coli* (Sujana *et al*., 2013). *Musa sapientum* (Banana) is a herbaceous plant and its skin has been referred to as nature's bacteria-proof. Its leaves have been found to be effective against bacteria (Agarwal, 2011).

As infections caused by biofilm forming isolates are difficult to treat, mainly because of their high resistance against different antibiotics used, the purpose of this study is to check the effects of different medicinal and herbal plant extracts against biofilm formation of isolates in order to provide an alternative to treat fatal infections caused by these bacteria.

MATERIALS AND METHODS

Sample collection: 30 oral samples were collected by swabbing across the tooth surfaces as well as from the roof and floor of the buccal cavity, supragingival and subgingival regions of patients at Punjab dental hospital Lahore. Also, 30 clinical and 15 urine samples were collected from Ganga Ram hospital and Mayo hospital, Lahore. All samples were immediately transferred to 1 ml saline solution (0.85%) and spread on nutrient agar plate except for urine samples, which were spread on CLED agar plates. Morphologically different strains were isolated and purified.

Morphological, biochemical, Physiological and genetic Characterization of isolated strains: Cell morphology was observed by gram staining and acid fast staining. Following Gerhardt *et al*. (1999), different biochemical tests such as catalase, citrate utilization, H_2S production etc. were performed to identify bacterial isolates. Bacterial strains were characterized physiologically on the basis of growth curve, temperatures (25, 37 and 45°C) and pH (5, 7 and 9).

Antibiotic resistance profile (Kirby Baeur method) and 16S rRNA gene sequencing: Antibiotic resistance profile of selected strains was performed using disc diffusion assays. 16S rRNA gene sequencing of six strains was performed. Genomic DNA was isolated and amplified using Universal primers 16S-27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16S-1522R (5′-AAGGAGGTGATCCAGCCGCA-3′) (Penicon). PCR reaction was performed under standard conditions. Sequence data obtained was examined using BLAST and Phylogenetic tree were constructed.

Biofilm formation: Biofilm forming capability of isolates was assessed by three methods i.e., Congo red Assay (Mathur *et al.,* 2006), tube method (Liaqat *et al.,* 2009) and air- liquid interface coverslip assay (Mathur *et al.,* 2006). Experiments were run in triplicates.

Preparation of plant extracts: Five different plants including *M. piperita*, *M. sapientum*, *C. sinensis*, *S. aromaticum* and *A. sativum* were used for the preparation of plant extracts. Both aqueous and methanolic extracts of aforementioned plants were prepared. The aqueous extracts of *M. piperita*, *C. sinensis* and *M. sapientum* were prepared following Somchit *et al*. (2003). Method by Badhe *et al*. (2013) was used to prepare aqueous extract of *S. aromaticum*. *A. sativum* extract was prepared by crushing *A. sativum* cloves using mortar and pestle by adding autoclaved distilled water (Saravanan *et al*., 2010). Methanolic extracts of plants were prepared by dissolving 60 g of plant powder in 360 ml methanol. All prepared extracts were stored at 4°C (Saravanan *et al*., 2010; Agrawal, 2011).

Antibacterial activity of plant extracts: Antibacterial activity of plant extracts was determined by Agar well diffusion method (Milyani and Ashy, 2011). In addition the antibacterial activity of combinations of plants extracts was also tested by preparing mixtures of plants by taking equal concentration of each plant extract.

Minimum inhibitory (MIC) and minimum bactericidal concentration (MBC) determination: The plant extracts which showed antibacterial activity were tested for MIC and MBC. MIC of the extracts was determined by broth dilution method. MIC was recorded as the lowest concentration which showed no visible growth. The concentration at which 99% of the growth was inhibited was recorded as MBC.

Susceptibility of biofilms against plant extracts: 3ml nutrient broth was prepared and added to test tubes. Plant extracts equal to their MIC concentrations were added in nutrient broth. O.D was measured at 523 nm. Experiment was run in duplicates.

Statistical Analysis: Means and SDs of whole data were calculated. Results obtained in these experiments were analyzed statistically according to Steel and Torrie (1981), and means and SEMs were calculated using Microsoft Excel software (Microsoft Corporation). Student ‘t’ test was applied to analyze the data.

RESULTS

Bacterial characterization: Out of 50 morphologically different strains, 20 highly antibiotic resistant strains were characterized morphologically and biochemically. Strains were identified as genera of *Enterobacter, Pseudomonas, Escherichia, Shigella, Yersinia, Klebsiella, Salmonella, Enterococcus, Proteus, Providencia, Staphylococcus, Streptococcus, Lactobacillus* and *Bacillus.* Six strains

exhibiting significant biofilm formation were characterized physiologically. All strains showed optimum growth temperature at 37°C and pH 7 except *E. aerogenes* (best growth at pH 6). Growth curve of these strains was noted for 14 hours. All the strains exhibited the lag phase for 1 hour followed by log phase. In *E. aerogenes* and *P. staurtii* log phase was observed till 9 hours after that stationary phase started. *M. caseolyticus* and *B. cereus* exhibited log phase for 7 hours (Fig. 1).

Antibiotic resistance profile and 16S rRNA gene sequencing: *P. stuartii, S. sonnei, E. coli* showed antibiotic resistance to all antibiotic discs used and *B. cereus, M. caseolyticus*, showed sensitivity to tetracycline only with zone of susceptibility (2mm, 3.5mm). *E. aerogenes* showed sensitivity to tetracycline and carbenicillin (2mm, 1mm). The isolates were identified as *B. cereus* (ATCC: 658270), *M. caseolyticus* (AC: KM658271), *E. aerogenes* (ATCC 658272), *E. coli* (ATCC 658276), *S. sonnei* (ATCC 658277) and *P. stuartii* (ATCC 658278).

Biofilm formation: Three methods were used to measure biofilm formation. All the strains showed positive result (black crystalline colony) on Congo red agar emphasizing their capability of biofilm formation except *P. stuartii* and *E. coli*, which showed negative results. Test tube method revealed biofilm formation ability in all six strains. Significant biofilm formation was observed after 72 hours. *E. aerogenes* showed highest biofilm forming capability (Fig. 2a). Biofilm formation on the coverslips showed *B. cereus* as strong biofilm former (Fig. 2b).

Antibacterial activity of plant extracts: Among all dilutions of extracts tested, 100% dilution was found to be most effective. Bacterial susceptibility against aqueous extract of *A. sativum* was highest (4.5-10 mm). The largest antibacterial zone of inhibition was 10±0.816 mm ($P<0.05$) against *E. aerogenes* while *S. sonnei* showed smallest zone of inhibition of 4.5±0.577 mm. Among methanolic extracts, *C. sinensis* showed zone of inhibition in the range of 3-4.25 mm. Maximum ZI noted to be 4.25±0.5 mm was shown by *P. stuartii*. It was found that aqueous extract of *C. sinensis*, *M. sapientum* and *A. sativum* were more effective in their antibacterial activity on studied strains as compared to methanolic ones (Table 1). The combination of *A. sativum*, *M. piperita* aqueous extracts and *A. sativum*, *M. sapientum* aqueous extracts showed increased antibacterial activity (with ZI 4-7.5 mm, 3.5-7.5 mm). Strong synergistic effect was studied by applying combination of *A. sativum* with *M. sapientum* and *M. piperit* (Table 1).

Minimum inhibitory (MIC) and minimum bactericidal concentration (MBC) determination: The range of MIC for all aqueous extracts of plants except *A. sativum* ranged between 5mg ml^{-1} to 30 mg ml^{-1}, while the MIC of methanolic extracts of plants were in the range of 10 mg ml^{-1} to 35 mg ml^{-1}. The lowest MIC value was determined against *M. caseolyticus* (5 mg ml^{-1}) using aqueous extract of *M. piperita*. The range of MBC of all plant extracts except for *A. sativum* was 20 mg ml^{-1} to 40 mg ml^{-1}. The MIC of *A. sativum* against tested strains ranged from 75 to 735 mg ml^{-1} and MBC ranged from 255 mg ml^{-1} to 740 mg ml^{-1} (Table 2; Fig. 3).

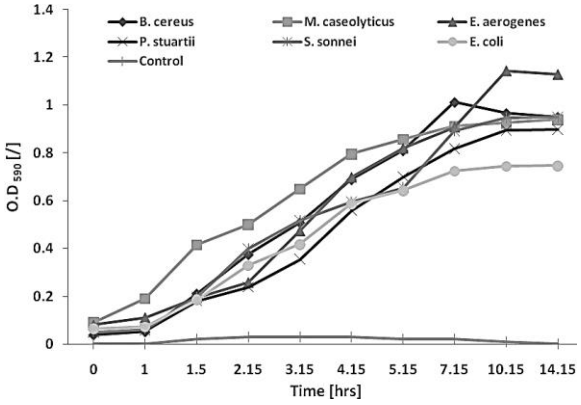

Fig. 1: Growth curve of Bacterial strains

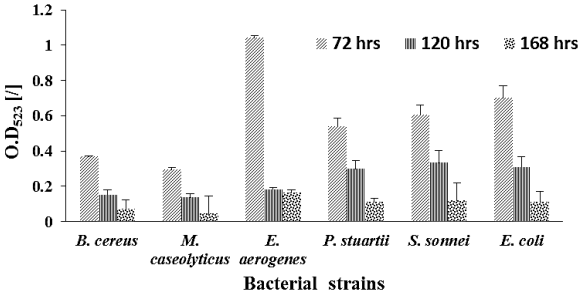

Fig. 2a: Quantification of Biofilm formation in selected strains by tube method.

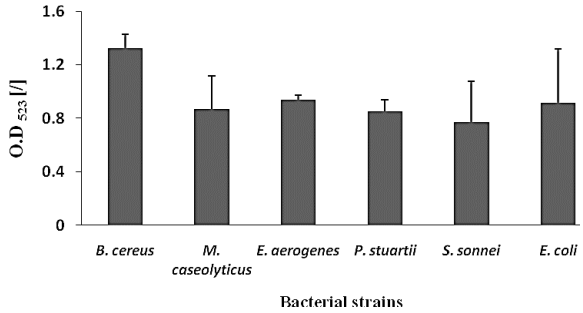

Fig. 2b: Estimation of biofilm formation in selected strains by coverslip method

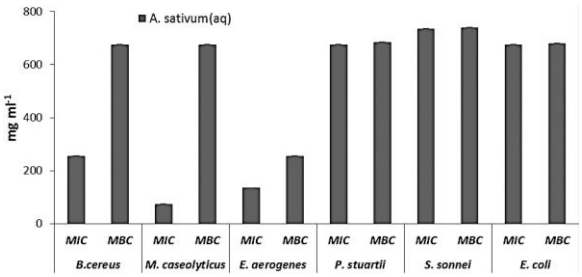

Fig. 3: The minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of *A. sativum*.

Susceptibility of biofilms against plant extract: Susceptibility of biofilms against plant extracts was also tested which showed that the effect of tested plants extracts both aqueous and methanolic inhibited the biofilm formation (Table 3). The extracts were found to be effective at their MIC concentration to significantly decrease the capacity to form biofilm. The maximum biofilm inhibition was observed in *B. cereus* by aqueous

extract of *M. sapientum*. The aqueous extract of *A. sativum* significantly reduced the biofilm formation in all the tested strains except for *P. staurtii* (Table 3).

DISCUSSION

Biofilms can cause serious hazards to human health. Urinary tract infections are the most frequent type of nosocomial infections accounting for 25-40% of these infections caused by number of biofilm forming bacteria (Bagshaw and Laupland, 2006). Dental plaque biofilms play an integral role in development of several oral infections as dental caries, periodontal diseases and gingivitis, so its removal is so crucial to maintain oral hygiene (Hasnor *et al*., 2013).

In order to determine the biofilm forming capacity of clinical isolates, and detect the antimicrobial activity of plant extracts on these isolates, this study was done on 60 clinical samples. From 60 clinical samples, 50 morphologically different strains were isolated and purified while 20 highly antibiotic resistant strains were selected for further characterization.

The bacterial ability to exhibit morphological variation may be an adaptation to thrive in a wide range of environmental conditions. On the basis of morphological and biochemical testing, the strains isolated from urine and wound samples belonged to the genus *Shigella, Yersinia, Pseudomonas, Klebsiella, Escherichia, Salmonella, Enterococcus, Proteus, Providencia* and *Staphylococcus spp.* Similar results were reported by Kunin (1997), who found that gram negative bacteria belonging to the family Enterobacteriaceae including *E. coli, Klebsiella, Enterobacter* and *Proteus* were the most common cause of urinary tract infections. Oral isolates showed similarity to *Staphylococcus* spp., *E. coli*, *Streptococcus* spp., *Lactobacillus* spp., *Enterobacter* spp., *Bacillus* spp. and *Enterococcus* spp which is in relevance with study by Smullen *et al*. (2012).

With frequent application of antibiotics, the threat of microbial resistance has become worse. Strains isolated from clinical samples also showed significant resistance against various antibiotics. Most of them were resistant to ampicillin, oxacillin and carbenicillin antibiotics. According to Liaqat *et al*. (2009), increase in bacterial resistance against frequently used antibiotics e.g. tetracycline and ampicillin has caused an alarming situation.

Phenotypic characteristics used for bacterial identification are not precisely sensitive to distinguish between species; Comparison of 16S rRNA gene sequences is one of the most powerful tool for classification of microorganisms. According to sequencing results, molecular identification of six strains W12(B), U3(H), U15(A), B_4, SpG_6 and F_{22} was done. Wound strains showed homology with *P. stuartii*, urine strains showed homology with *S. sonnei* and *E. coli*, as was found in study by Savas *et al*. (2006), while oral strains were found to be *B. cereus*, *E. aerogenes* and *M. caseolyticus*.

Table 1: Bactericidal activity of plant extracts alone and in combinations against isolated strains

Bacterial Strains	*M. piperita (aq)*	*M. piperita+A. sativum (aq)*	*A. sativum (aq)*	*M. sapientum +A. sativum(aq)*	*M. sapientum (aq)*	*M. piperita (met)*	*M. piperita+ M. sapientum (met)*	*M. sapientum (met)*	*M. piperita+A. sativum+M. sapientum (aq)*	*A. sativum (met)*
B. cereus	3.6±0.57	7.5±0.71	7.5±0.7	6.6±0.57	5.5±0.12	4.5±0.71	4.5±0.0.72	3.5±0.71	6±0.95	1.2±0.89
E. aerogens	2.6±0.54	5±0.82	10±0.82*	7.5±0.71	4.6±0.58	4.3±1.52	5±0.71	2±0.82	9±0.01*	1±0.31
M. caseolyticus	4±0.37	4±0.95	7±0.5	3.5±1.52	3±0.96	5±0.57	5±0.62	2.6±0.57	5±0.82	1±0.12
Bacterial Strains	*C. sinensis (aq)*	*C. sinensis +A. sativum (aq)*	*S. aromaticum +A. sativum (aq)*	*S. aromaticum (aq)*	*C. sinensis +Clove (aq)*	*S. aromaticum +C. sinensis +A. sativum (aq)*	*S. aromaticum +C. sinensis (met)*	*C. sinensis (met)*	*S. aromaticum (met)*	
P. stuartii	6.3±0.12	6.8±0.53	6.3±0.52	4±0.5	6.3±0.82	7.3±0.5	7.8±0.5	4.3±0.54	3.8±0.51	
S. sonnei	4.3±0.5	6.5±0.52	5.5±0.58	3.3±0.58	5±0.5	6.3±0.82	5.3±0.52	3±0.53	4±0.82	
E. coli	6±0.23	5.8±0.82	6.75±0.5	4.5±0.95	5.3±0.58	7.5±0.5	6.8±0.58	4.3±0.95	4.5±0.5	

Values bearing asterisk in column differ significantly (P<0.05).

Table 2: The minimum inhibitory concentration (MIC; mg ml^{-1}) and minimum bactericidal concentration (MBC; mg ml^{-1}) of different plant extracts

Plant Extracts	*B. cereus*		*M. caseolyticus*		*E. aerogenes*	
	MIC	MBC	MIC	MBC	MIC	MBC
Peppermint (aq)	15±0.13	40±0.14	5±0.07	40±0.14	25±0.13	40±0.13
Peppermint (met)	10±0.15	35±0.15	20±0.05	30±0.18	15±0.14	25±0.17
Banana (aq)	15±0.20	40±0.17	20±0.09	30±0.13	10±0.15	35±0.18
Banana (met)	20±0.14	30±0.15	35±0.05	35±0.12	10±0.16	30±0.14
Plant Extracts	*P. stuartii*		*S. sonnei*		*E. coli*	
	MIC	MBC	MIC	MBC	MIC	MBC
Green tea (aq)	20±0.13	25±0.12	15±0.13	20±0.13	25±0.14	30±0.13
Green tea (met)	20±0.14	25±0.13	30±0.14	35±0.16	20±0.15	25±0.14
Clove (aq)	20±0.15	25±0.14	30±0.15	35±0.14	25±0.15	30±0.15
Clove (met)	25±0.16	30±0.15	20±0.14	25±0.15	20±0.14	30±0.14

Table 3: Inhibitory effect of plant extracts on biofilm by bacterial strains

Bacterial strains	Control	*C. sinensis (aq)*	*C. sinensis (meth)*	*S. aromaticum (aq)*	*S. aromaticum (meth)*	*A. sativum (aq)*
P. stuartii	0.43±0.05	0.374±0.04	0.239±0.09	0.366±0.05	0.245±0.06	0.328±0.08
S. sonnei	0.68±0.07	0.421±0.06	0.371±0.05	0.298±0.05*	0.302±0.07*	0.343±0.06
E. coli	0.54±0.07	0.395±0.08	0.471±0.06	0.298±0.06	0.308±0.07	0.284±0.05
Bacterial strains	Control	*M. piperita (aq)*	*M. piperita (meth)*	*M. sapientum (aq)*	*M. sapientum (meth)*	*A. sativum (aq)*
B. cereus	0.40±0.02	0.075±0.07*	0.103±0.07*	0.052±0.03*	0.374±0.06	0.214±0.05
M. caseolyticus	0.56±0.06	0.329±0.09	0.42±0.04	0.141±0.02	0.52±0.19	0.142±0.01*
E. aerogenes	0.55±0.05	0.368±0.10	0.137±0.14*	0.263±0.06	0.256±0.08	0.206±0.05*

Values bearing asterisk in row differ significantly (P<0.05) than control.

Biofilm formation is an important factor that determines pathogenicity of bacteria and it was assessed by three methods i.e. Tube method, Congo red assay and Air- liquid interface assay. *E. aerogenes* and *E. coli* showed strongest biofilm formation in tube method while *B. cereus* and *E. aerogenes* showed maximum biofilm formation through liquid-interface coverslip method compared to other four strains. Congo red assay was found negative for *E. coli* and *P. stuartii*. This is in agreement with the study by Taj *et al*. (2012) who reported Tube method more reliable for the detection of biofilm formation.

Antibacterial activity of aqueous and methanolic extracts of *C. sinensis* , *S. aromaticum* and *A. sativum* was determined individually and in combination against *P. stuartii, S. sonnei* and *E. coli,* while that of *M. sapientum, M. piperita* and *A. sativum* extracts was investigated against *B. cereus, E. aerogenes* and *M. caseolyticus*. Except for *A. sativum* methanolic extract, all other extracts showed significant antibacterial activity against all six strains. The ZI of all aqueous extracts were found in range of 2.6-10 mm and the diameters of methanolic extracts were in the range of 1-5.5 mm. In case of combination of plant extracts, aqueous extract combinations showed ZI in the range of 3.5-9 mm while ZI showed by methanolic plant extract combinations were in the range of 2.75-5 mm. This study correlates with Bupesh *et al*. (2007), who observed the activity of aqueous extract of *M. piperita* against *P. aerogenosa, S. aureus, B. subtilis* and found ZI within the range of 2.3-4.2 mm. According to Milyani and Ashy (2011), *C. sinensis* aqueous extract was found very effective against clinical isolates of *S. aureus* showing ZI of 16-20 mm in diameter. In another research by Satyan *et al*. (2011), *S. aromaticum* aqueous extract showed significant inhibitory effect against *Shigella* with ZI of 15.6 mm. In this study, *A. sativum* methanolic extract was found to be least effective among all other extracts as previously shown in a study by Gull *et al*. (2012) where *A. sativum* methanolic extract was found to be least effective as compared to its aqueous and ethanolic extracts.

MIC and MBC of plant extracts were determined against six isolates. Except for *A. sativum*, the MIC values of aqueous and methanolic plant extracts against six isolates were in range of 5-35 mg ml^{-1} and MBC values were found in range of 20-40 mg ml^{-1}.The results correlates with that of Fagbemi *et al*. (2009), who found the MIC of ethanolic and aqueous extracts of *M. sapientum* within the range of 2-512 mg ml^{-1} and 32-512 mg ml^{-1}, respectively against *S. aureus, K. pneumoniae, E. coli, S. paratyphi, S. flexnerii, B. cereus* and *P. aeruginosa*. In another study by Bupesh *et al*. (2007), MIC of aqueous extract of *M. piperita* was 10 mg ml^{-1} against *B. substillis* and *P. aerogenes.* In the present study, the *A. sativum* aqueous extract showed MIC values in the range of 75-735 mg ml^{-1} while the range of its MBC values was found to be 255-740 mg ml^{-1}. The results are in accordance with the study by Bakri and Douglas, (2005), who reported MIC within the range of 35.7 to 142 mg ml^{-1} for the gram positive oral isolates and 1.1-35.7 mg ml^{-1} for gram negative oral isolates. The MBC ranges from 284 to >571 mg ml^{-1}.

Susceptibility of biofilms against plant extracts was also tested which showed that all aqueous and methanolic extracts were effective in decreasing the capacity of biofilm formation. In case of *B. cereus*, the O.D value of control was 0.401 while addition of *M. sapientum* aqueous extract upto its MIC value, decreased the O.D value to 0.075. In *S. sonnei*, the O.D value of control was 0.680 and it decreased to 0.302 after addition of *S. aromaticum* methanolic extract. Agrawal (2011) determined that aqueous extracts of *C. sinensis* and *M. sapientum* were very effective in inhibiting biofilm formation by clinically important *E. coli, B. subtilis, S. aureus* and *P. aeruginosa*. Similarly Mathur *et al*. (2013) found that essential oils of *S. aromaticum* and *A. sativum* remarkably reduced biofilm formation in clinically important *K. pneumoniae*. In another study by Wolkinsky *et al*. (2000), *M. sapientum* aqueous extract showed remarkable antibiofilm activity against *S. aureus* biofilms.

Conclusions: Biofilm forming clinical isolates usually cause serious infections and are very tough to eradicate mainly because of the development of high resistance against commonly used antibiotics. There is an immediate need to find out an alternative to treat these microbial infections. In this study, clinical isolates were found to have potential biofilm forming capacity and different plant extracts were tested for their antibiofilm properties against these isolates. These plant extracts showed profound ability to inhibit biofilm formation and thus may provide a way to use them as an alternative to treat different infections caused by antibiotic resistant biofilm forming clinical isolates.

Author's contribution: IL conceived and designed the study and experimental protocols. IL, QP and SJB executed the experiments. SIA and IL analyzed the data. IL interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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