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RESEARCH ARTICLE OPEN ACCESS

In-vitro Evaluation of Anti-Bacterial, Anti-biofilm and Cytotoxic Activity of Naturally Inspired *Juglans regia, Tamarix aphylla* L., and *Acacia modesta* with Medicinal Potentialities

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Abstract

The usefulness of medicinal plants has evoked increasing research interests to combat antimicrobial resistance. The current work reports on the *in vitro* bioactive activities, i.e., antibacterial, anti-biofilm and cytotoxicity evaluation of three medicinal plants. Extracts from different plant parts (leaves, stems, and root barks) were obtained using different solvents, such as methanol, ethanol, and water. The methanolic extracts of bark of *Juglans regia* and *Tamarix aphylla L.*, whereas the stem of *Acacia modesta (*Wall.*)* exhibited the highest antibacterial activity. The maximum zone of inhibition, i.e., 21.8±0.76 mm was observed against *H. influenza* for a methanolic extract of *J. regia*. Similarly, the maximum inhibition zones of ethanolic extract of *T. aphylla L.* against *A. baumannii* and methanolic extract of *A. modesta* against *S. aureus* were recorded. Notably, a higher anti-biofilm potential of the methanolic extract of *J. regia* was recorded against six pathogenic strains. Finally, the cytotoxicity of plants extracts was evaluated by testing the hemolytic activity against human erythrocytes, that displayed the negligible percent lysis of RBCs. In conclusion, the results of this study provided information related to the possible use of some medicinal plants in the treatment of microbial related ailments.

Keywords: Medicinal plants, methanolic extract, bacterial pathogens, antibacterial, anti-biofilm, cytotoxicity

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INTRODUCTION

The emergence and spread of multidrug-resistant (MDR) pathogens are one of the most serious threats to successful treatment of microbial diseases[1]. According to the World Health Organization (WHO), infectious diseases are the 3rd most significant cause of mortality worldwide. Strains such as-β lactamase-producing *Escherichia coli* (BL-EC) and *Klebsiella pneumoniae* (BL-KP), carbapenem-resistant *Enterobacteriaceae, Pseudomonas aeruginosa*, hospital-acquired methicillin resistant *Staphylococcus aureus* (MRSA), and vancomycin resistant *Enterococcus* (VRE) have been identified as utmost notorious pathogens, thus effective therapies are urgently required for these pathogenic microbes[1,2]. The healing choices for these pathogens are enormously limited and Clinicians are imposed to use expensive drugs associated with noteworthy side consequence to the patients' health[1]. Hence, it is indispensable to find out some substitutions that can possibly be efficient in the treatment of these infections. Practicality, the health beneficial aspects of medicinal components from plants have evoked increasing interest for antimicrobial therapy since they have been evaluated for their probable usages as substitute medications for the treatment of numerous infectious diseases[3]. The WHO estimated that nearly eighty percent of the world's population depend on conventional medication for their central healthcare necessities. The beneficial value of plants lies in bioactive compounds including alkaloids, tannins and phenolic etc.[4].

Bacteria predominantly grow as either free-living planktonic cells or surface attached biofilms; a highly populated assemblage of bacterial cells surrounded in self-produced polymeric substances including bacterial consortia[4]. This microenvironment of bacteria (biofilm) is one of the foremost pathogenic approaches by infection-causing microbes. Since, it makes them extremely resistant to currently available antibiotics and biocides, as well as immune responses[4]. Biofilm development is facilitated by mechanical, biochemical and genetic factors in bacteria strains[5]. Other than bacterial cells, the major constituent of biofilm is an extracellular polymeric matrix (EPM), composed of polysaccharides, proteins, lipids, polychronic acids and nucleic acids[5-7]. All properties of biofilms, viz. attachment, antibiotic resistance, and phagocytosis may be ascribed to EPM[8].

Walnut (*Juglans regia* L.) is the furthermost prevalent tree nut in the world. It belongs to juglandaceae and has the scientific name *Juglans regia*. *J. regia* is a medicinal plant that has been extensively used in traditional medicine for a wide array of ailments[8-11]. *Tamarix aphylla* (L.) Karst., is another important medicinal plant and belong to the Tamaricaceae family. *Acacia* belongs to the family *Fabaceae* or *Leguminosae*[3,4] and displayed noticeable antimicrobial activity against numerous pathogenic microbes[5,6].

MATERIALS AND METHODS

Plant material and extract preparation

Different parts (leaf, stem, and bark) of the plants were locally collected and washed thoroughly (3-4 times) with tap water (to eliminate dirt from the surface). The washed plant materials were dried at room temperature (25°C) on filter papers. The dried plant materials were ground to fine powder and preserved in polyethylene bags for further use. Two hundred grams of dried powder of each plant was subjected to a maceration process three times with various solvents, i.e., methanol, ethanol, and water at 25°C. The solvents were removed by subjecting to the rotary evaporator at 45°C followed by lyophilization to obtain pure extracts. The extracts were then stored in a refrigerator at 4°C for further analysis.

Microbial cultures and inoculum development

Six clinically proven pathogenic microbes, i.e., (1) *Staphylococcus aureus* (*S. aureus*), (2) *Pseudomonas aeruginosa* (*P. aeruginosa*), (3) *Klebsiella pneumoniae* (*K. pneumoniae*), (4) *Acenetobacter baumannii* (*A. baumannii*), (5) *Marginella morganii* (*M. morganii*) and (6) *Haemophilus influenza* (*H. influenza*) were used to test the bioactivities of the plant extracts. As collected microbial strains were incubated for 18 h on a rotary shaker at 37°C. The 18-h grown cultures were then diluted appropriately to yield a culture density of approximately 1.5×10^8 CFU/mL.

Antimicrobial and antibiofilm assay

The antimicrobial activity of the plant extracts was evaluated using agar well diffusion method[12]. Twenty microliters of plant extracts

Table 1. Antibacterial activity of *Juglans regia* extracts against bacterial pathogens

Leaf Microorganisms	Origin	*Juglans regia* Methanol Zone mm	Methanol MIC mg/mL	Ethanol Zone mm	Ethanol MIC mg/mL	Water Zone mm	Water MIC mg/mL	N. C	P.C
M. morganii	Clinical	17.4±0.45	3.4±0.75	15.6±0.65	3.2±0.44	10.6±0.54	1.7±0.76	N.D	16.8±0.27
P. aeruginosa	Clinical	13.2±0.21	6.5±0.32	11.3±0.14	0.7±0.87	6.5±0.76	2.3±0.58	N.D	12.0±0.21
A. baumannii	Clinical	11.3±0.35	1.3±0.45	14.3±0.45	6.3±0.34	5.3±0.87	0.7±0.86	N.D	15.8±0.13
H. influenza	Clinical	9.5±0.55	5.4±0.35	8.6±0.17	1.6±0.56	4.8±0.49	3.7±0.68	N.D	17.5±0.17
k. pneumoniae	Clinical	15.6±0.15	8.2±0.15	14.3±0.55	5.4±0.65	10.9±0.77	4.3±0.31	N.D	20.7±1.00
S. aureus	Clinical	7.3±0.14	2.5±0.11	9.1±0.76	0.8±0.66	9.8±0.64	3.2±0.65	N.D	15.2±0.11
Stem									
M. morganii	Clinical	16.3±0.75	5.6±0.18	17.5±0.36	2.7±0.57	13.2±0.71	1.4±0.45	N.D	19.2±0.21
P. aeruginosa	Clinical	14.6±0.53	6.3±0.36	15.3±0.32	9.2±0.81	12.3±0.31	5.3±0.41	N.D	16.3±0.20
A. baumannii	Clinical	10.7±0.33	7.2±0.71	13.2±0.12	8.5±0.83	14.6±0.73	0.9±0.45	N.D	17.5±0.26
H. influenza	Clinical	10.2±0.76	1.4±0.51	16.5±0.69	0.3±0.83	8.4±0.73	0.7±0.55	N.D	14.3±0.21
k. pneumoniae	Clinical	16.6±0.47	2.4±0.15	18.3±0.43	5.2±0.84	11.2±0.21	6.2±0.74	N.D	19.3±0.21
S. aureus	Clinical	11.8±0.43	6.4±0.17	8.2±0.53	3.6±0.13	7.3±0.65	2.5±0.56	N.D	11.7±0.17
Bark									
M. morganii	Clinical	18.5±0.57	8.3±0.55	19.4±0.51	5.2±0.25	8.3±0.87	5.8±0.38	N.D	16.8±0.27
P. aeruginosa	Clinical	15.3±0.51	5.4±0.71	17.3±0.61	2.4±0.57	11.3±0.59	4.7±0.59	N.D	11.0±0.21
A. baumannii	Clinical	16.5±0.48	4.6±0.75	15.7±0.53	6.5±0.85	15.8±0.49	6.4±0.91	N.D	15.8±0.13
H. influenza	Clinical	21.8±0.76	0.5±0.19	19.4±0.38	1.4±0.96	18.9±0.81	7.2±0.61	N.D	17.5±0.17
k. pneumoniae	Clinical	14.6±0.37	7.7±0.81	15.8±0.57	6.8±0.69	10.3±0.57	1.4±0.28	N.D	20.7±1.00
S. aureus	Clinical	18.9±0.41	8.3±0.57	19.6±0.71	7.4±0.79	15.2±0.58	3.3±0.87	N.D	9.5±0.40

Table 2. Antibacterial activity of *Tamarix aphylla L.* extracts against bacterial pathogens

Leaf Microorganisms	Origin	*Tamarix aphylla L.* Methanol Zone mm	Methanol MIC mg/mL	Ethanol Zone mm	Ethanol MIC mg/mL	Water Zone mm	Water MIC mg/mL	N. C	P.C
M. morganii	Clinical	15.2±0.43	7.3±0.57	12.6±0.54	5.7±0.87	9.2±0.58	4.5±0.85	N.D	15.2±0.11
P. aeruginosa	Clinical	14.2±0.48	5.4±0.68	11.4±0.84	6.3±0.67	6.7±0.81	3.8±0.65	N.D	19.2±0.21
A. baumannii	Clinical	11.1±0.89	3.7±0.69	9.3±0.59	4.7±0.46	8.5±0.47	5.9±0.29	N.D	16.3±0.20
H. influenza	Clinical	12.3±0.41	5.2±0.81	10.1±0.28	2.3±0.58	6.1±0.44	0.6±0.71	N.D	17.5±0.26
k. pneumoniae	Clinical	9.6±0.53	3.4±0.45	9.8±0.57	1.4±0.76	5.2±0.55	2.7±0.57	N.D	14.3±0.21
S. aureus	Clinical	8.3±0.54	3.2±0.15	6.4±0.53	4.3±0.25	4.3±0.75	2.2±0.95	N.D	19.3±0.21
Stem									
M. morganii	Clinical	16.5±0.11	1.3±0.41	15.3±0.58	0.3±0.85	14.2±0.48	3.1±0.14	N.D	16.8±0.27
P. aeruginosa	Clinical	12.4±0.67	0.4±0.15	13.1±0.25	3.4±0.26	9.2±0.57	1.4±1.00	N.D	17.0±0.21
A. baumannii	Clinical	14.6±0.87	7.3±0.88	11.3±0.87	5.2±0.86	6.2±0.41	7.6±0.27	N.D	15.8±0.13
H. influenza	Clinical	11.8±0.97	6.1±0.57	14.1±0.28	7.3±0.41	10.3±0.46	5.4±0.58	N.D	17.5±0.17
k. pneumoniae	Clinical	13.8±0.88	7.3±0.78	15.8±0.58	4.1±0.43	12.7±0.71	2.3±0.86	N.D	20.7±1.00
S. aureus	Clinical	10.1±0.29	0.4±0.65	9.5±0.78	7.3±0.41	5.3±0.57	8.1±0.57	N.D	15.2±0.11
Bark									
M. morganii	Clinical	18.3±0.41	2.8±0.43	17.6±0.44	1.2±0.27	12.2±0.55	4.2±0.42	N.D	19.2±0.21
P. aeruginosa	Clinical	16.2±0.48	1.2±0.57	14.3±0.24	3.3±0.47	11.4±0.64	3.4±0.58	N.D	16.3±0.20
A. baumannii	Clinical	17.1±0.89	7.8±0.24	21.5±0.78	1.4±0.24	14.8±0.81	5.6±0.15	N.D	17.5±0.26
H. influenza	Clinical	19.3±0.41	8.4±0.23	15.4±0.25	3.9±0.26	12.4±0.45	0.8±0.96	N.D	14.3±0.21
k. pneumoniae	Clinical	17.6±0.55	5.1±0.87	14.8±0.88	4.3±0.22	11.3±0.65	3.7±0.41	N.D	19.3±0.21
S. aureus	Clinical	15.3±0.57	3.8±0.36	16.4±0.55	2.8±0.44	14.4±0.24	4.8±0.41	N.D	16.8±0.27

Table 3. Antibacterial activity of *Acacia modesta* (Wall.) extracts against bacterial pathogens

Leaf Microorganisms	Origin	*Acacia modesta* (Wall.) Methanol		Ethanol		Water		N. C	P.C
		Zone mm	MIC mg/mL	Zone mm	MIC mg/mL	Zone mm	MIC mg/mL		
M. morganii	Clinical	8.2±0.14	2.7±0.51	11.4±0.74	2.3±0.43	6.4±0.48	2.3±0.21	N.D	17.0±0.21
P. aeruginosa	Clinical	9.8±0.58	4.3±0.61	9.3±0.54	5.2±0.28	5.8±0.91	4.3±0.14	N.D	15.8±0.13
A. baumannii	Clinical	11.5±0.47	1.8±0.21	8.2±0.28	0.4±0.62	7.3±0.41	0.8±0.32	N.D	17.5±0.17
H. influenza	Clinical	15.5±0.58	7.4±0.57	12.6±0.41	6.1±0.12	8.1±0.22	2.2±0.54	N.D	20.7±1.00
k. pneumoniae	Clinical	10.1±0.47	5.8±0.41	13.3±0.74	3.7±0.58	10.3±0.32	5.3±0.47	N.D	15.2±0.11
S. aureus	Clinical	8.5±0.87	3.1±0.32	9.8±0.45	1.4±0.66	5.1±0.27	1.4±0.45	N.D	19.2±0.21
Stem									
M. morganii	Clinical	17.4±0.45	9.5±0.15	16.8±0.43	8.1±0.54	12.4±0.45	2.3±0.43	N.D	16.3±0.20
P. aeruginosa	Clinical	19.8±0.91	8.4±0.26	19.3±0.91	7.3±0.41	14.5±0.57	5.3±0.51	N.D	17.5±0.26
A. baumannii	Clinical	17.3±0.22	7.2±0.29	20.4±0.22	5.8±0.52	18.2±0.44	4.3±0.14	N.D	14.3±0.21
H. influenza	Clinical	16.3±0.41	5.2±0.41	14.2±0.26	3.7±0.61	9.3±0.71	0.5±0.61	N.D	19.3±0.21
k. pneumoniae	Clinical	13.4±0.95	4.2±0.88	17.4±0.56	8.2±0.74	11.6±0.57	6.4±0.45	N.D	16.8±0.27
S. aureus	Clinical	20.1±0.47	8.5±0.31	15.6±0.65	6.7±0.85	13.6±0.41	1.8±0.58	N.D	17.0±0.21
Bark									
M. morganii	Clinical	11.1±0.44	5.3±0.41	14.3±0.57	7.3±0.25	9.3±0.44	3.4±0.41	N.D	15.2±0.11
P. aeruginosa	Clinical	9.2±0.25	1.4±0.43	10.5±0.47	5.2±0.12	7.8±0.58	3.5±0.25	N.D	19.2±0.21
A. baumannii	Clinical	7.3±0.87	3.7±0.58	7.2±0.66	1.3±0.84	4.6±0.47	2.5±0.55	N.D	16.3±0.20
H. influenza	Clinical	3.2±0.25	0.1±0.81	2.4±0.85	0.4±0.21	6.3±0.24	4.3±0.67	N.D	17.5±0.26
k. pneumoniae	Clinical	2.8±0.47	1.9±0.91	5.4±0.77	2.8±0.57	3.4±0.92	1.3±0.47	N.D	14.3±0.21
S. aureus	Clinical	9.3±0.87	14.4±0.47	8.3±0.41	5.1±0.32	6.3±0.41	1.9±0.81	N.D	19.3±0.21

ND-antimicrobial activity not detected, NC- Negative control (DMSO), PC-positive control (Ampicillin 30µg), MIC-minimal inhibitory concentration, *M. morganii - Marginella morganii, P. aeruginosa Pseudomonas aeruginosa, A. baumannii - Acinetobacter baumannii, H. influenza - Haemophilus influenza, K. pneumonia - Klebsiella pneumoniae, S. aureus - Staphylococcus aureus.*

were dispensed into each well and plates were incubated at 37°C aerobically for 18 h. At the end of each incubation period, the zone of bacterial inhibition of the extract material was measured[13]. The minimum inhibitory concentrations (MICs) were recorded by agar dilution and micro-broth dilution assays[14], using around 5000 µg/mL as an extract concentration. Plant extracts showing promising antimicrobial activity were also tested to check the formation of biofilm through *in vitro* assays.

***In vitro* assays**

The biofilm forming ability of pathogenic strains were assessed quantitatively as well as qualitatively using microtitre plate assay and by tube assay[5], respectively.

Microtiter plate assay

The bacterial strains were transferred to LB broth from nutrient agar plates and incubated for 72 h at 37°C under static conditions. Each sterile well of the flat-bottom microtiter plate was filled with 200 uL overnight culture. LB broth without culture served as a control for each strain. After designated time, the plates were emptied by tapping and washed three times with phosphate-buffered saline (PBS; pH 7.3) to remove free floating cells. The plates were then stained with 0.1% crystal violet solution (CVS) and kept for drying after washing with 95% ethanol to remove excess color. Micro-Plate reader (Perkin Elmer, Victor X3 2030 multi-label reader) was used to record the optical density (OD_{595}) of wells. The average values of the control wells were subtracted from the mean value of each strain. The standard deviation was calculated with average values after repeating experiments in triplicate, and the OD values ≥ 0.01 were reflected in the attachment index to the walls and biofilm formation.

Tube assay

Qualitative evaluation of biofilm formation was carried out through tube assay with some modifications. A loopful of each strain

was inoculated in tubes containing two mL of appropriately sterilized LB broth and incubated at 37°C for 72 h. The tubes were decanted, washed with PBS (PH 7.3) and air-dried. CVS [0.1%, w/v] was used to stain the dried tubes to visually observe the adherent layer on the wall and bottom of the tubes.

Cytotoxicity analysis

Hemolytic activity of plant extracts was conducted by following the method used by[15]. Briefly, freshly collected human blood (3 mL) was gently mixed in heparinized tubes (to avoid coagulation) and transferred into a sterile Falcon tube (15 mL) followed by centrifugation for 5

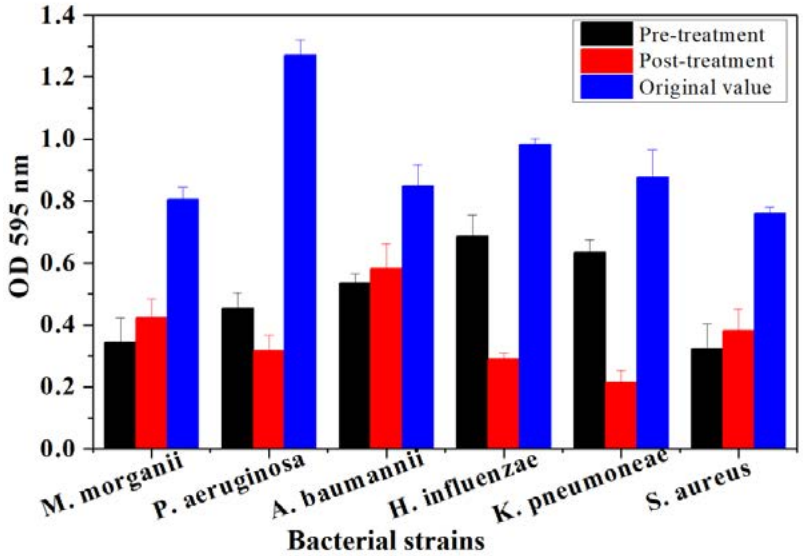

Fig. 1. Comparative analysis of biofilm growth of six bacterial strains on a microtiter plate before and after pre and post treatment by *Juglans regia* bark. At the end of 72-h controlled incubation, the biofilm formation was quantified via staining with crystal violet. Following staining, the spectrophotometric absorbances were recorded at OD_{595}. Bars represent means ± standard errors for three replicates.

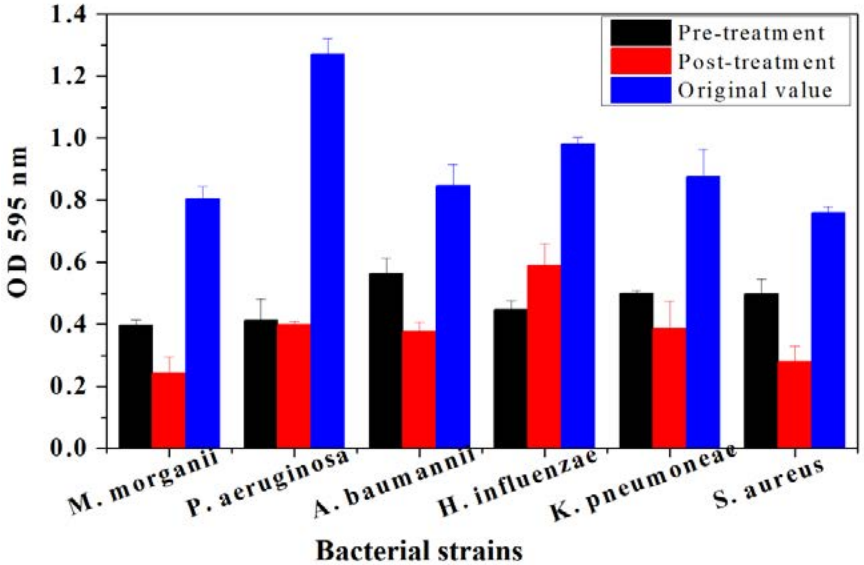

Fig. 2. Comparative analysis of biofilm growth of six bacterial strains on a microtiter plate before and after pre and post treatment by *Tamarix aphylla* bark. Bars represent means ± standard errors for three replicates.

min at 850 *g*. The supernatant was decanted, and RBCs were washed thrice with chilled sterile PBS solution. Various extracts of the plant (20 µL) were taken in 2 mL Eppendorf tubes; diluted ten times with RBCs suspension (7.068×10^8 cell/mL) and incubated at 37°C for 30 min. After brief centrifugation, collected supernatants (100 µL) were diluted with chilled PBS (900 µL) and added to 96 well plates. Triton X-100 (0.1%) and PBS were taken as positive and negative controls, respectively, for each assay.

RESULTS

Antibacterial activity of selected medicinal plants

The antibacterial activities of extracts of different parts (leaves, stems, and barks) of three medicinal plants such as *Juglans regia*, *Tamarix aphylla L.* and *Acacia modesta (Wall.)* were investigated against six bacterial pathogens by well diffusion method, and the results are summarized in Tables 1, 2 and 3. The results revealed that plants extracts exhibited different degrees of antibacterial activities against all tested microbes. Table 1 demonstrates the results of antibacterial activity of *J. regia* extracts. Maximum inhibitory effect of *J. regia* extract was recorded against *H. influenza* strain with MIC 0.5±0.19 mg/mL and inhibition zone of 21.8±0.76 mm. In contrast with bark tissue, the leaf and stem showed relatively less antibacterial activities against all tested strains. In the case of *T. aphylla L.* plant, the maximum zone of inhibition, i.e., 21.5±0.78 mm was recorded against *A. baumannii* and least activity was measured to be 14.3±0.24 against *P. aeruginosa* (Table 2). Noticeably, among the plant extracts tested, ethanolic extract of bark of *T. aphylla L.* displayed the superior antibacterial potential when compared with other parts. The *A. modesta* stem extract displayed the zone inhibition, i.e., 20.1±0.47 mm against *S. aureus* strain and the lowest value of 13.4±0.95 mm against *k. pneumoniae* was recorded.

Biofilm formation and anti-biofilm activity of selected medicinal plants

In order to determine the extent to which selected bacterial isolates formed biofilms, the strains were subjected to Microtiter plate, and tube assay and biofilm growth were monitored after 72 h. Comparative analysis of biofilm formation revealed that *P. aeruginosa* strain exhibited the highest optical density (OD_{595}=1.2713), whereas the least biofilm/planktonic OD_{595} value (OD=0.7598) was recorded in case of *S. aureus.* The observation of biofilm growth through tube assay further confirmed the biofilm formation capability of the tested strains.

The plant tissues presenting maximum antimicrobial activity were used for both pre

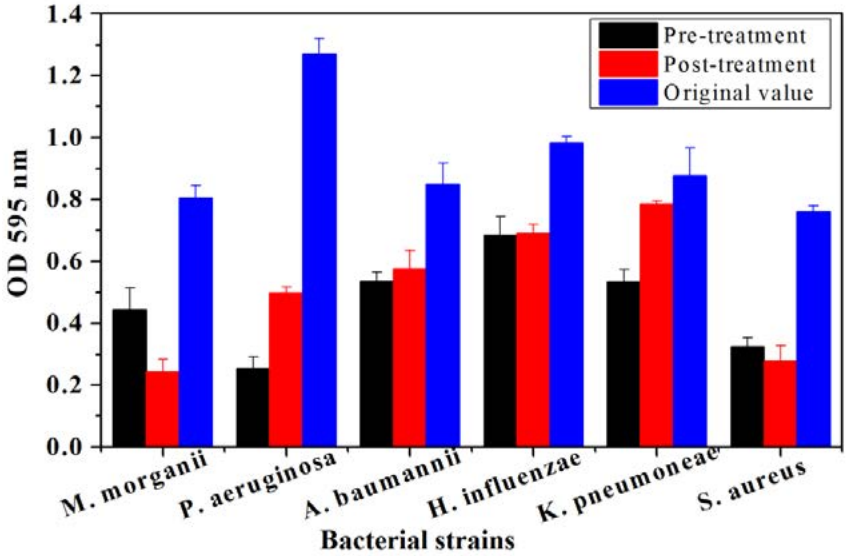

Fig. 3. Comparative analysis of biofilm growth of six bacterial strains on a microtiter plate before and after pre and post treatment by *Acacia modesta* stem. Bars represent means ± standard errors for three replicates.

and post treatment in-vitro assays. From Fig. 1, it is evident, that root bark extract of *J. regia* significantly decreased the biofilm forming activity for all pathogenic strains compared to the original OD recorded for the six strains. The maximum OD reductions were found in the case of *P. aeruginosa.* The OD percentage reductions recorded were 64.35 and 75.2% after pre- and post-treatment assays, respectively. Similarly, pre and post-treatment *P. aeruginosa* with the bark extract of *T. aphylla* led to 67.51 and 68.64% reduction in absorbance as compared to OD without any treatment (Fig. 2). The treatments with stem extract of *A. modesta* also considerably inhibited the biofilm capability of *P. aeruginosa* (Fig. 3).

Cytotoxicity study

The cytotoxic potential of plant extracts was analyzed by carrying out the hemolytic activity against human erythrocytes. The results are shown in Fig. 4 as percentage lysis of erythrocytes by comparing the absorbance of samples with the positive control (Triton X-100). The positive control displayed almost 100% erythrocytes lysis; whereas the negative control [phosphate buffer saline (PBS)] showed no RBCs lysis. The results indicated that hemolytic activities of tested plants extracts were found to be non-significant. In comparison to positive control, *J. regia* bark, *T. aphylla* bark, and *A. modesta* stem extract showed 2.52±1.03, 3.48±1.08 and 4.79±1.09 hemolytic activity, respectively.

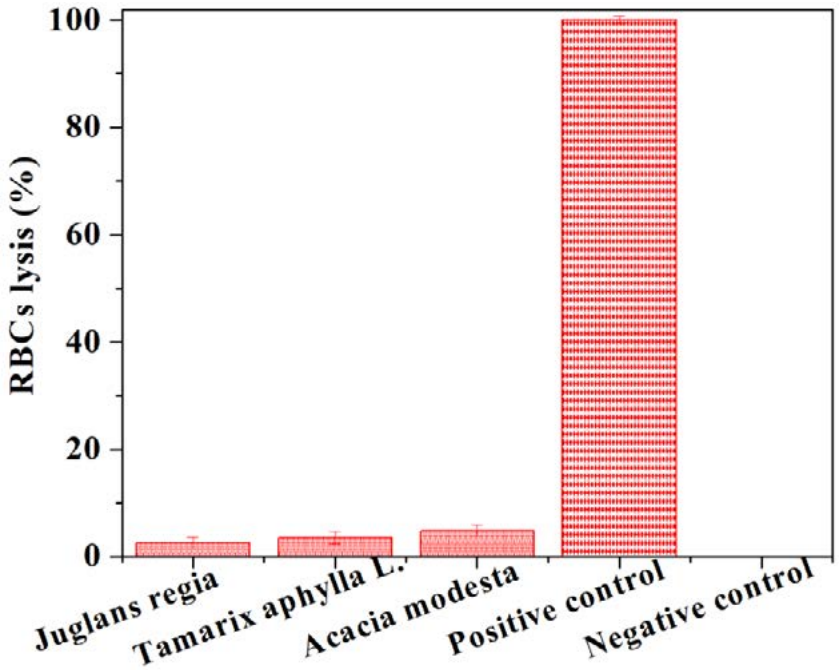

Fig. 4. Cytotoxicity assay by heamolytic activity of various plant extracts. Each bar is determinant of mean of three readings.

DISCUSSION

Herein, three ethnobotanical plants including *J. regia, T. aphyla* and *A. modesta* were assessed for antibacterial activity against bacterial pathogens. All the tested plants tested presented the significant antibacterial efficiencies against bacterial isolates. The high antimicrobial potential of *J. regia* aqueous and methanolic extracts was recorded against MRSA strains with zones of inhibition more than 20 mm and MICs values of 0.31 and 0.53 mg/mL, respectively. *J. regia* (walnut tree) is a temperate forest tree commonly found in Asia. The dried bark of this tree is conventionally used in Pakistan for improving oral hygiene and promoting teeth brightening. Earlier studies reported the antimicrobial activity of this plant against bacteria and fungi that may be due to the presence of polyphenols[1,2,9-15].

Noticeably, among the plant extracts tested, the bark of *J. regia* and *T. aphylla* showed the higher antimicrobial potential that might be due the accumulation of flavonoids content in the bark as resported earleir[16,17]. Like that, the bark of *J. regia* and *T. aphylla* has been particularly demontrated previously to have a higher antimicrobial potential as compared to other parts[18,19]. Furthermore, Stem extract of *A. modesta* also showed the superior antibacterial potential when compared with other parts. Comparable potential of stem extract of *A. modesta* has also been extensively reported previously[20-23].

The ethanolic extract of *T. aphyla* showed remarkable activity against sixteen Gram-positive and Gram -negative bacterial strains. As compared to the standard drug, the highly significant activity of *Acacia modesta* and other medicinal plants extracts was recorded by Napar *et al.*[15] against *S. aureus*, *B. subtilis* and *E. coli*. The stem extracts of *A. modesta* with five bacterial strains showed that this specific part of the plant has profound antibacterial activity, and same results were noted by[17]. The methanolic extract of *A. modesta* displayed maximum zone of inhibition of 8 mm against *E. faecalis, S. aureus,* and *S. typhi,* and did not show any activity against *B. subtalis* and *P. aeruginosa*[18].

It has been demonstrated that overall antimicrobial efficacy primarily accredited to the occurrence of secondary metabolites such as alkaloids, tannins, steroids, flavonoids and terpenoids in plant extracts[19,20]. Although, the precise mode of action of plant extracts is not yet fully explored; it is known that the efficacy of the extract mainly depends on the type of solvent used. Moreover, Gram-negative bacteria are more susceptible to antimicrobials as compared to gram-positive bacteria presumably due to non-diffusible lipopolysaccharide membrane surrounding the cell wall in gram-negative bacteria[21]. Additionally, the periplasmatic space contains enzymes which are capable of engulfing foreign molecules incorporated from the outside[21]. Results of biofilm growth pointed out the fact that *P. aeruginosa* displayed the highest ability to form biofilm among the strains tested. The comparable result has also been demonstrated earlier by several investigators[22-24]. *P. aeruginosa*, an opportunistic human pathogen, is a commonly studied biofilm forming model organism[25-27]. Nevertheless, less than 4.0 % cases, the oral cavity are occupied by *Pseudomonas*[28], and patients with cystic fibrosis are most often a host of this strain[29]. Tightly regulated biofilm development by pathogenic strain *S. aureus* has also demonstrated by prior reports[30]. Moreover, a higher anti-biofilm potential for the root bark extract of *J. regia,* bark extract of *T. aphylla* and stem extract of *A. modesta* plants was recorded against six pathogenic strains. The same anti-biofilm activity of these plants has been recorded for the number of biofilm forming strains[22,24-27]. Similarly the anti-biofilm effect of several other medicinal plant has also been extensively reported[28-31].

The cytotoxicity test (hemolytic test) was employed to evaluate the cytotoxic effect of plants extracts. The percent hemolysis remained in the range of 2.52-4.79 that was lower than the previously reported values of percent hemolysis of human erythrocytes by various other plant extracts[31-33]. Radiation-induced hemolysis of human erythrocytes by *Psoralea corylifolia* extract was studied[34], and significant protection was recorded against radiation in the concentration range of 25-50 µg/mL. Priya *et al.*[35] reported that *Achyranthes aspera* display very low hemolytic activity (1.3%) to human erythrocytes. Less hemolytic potential of tested plant extracts as shown in Fig. 2 makes it safe for their exploitation in pharmacological uses. Overall, all extracts investigated possess only small hemolytic activity which favors the use of these plants in safe measures.

CONCLUSIONS

Given the facts mentioned above, we can suggest that the medicinal plants (commonly available in Pakistan) are a natural source of biologically active constituents of high value that could be used as potential raw material in pharmaceutical industries for the effective control of infectious diseases. In conclusion, the tested plants-based extract exhibited appropriate antimicrobial and anti-biofilm activities against the tested microorganisms. The cytotoxicity of various plants extract assessed through hemolytic test revealed that the tested plants show only negligible cytotoxicity compared to the positive control. Therefore, the results of the present

study highlight the therapeutic prospect of plants under study as general therapy amongst numerous ethnic groups and conventional therapeutic and remedial experts for the treatment of various infectious diseases.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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AUTHORS' CONTRIBUTION

All listed author(s) have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

DATA AVAILABILITY

Not applicable.

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