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

I Liaqat1*, Q Pervaiz1, S Jamil Bukhsh1, SI Ahmed2 and N Jahan1

1Department of Zoology, Govt. College University, Lahore, Pakistan; 2Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan
*Corresponding author: iramliaq@hotmail.com

A B S T R A C T

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 sapientum (banana), Mentha piperita (peppermint) and Allium sativum (Garlic) was determined both individually and in combination against selected strains in both planktonic and biofilm mode. 16sRNA 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.

©2015 PVJ. All rights reserved

To Cite This Article: Liaqat I, Pervaiz Q, Bukhsh SJ, Ahmed SI and Jahan N, 2016, Investigation of bactericidal effects of medicinal plant extracts on clinical isolates and monitoring their biofilm forming potential. Pak Vet J, 36(2): 159-164.

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 pyogenes*, *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, H2S 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'-AGAGTTTGATCCTGCTCAG-3') and 16S-1522R (5'-AAGGAGGTGATCAGCCGCA-3') (Peniciol). 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. stuartii 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 qualitative 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. piperita (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).

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

---

**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.
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 Liaquat 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), B5, SpG6 and F22 was done. Wound strains showed homology with *P. staurtii*, 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:** Bacteriocal activity of plant extracts alone and in combinations against isolated strains

<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>M. piperita (aq)</th>
<th>M. piperita*+A. sativum (aq)</th>
<th>M. sapientum (aq)</th>
<th>M. sapientum*+A. sativum (aq)</th>
<th>M. piperita (met)</th>
<th>M. piperita*+C. sinensis (met)</th>
<th>M. sapientum (met)</th>
<th>M. sapientum*+C. sinensis (met)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. cereus</td>
<td>3.6±0.57</td>
<td>7.5±0.71</td>
<td>7.5±0.7</td>
<td>6.6±0.57</td>
<td>5.5±0.12</td>
<td>4.5±0.71</td>
<td>4.5±0.72</td>
<td>3.5±0.71</td>
</tr>
<tr>
<td>E. aerogenes</td>
<td>2.6±0.54</td>
<td>5±0.82</td>
<td>10±0.82*</td>
<td>7.5±0.71</td>
<td>4.6±0.58</td>
<td>4.3±1.52</td>
<td>5±0.71</td>
<td>2±0.02</td>
</tr>
<tr>
<td>M. caseolyticus</td>
<td>4.0±0.37</td>
<td>4±0.95</td>
<td>7±0.5</td>
<td>3.5±1.52</td>
<td>3±0.96</td>
<td>5±0.57</td>
<td>5±0.62</td>
<td>2±0.07</td>
</tr>
</tbody>
</table>

**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

<table>
<thead>
<tr>
<th>Plant Extracts</th>
<th>B. cereus</th>
<th>M. caseolyticus</th>
<th>E. aerogenes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MIC</strong></td>
<td><strong>MBC</strong></td>
<td><strong>MIC</strong></td>
<td><strong>MBC</strong></td>
</tr>
<tr>
<td>Peppermint (aq)</td>
<td>15±0.13</td>
<td>40±0.14</td>
<td>5±0.07</td>
</tr>
<tr>
<td>Peppermint (met)</td>
<td>10±0.15</td>
<td>35±0.15</td>
<td>20±0.05</td>
</tr>
<tr>
<td>Banana (aq)</td>
<td>15±0.20</td>
<td>40±0.17</td>
<td>20±0.09</td>
</tr>
<tr>
<td>Banana (met)</td>
<td>20±0.14</td>
<td>30±0.15</td>
<td>35±0.05</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>Control</th>
<th>M. piperita (aq)</th>
<th>M. piperita (met)</th>
<th>M. sapientum (aq)</th>
<th>M. sapientum (met)</th>
<th>A. sativum (aq)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. staurtii</td>
<td>0.43±0.05</td>
<td>0.37±0.04</td>
<td>0.23±0.09</td>
<td>0.36±0.05</td>
<td>0.24±0.06</td>
<td>0.32±0.08</td>
</tr>
<tr>
<td>S. sonnei</td>
<td>0.66±0.07</td>
<td>0.4±0.06</td>
<td>0.37±0.05</td>
<td>0.29±0.05*</td>
<td>0.32±0.07*</td>
<td>0.34±0.06</td>
</tr>
<tr>
<td>E. coli</td>
<td>0.56±0.07</td>
<td>0.35±0.08</td>
<td>0.47±0.06</td>
<td>0.29±0.06</td>
<td>0.39±0.06</td>
<td>0.29±0.05</td>
</tr>
</tbody>
</table>

**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 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. subtilis 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 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. sapientium aqueous extract showed remarkable antibiofilm activity against S. aureus biofilms.

Conclusions: Biofilm forming clinical isolates usually causeserious 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.

REFERENCES


