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Impact of Plant Extracts and Antibiotics on Biofilm Formation of Clinical Isolates From Otitis Media

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Abstract

Background: Otitis media can lead to severe health consequences, and is the most common reason for antibiotic prescriptions and biofilm-mediated infections. However, the increased pattern of drug resistance in biofilm forming bacteria complicates the treatment of such infections.

Objectives: This study was aimed to estimate the biofilm formation potential of the clinical isolates of otitis media, and to evaluate the efficacy of antibiotics and plant extracts as alternative therapeutic agents in biofilm eradication.

Materials and Methods: The ear swab samples collected from the otitis media patients visiting the Mayo Hospital in Lahore were processed to isolate the bacteria, which were characterized using morphological, biochemical, and molecular (16S rRNA ribotyping) techniques. Then, the minimum inhibitory concentrations (MICs) of the antibiotics and crude plant extracts were measured against the isolates. The cell surface hydrophobicity and biofilm formation potential were determined, both qualitatively and quantitatively, with and without antibiotics. Finally, the molecular characterization of the biofilm forming proteins was done by amplifying the ica operon.

Results: *Pseudomonas aeruginosa* (KC417303-05), *Staphylococcus hemolyticus* (KC417306), and *Staphylococcus hominis* (KC417307) were isolated from the otitis media specimens. Among the crude plant extracts, *Acacia arabica* showed significant antibacterial characteristics (MIC up to 13 mg/ml), while these isolates exhibited sensitivity towards ciprofloxacin (MIC 0.2 μg/mL). All of the bacterial strains had hydrophobic cellular surfaces that helped in their adherence to abiotic surfaces, leading to strong biofilm formation potential (up to 7 days). Furthermore, the *icaC* gene encoding polysaccharide intercellular adhesion protein was amplified from *S. hemolyticus*.

Conclusions: The bacterial isolates exhibited strong biofilm formation potential, while the extracts of *Acacia arabica* significantly inhibited biofilm formation among the isolates and, therefore, could be executed in the development of cost-effective biofilm inhibitor medicines.

Keywords: Biofilms, Plant Extracts, Bacterial Adhesion, Bacterial Infections, Anti-Bacterial Agents

1. Background

Permanent hearing loss in childhood following antibiotic consumption is largely attributed to otitis media throughout the world. Otitis media is an inflammation of the middle ear that prevails in two major forms: acute and chronic otitis media (1-3). Otitis media is known to have a very high incidence, up to 43% of the children in the United States, while one of the studies in Pakistan reported an incidence of 11.5% (4). Otitis media can lead to the bulging of the tympanic membrane, with purulent fluid behind it, which often leads to the morphological disruption of the tissues and, ultimately, permanent hearing loss (1). Among the bacterial agents, *Pseudomonas aeruginosa*, *Escherichia coli*, and members of the genera *Staphylococcus*, *Haemophilus*, *Streptococcus*, *Moraxella*, and *Klebsiella* are typically associated with the disease (2-5).

Bacterial cells adhered to the mucosal cell lining of the middle ear through their specific hydrophobic cellular surfaces tend to form biofilms. Therefore, these drug resistant and slowly dividing bacterial cells can lead to prolonged infections, even after antibiotic regimens. In addition, these biofilm-mediated infections cannot be easily eliminated, because of the protection from the host defense system that is provided by the continuous secretion of the bacterial extracellular polymeric substance (EPS) (6). Among the two principal requirements of biofilm formation, the potential of bacterial cells to attach to a surface is followed by their tendency to aggregate themselves with the help of slime production. This slime is mainly composed of the polysaccharide intercellular adhesion (PIA) protein, which is genetically expressed by the ica operon consisting of four genes: *icaA*, *D*, *B*, and *C* (7, 8).

There are no successful preventative measures available for otitis media, but antibiotic prophylaxis and the insertion of a tympanostomy tube may help in disease prevention (9). Depending on the etiological agents, fluoroquinolones and aminoglycosides are recommended for the disease treatment.

However, the increased drug resistance of bacterial pathogens has paved the way to search for some alternative means of treatment. Natural crude plant extracts have long been used in medication, so they can be targeted as substitutes for the effective eradication of drug resistant bacterial pathogens. Plant crude extracts, such as those of *Acacia arabica*, display anti-bacterial, anti-inflammatory, and anti-pyretic characteristics, due to the presence of flavonoids and saponins (10).

2. Objectives

Because of increased drug resistance in biofilm forming bacteria, this study was designed to investigate the biofilm formation potential of bacterial strains isolated from otitis media. The inhibitory effects of antibiotics and natural plant crude extracts were determined in biofilm formation and detachment, as well as in planktonic cells. Furthermore, the role of the cell surface hydrophobicity, and the genetic factors responsible for biofilm formation among the isolates were also studied.

3. Materials and Methods

3.1. Isolation and Characterization of Bacterial Strains

Ear swab samples from the otitis media patients visiting the outpatient Ear, Nose, and Throat (ENT) Department of the Mayo Hospital, Lahore were collected under the supervision of an ENT specialist, and transported immediately to the laboratory for microbiological processing. The isolated bacterial strains were identified using the morphology, biochemical analytical profile index (API 20E; Biomerieux, France), and molecular characterization (16S rRNA ribotyping; Macrogen, Inc., Seoul, South Korea). The sequences of the 16S rRNA gene product were aligned using FinchTV software (Geospiza, Inc., Seattle, WA). These gene sequences, along with the names of highly similar bacterial strains pre-reported in the National Center for Biotechnology Information (NCBI) databases, were submitted to GenBank in order to obtain accession numbers.

3.2. Minimum Inhibitory Concentrations (MICs) of Antibiotics and Plant Crude Extracts

The broth dilution method was used to determine the MICs of the antibiotics (ciprofloxacin, tobramycin, and ofloxacin; Alcon, USA) against the bacterial isolates. A standardized culture (50 μL, 0.2 A, 600 nm) of each strain was inoculated into a 5 mL sterile LB broth (HiMedia, India) that was supplemented with antibiotics (0.1 to 9.6 μg/mL concentrations), followed by 24 hours of incubation. One antibiotic supplemented broth tube from each concentration was considered to be a negative control, while the non-antibiotic supplemented tubes inoculated with the bacteria were positive controls for the experiment. The MIC was then determined by observing the visible bacterial growth in the tubes.

Plant crude extracts (100 mg/mL) of *Aloe barbadensis* (aloe vera), *Zingiber officinale* (ginger), *Curcuma longa* (turmeric), and *Acacia arabica* (kikar), prepared using the method described by Odey et al. (2012), were used in the present study (11). The MICs of the plant crude extracts against the clinical isolates were also determined by the broth dilution method. Moreover, the concentrations of the plant crude extracts were extended from 1 to 15 mg/mL.

3.3. Cell Surface Hydrophobicity

The salt aggregation test (SAT) and bacterial adherence to hydrophobic carbon (BATH) test were performed, to determine the nature and effects of the antibiotics (0.1 μg/mL) on the surfaces of the bacterial cells (12).

3.4. Qualitative Assay for Biofilm

3.4.1. Slime Production Test

The production of slime was checked qualitatively, with some modifications, in both the presence and absence of 20% glucose (BDH, England), using the Congo red agar (CRA) (BDH, England) assay (13). The inoculated CRA plates were incubated at 37°C for 24 hours, and the color of the colonies was observed for slime productivity.

3.5. Quantitative Assays for Biofilm

3.5.1. Microtiter Plate Assay

A microtiter plate assay was performed in 96-well polystyrene microtiter plates (Orange Scientific, Belgium), to quantitatively determine the biofilm formation at different time intervals (72, 120, and 172 hours) in the presence and absence of antibiotics (0.1 μg/mL) and plant crude extracts (2 mg/mL) (14, 15). After the processing of the assay, the OD was measured at 578 nm, with the help of a microplate reader (BIO-RAD Model 680 XR). Those microtiter wells containing un-inoculated LB broth were considered to be the negative controls, while the inoculated wells were positive controls.

3.5.2. Effect of Abiotic Surface on Biofilm Growth and Detachment

The effects of an abiotic surface (glass tubes) and antibiotic stress on the growth and detachment of biofilm were determined by the estimation of the planktonic, loosely attached, and tightly bound cells of the bacterial strains (16). A standardized bacterial culture (50 μL, 0.2 A, 600 nm) was inoculated into a test tube containing 5 mL sterile LB broth, along with antibiotics (0.1 μg/mL), in three sets of test tubes. The sets of tubes were then incubated at 37°C for 72, 120, and 172 hours, respectively, in static conditions. Following incubation, the tubes were processed according to the aforementioned protocol.

3.6. Amplification of ica Operon

To detect and amplify the ica operon from the bacterial isolates, the genomic DNA was extracted from the bac-

terial cells (17). This genomic DNA was then targeted for the detection of the ica operon, consisting of four genes (*icaA*, *icaB*, *icaC*, and *icaD*), via the polymerase chain reaction (PCR), by using previously reported primers that were synthesized by Gene Link, Inc., USA (8, 18, 19).

3.7. Data Analysis

All of the experiments were performed in triplicate, and the values of the mean, standard deviation, and standard error were determined with the help of Microsoft Office Excel 2010.

4. Results

A total of five morphologically distinct bacterial strains isolated from ear swabs were designated as P1, P2, P3, S1, and S2. Moreover, the morphological, biochemical, and molecular characterizations of these bacterial strains and their GenBank accession numbers are shown in Table 1. In addition, the phylogenetic linkages of these isolates determined using the neighbor-joining method are displayed in Figure 1. The MIC values of the antibiotics ranged from 0.2 to 1.0 μg/mL in concentration. All 3 strains of *Pseudomonas aeruginosa* (P1, P2, and P3) displayed MICs of ciprofloxacin at 0.2 μg/mL, and of ofloxacin and tobramycin up to 0.5 μg/mL. However, *Staphylococcus hemolyticus* (S1) and *Staphylococcus hominis* (S2) exhibited MICs of all three antibiotics at the 1 μg/mL concentration. In the case of plant crude extracts, a varied pattern of MIC values was observed against the bacterial isolates, which ranged between 5 and 13 mg/mL, as presented in Table 2.

The results of the SAT showed that all of the bacterial isolates exhibited hydrophobic cell surfaces, since they showed aggregation with salt $(NH_4)_2SO_4$ in the range of 0.1^{-1} M. In the case of the BATH test, the *P. aeruginosa* strains P1 and P2 exhibited slightly hydrophobic behavior, while the *P. aeruginosa* strain P3 and both strains of *Staphylococcus* were moderately hydrophobic in nature. However, under stress conditions, all of the strains displayed increases in their percentages of hydrophobicity. Contrarily, a hydrophobic reduction was found in the *P. aeruginosa* strain P3 and *S. hemolyticus* strain S1 in the presence of ofloxacin, when compared to their control strains not supplemented with antibiotics.

The qualitative test for biofilm formation showed that among the tested isolates, both strains of Staphylococci produced black colored colonies on the CRA with glucose. Only the *S. hominis* strain S2 indicated positive results on the slime test, through the production of black colored colonies on the CRA in the absence of glucose. The results of the quantitative estimation of the microtiter plate assay revealed that all of the bacterial isolates exhibited maximum biofilm formation after 172 hours of incubation in the hydrophobic polystyrene micro-wells. Among the various plant crude extracts and antibiotics tested, the ciprofloxacin and crude extract of A. arabica were found to be most effective in the inhibition of bacterial biofilm formation (Figure 2).

Table 1. Morphological and biochemical Characterizations of Bacterial Strains Isolated From Otitis Media

Bacterial Strain	Morphological characteristics							Biochemical characteristics					Name of organism	% Similarity	GenBank accession number
	Size, mm	Color	Surface texture	Elevation	Margins/ opacity	Gram reaction	Shape	Oxidase	Catalase	Coagulase bound	Coagulase free	DNase	API 20E code			
P1	2	Off-white	Rough, dry	Wavy	Irregular, translucent	-ve	Rods	+ve	+ve	NA	NA	NA	2202004	*Pseudomonas aeruginosa*	99	KC417303
P2	3	Yellow	Mucoid	Raised	Smooth, translucent	-ve	Rods	+ve	+ve	NA	NA	NA	2206004	*Pseudomonas aeruginosa*	99	KC417304
P3	2	Yellow	Shiny	Convex	Smooth, translucent	-ve	Rods	+ve	+ve	NA	NA	NA	2204004	*Pseudomonas aeruginosa*	100	KC417305
S1	1	White	Smooth	Raised	Smooth, opaque	+ve	Cocci	-ve	+ve	-ve	-ve	-ve	NA	*Staphylococcus hemolyticus*	99	KC417306
S2	Punctiform	White	Smooth	Flat	Smooth, opaque	+ve	Cocci	-ve	+ve	-ve	-ve	-ve	NA	*Staphylococcus hominis*	99	KC417307

Abbreviations: NA, not available; +ve, positive; -ve, negative; %: percentage.

The estimation of the planktonic cells demonstrated an increase in their number after 72 hours, which decreased after 120 hours. But the planktonic (free cells) count was raised again after 172 hours. With regard to the loosely attached cells, they were greater after 72 hours of incubation, followed by a decrease in their number after 120 hours, which rose again after 172 hours in all of the strains except the *P. aeruginosa* strain P1. The tightly bound cells actually represented the biofilm formation and adherence to the abiotic surfaces. In general, the tightly bound cells were greater after 120 hours, when compared to 72 and 172 hours. An overall decrease in the number of planktonic, loosely attached, as well as tightly bound cells under antibiotic stress among all of the strains indicated that the antibiotics inhibited the bacterial adherence to a hydrophilic surface (glass tubes) (Figure 3).

Among the four ica operon genes, only *icaC* was amplified in the *S. hemolyticus* strain, and the product was approximately 990 bp, when compared to a parallel run 1 kb DNA ladder.

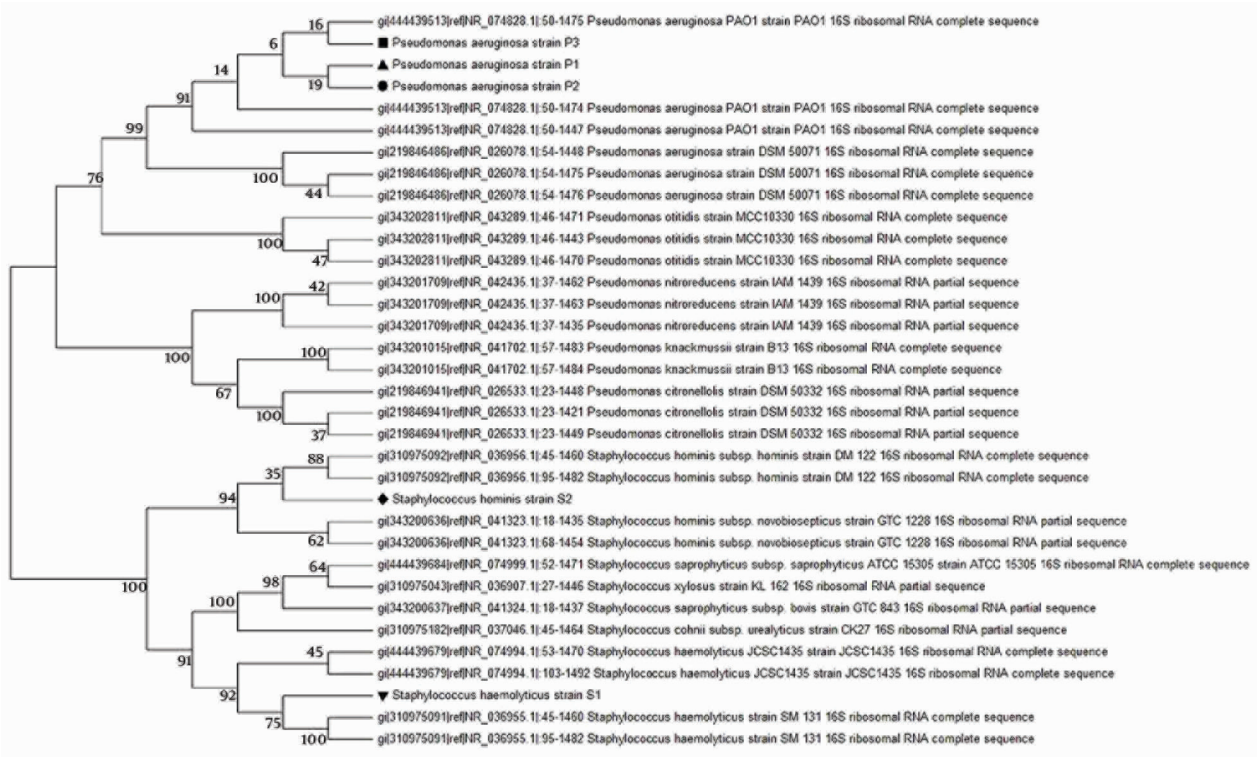
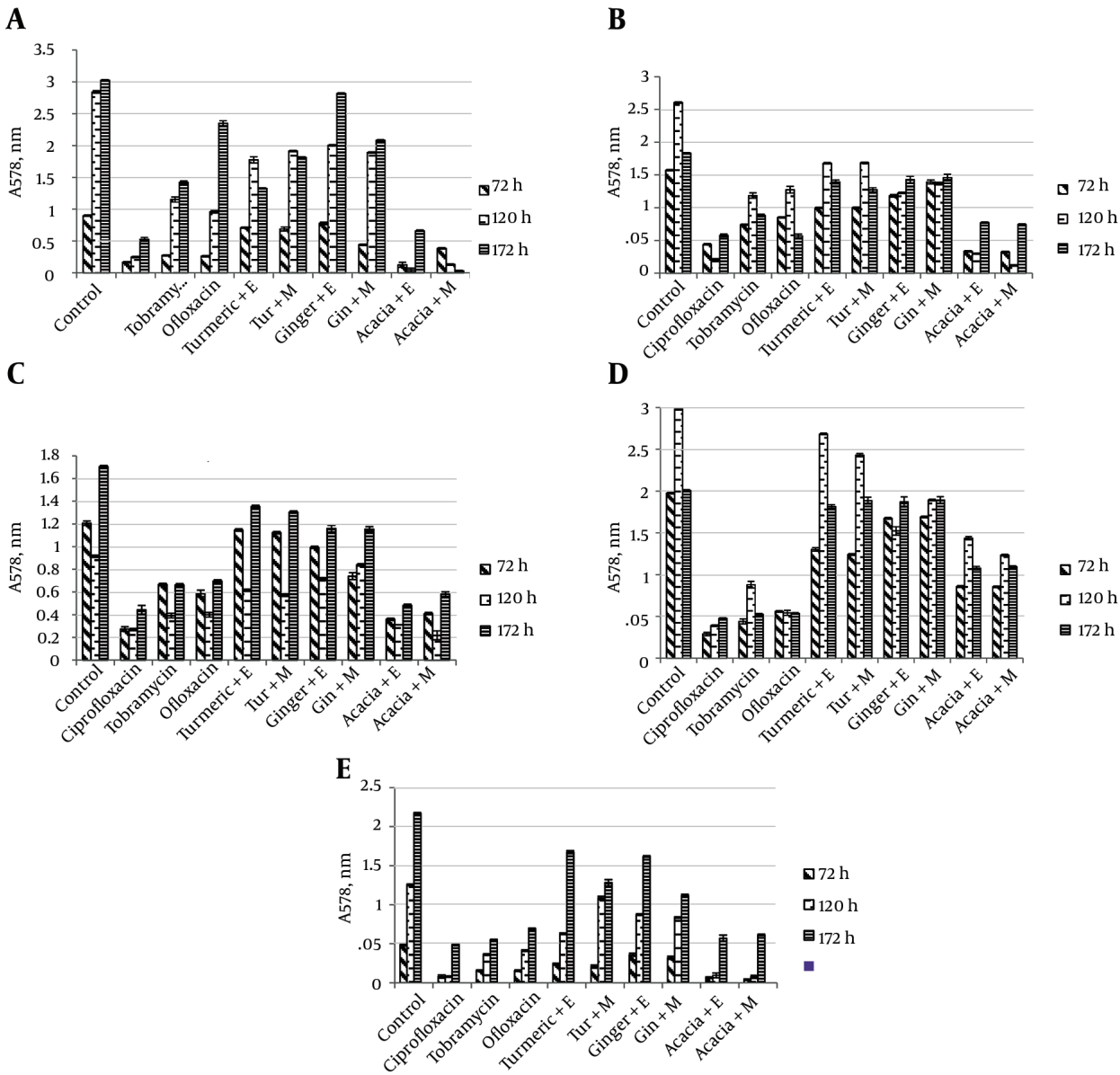


Figure 1. Phylogenetic Tree of Clinical Isolates (MEGA 5.2)

Table 2. MIC Values of Antibiotics and Plant Extracts Against Bacterial Strains Isolated From Otitis Media

Sr. No.	Bacterial strain	Concentration of Antibiotic at Which MIC was Obtained (μg mL^{-1})			Concentration of Crude Plant Extracts at Which MIC was Obtained (mg mL^{-1})			
		Ciprofloxacin	Tobramycin	Ofloxacin	*Aloe barbadensis*	*Zingiber officinale*	*Curcuma longa*	*Acacia arabica*
1	P1	0.2	0.5	0.5	12	7	8	9
2	P2	0.2	0.5	0.5	13	10	9	7
3	P3	0.2	0.5	0.5	13	8	9	6
4	S1	1.0	1.0	1.0	12	10	10	5
5	S2	1.0	1.0	1.0	11	8	7	6

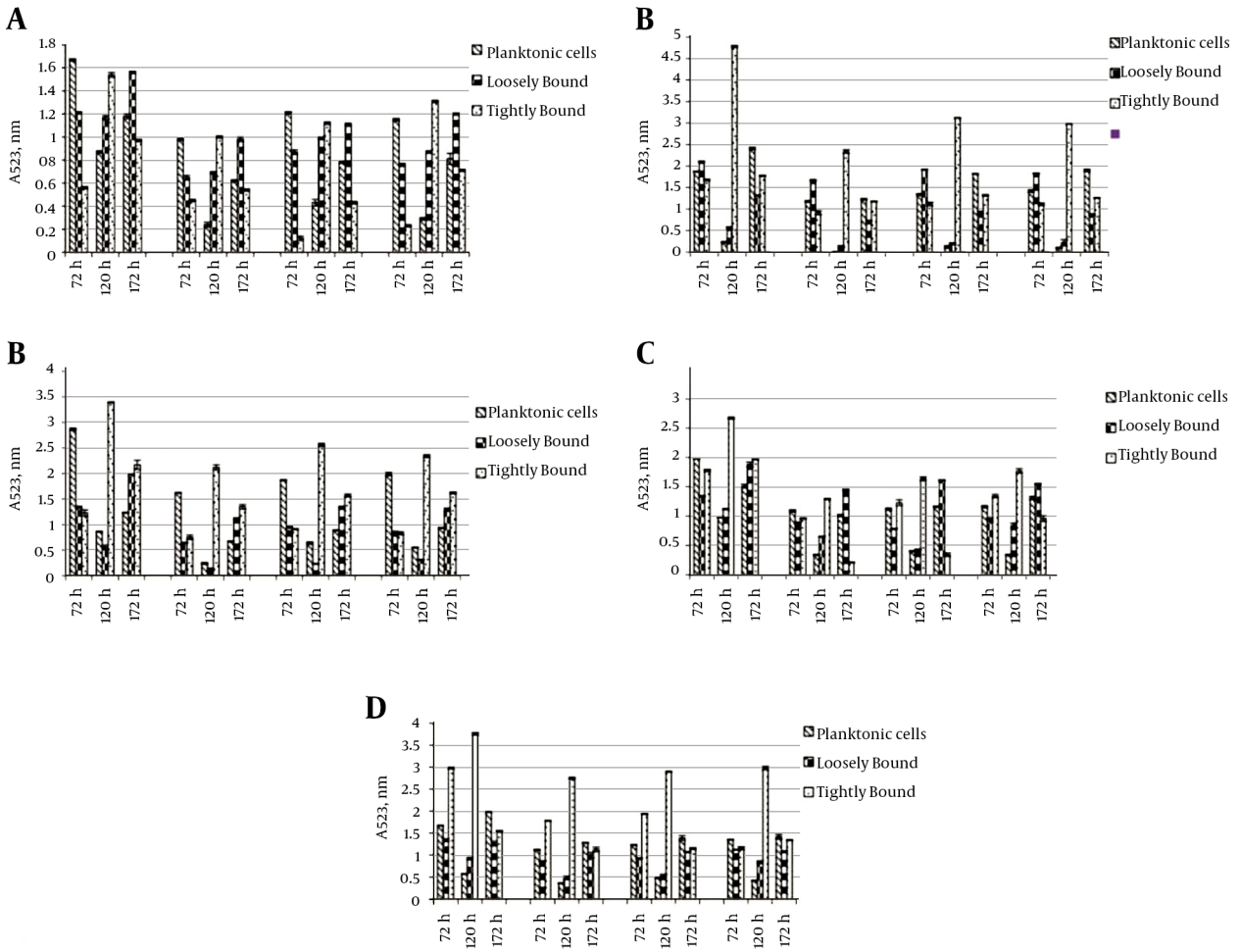
Figure 2. Microtiter Plate Assay for Biofilm



A, strain P1; B, strain P2; C, strain P3; D, strain S1; E, strain strain S2.

Turmeric + E, ethanolic extract of turmeric; Tur + M, methanolic extract of turmeric; Ginger + E, ethanolic extract of ginger; Gin + M, methanolic extract of ginger; Acacia + E, ethanolic extract of Acacia; Acacia + M, methanolic extract of Acacia; A, strain P1; B, strain P2; C, strain P3; D, strain S1; E, strain strain S2.

Figure 3. Effect of Abiotic Surface on Biofilm Growth and Detachment at Different Time Intervals



A, strain P1; B, strain P2; C, strain P3; D, strain S1; E, strain strain S2.

A, strain P1; B, strain P2; C, strain P3; D, strain S1; E, strain S2.

5. Discussion

Otitis media is the most common reason for childhood antibiotic regimens, and may lead to deafness in cases of mistreatment (20). In addition to the disease severity and complex diagnosis, the potentially increased tendency of bacterial pathogens to become drug resistant contributes significantly towards the consequences of the ailment (21). Therefore, researchers need to focus on some alternative, effective, and harmless remedies for bacterial infections. The present study has revealed the antibacterial activity of commercially available antibiotics and natural plant crude extracts against the planktonic and biofilm forming clinical isolates of otitis media. In this study, three different strains of *P. aeruginosa*, one strain of *S. hemolyticus*, and one of *S. hominis* were isolated from ear swab samples of otitis media. These results were in harmony with those of another study, which reported that *P. aeruginosa* and *S. aureus* are among the most common causative agents of otitis media in different areas of Pakistan (2).

The MICs of ciprofloxacin against the strains of *P. aeruginosa* were less than those for Staphylococci. Whereas, the MIC values of the ofloxacin and tobramycin for the Staphylococci were almost double those for the *P. aeruginosa*. An overall trend of antibiotic susceptibility among these strains has shown that *P. aeruginosa* is relatively sensitive to antibiotics, and most susceptible to ciprofloxacin, which shows harmony with another study (22). The MIC values of the plant crude extracts showed that *A. arabica* required the lowest concentration to inhibit bacterial growth. A previous study reporting *A. arabica* as the most noteworthy antibacterial extract among 30 different medicinal plants tested against pathogenic bacteria also supported our results (23). Overall, the members

of the genus Acacia contain certain flavonols, aglycones, and flavone glycosides that play important roles in their antibacterial properties (24).

The capacity of bacterial cells to colonize as biofilms is largely dependent on their propensity to adhere to a certain surface. Therefore, cell surface hydrophobicity is the most important factor that determines bacterial adhesive properties (25). The results of the SAT indicated the hydrophobic behavior of all of the bacterial strains, executed by their aggregation with ammonium sulfate (0.1 - 1.0 M). The BATH test was carried out to check the bacterial adherence to hydrocarbons (xylene). Two strains of *P. aeruginosa* (P1 and P2) exhibited slightly hydrophobic properties, while strain P3, along with *S. hemolyticus* and *S. hominis*, showed moderately hydrophobic behavior. When these hydrophobic cells come into contact with another hydrophobic surface or cell, they adhere to one another. However, under the stress of antibiotics, an increase in the percentage of the hydrophobicity among all strains was observed, when compared to the non-stressed environment. It has also been suggested that bacterial hydrophobicity is due to the presence of particular proteins on the cell surface, called hydrophobins (26). Under antibiotic stress, the overexpression of these proteins might increase the hydrophobicity of the bacterial strains.

Among the qualitative assays for biofilm formation, the evaluation of slime production has shown that *S. hemolyticus* and *S. hominis* were strong slime producers, as revealed by their black colored colonies on the CRA medium. Those bacterial strains capable of forming biofilms produce slime that helps in their adherence to the surface, and also in protection from the host defense systems (27). The study by Boynukara et al. reported that *S. hemolyticus* and *S. hominis* isolated from various clinical specimens were the strongest slime producers, which gave very black colonies on the CRA medium, and these findings are in agreement with those of the recent study (28). The addition of glucose into the CRA medium enhanced the slime production, as indicated by the production of very black colonies by both strains, while only *S. hominis* grew black colonies on the non-glucose supplemented medium.

The results of the microtiter plate assay revealed maximum biofilm formation after 172 hours. It was evident that all of the bacterial strains exhibited a prolonged affinity for adherence to the polystyrene surface of the microtiter plates, when compared to the glass surface of the test tubes. Most investigators have found that hydrophobic substances, such as Teflon and plastic, provide better substrates for biofilm formation, when compared to hydrophilic substances like glass or metal (29, 30). Among the antibiotics, ciprofloxacin inhibited maximum biofilm formation, and *A. arabica* was found to be a significant inhibitor among the plant crude extracts.

The effects of a glass surface on biofilm formation and attachment revealed that planktonic cells (free-floating) were greater in number after 72 and 172 hours in all of the bacterial strains. Loosely attached bacterial cells were found excessively after 172 hours, in contrast to the tightly bound cells that were most abundant after 120 hours. Therefore, the results of this study suggest that the bacterial cells began attachment after 72 hours, and were strongly adhered to the glass surface after 120 hours, showing maximum biofilm formation. After 172 hours, the rise in the number of planktonic and loosely attached cells indicated biofilm detachment. Therefore, all of the strains acquired maximum biofilm maturation at 120 hours in the presence of the glass substrate, as reported by Liaqat et al. (16). However, in the case of antibiotic stress, all of the antibiotics inhibited the growth of planktonic and loosely attached cells, with few exceptions. The study by Liaqat et al. also stated that the planktonic and loosely attached cells were readily available to the antibiotics, but the tightly bound cells were densely packed and impenetrable to the antibiotics, exhibiting less reduction potential (16).

The bacterial biofilm formation checked by qualitative and quantitative assays was also investigated through the molecular technique of PCR amplification in order to target the ica operon. Among the four genes of the ica operon, only the *icaC* gene (approximately 990 bp) could be amplified in the *S. hemolyticus*. Similarly, the *icaC* gene was amplified in *S. hemolyticus* in the study of Bradford et al. (31).

In conclusion, this study proposed ciprofloxacin and crude extracts of *A. arabica* as effective antimicrobials, not only against planktonic, but also bacterial biofilm communities. Therefore, the compositional analysis of the crude extracts of *A. arabica* may be helpful in designing some new and effective drugs. Furthermore, the bacterial adherence to the hydrophobic surface of polystyrene microtiter plates for a longer duration suggests that tympanostomy tubes should be made up of hydrophilic substances in order to avoid bacterial adhesion and biofilm formation.

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Footnotes

Authors' Contribution: Saba Rehman carried out the research work and wrote this manuscript. Shahbaz Mujtaba Ghauri helped and guided in the acquisition of the clinical specimens. Anjum Nasim Sabri supervised the research work, critically analyzed the findings, and conducted the final check of this manuscript.

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