


# Antibiofilm and Quorum Sensing Inhibition (QSI) Potential of *Lagerstroemia speciosa* Leaves Extract

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## Abstract

Disruption of quorum sensing pathway of pathogenic microbes is considered as novel approach to fight against infectious diseases. The current study was planned to evaluate the antibiofilm and quorum sensing inhibitory potential of *Lagerstroemia speciosa*. Antibacterial and antibiofilm potential of *L. speciosa* extracts was determined through agar well diffusion and crystal violet assay against sinusitis isolates, that is, *Staphylococcus aureus*, *Enterococcus faecalis*, *Proteus mirabilis*, and *Klebsiella pneumoniae*, while quorum sensing inhibition efficacy of *L. speciosa* extracts was determined through violacein inhibition assay using *Chromobacterium pseudoviolaceum* as bacterial model. The methanolic extract of *L. speciosa* presented the highest antimicrobial activity against *E. faecalis* and antibiofilm activity against *K. pneumoniae* ( $77.42 \pm 1.51\%$ ), while *n*-hexane extract was found to be least active against all tested bacterial strains. Quorum sensing inhibition activity of *L. speciosa* extracts against *C. pseudoviolaceum* showed significant dose-dependent inhibition in violacein production by different concentrations of methanolic extract. Furthermore, none of the extracts of *L. speciosa* showed any hemolytic activity against human RBCs and hold considerable thrombolytic potential in comparison to streptokinase ( $75.9 \pm .46\%$ ). In conclusion, findings suggest that *L. speciosa* leaves are excellent source of phytochemicals with potent antibiofilm and quorum sensing inhibition potential.

## Keywords

biofilm, quorum sensing, sinusitis isolates, *Lagerstroemia speciosa*, *Chromobacterium pseudoviolaceum*

## Introduction

Sinusitis is inflammation of paranasal sinuses that occur due to autoimmune disorders, allergic reactions, and infections.<sup>1</sup> Inflammation in sinuses area can cause swelling and produce excess mucus which may lead to blockage of nasal drainage and cause breathing issues.<sup>2</sup> There may be several causes of nasal inflammation such as infection of respiratory tract, nasal polyps and deviated septum, facial trauma, immune system cells, allergic reactions like hay fever, and other diseases like human immunodeficiency virus (HIV), gastroesophageal reflux (GERD), and cystic fibrosis that can also result in nasal blockage.<sup>3</sup> Most of the reported sinusitis infections are viral but bacterial sinusitis is also common. Based upon duration of sinus infection, bacterial sinusitis is divided into two categories: acute if sinusitis infection lasts than

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3 weeks and chronic when the sinuses are affected for more than 3 months, and the treatment is ineffective.<sup>4</sup> The bacterial agents which are involved in chronic sinusitis are different from bacteria of acute sinusitis. *Staphylococcus aureus*, *Moraxella catarrhalis*, *Haemophilus influenza*, *Streptococcus pneumonia*, Coagulase-negative staphylococci Gram negative bacteria such as *Escherichia coli*, *Enterobacter* spp., *Klebsiella* spp., *Proteus* spp., *Pseudomonas aeruginosa* and anaerobic bacteria (*Fusobacterium* spp, *Bacteroides*, *Porphyromonas*, *Prevotella*, and *Peptostreptococcus*) are commonly isolated from chronic sinusitis patients through sinus puncture or endoscopy.<sup>5</sup>

Biofilms are surface attached complex population of microorganisms attached with self-produced polymer matrixes of extracellular DNA, proteins and polysaccharides.<sup>6</sup> A biofilm can contain only a single type of microbial species or a combination of different bacteria, fungi, yeast, algae, archaea, and protozoa that are strongly associated with each other and to abiotic and biotic surfaces.<sup>7</sup> Biofilms can protect bacteria and make them more suitable for the external environment under certain conditions. The capacity of microbes to build biofilms is reported to be an adaptable characteristic of microorganisms.<sup>8</sup> Biofilms formation seems to be an ancient survival mechanism that gives microorganisms more options than planktonic cells which include better environmental stability, better adaptation to oligotrophic environment, increased resistance to biocides, improved organism interaction and productivity, and increased access to nutritional resources.<sup>9</sup>

Quorum sensing or quorum signaling (QS) is ability of microorganisms to recognize and respond to change in population density by regulating their gene expression.<sup>10</sup> As biofilms have high density of microbes, quorum sensing is a crucial component of biofilm physiology.<sup>11</sup> Bacteria use quorum signaling or cell to cell communication mechanism for initiation, maturation, and dispersal of biofilm and also for the regulation of metabolic activity and controlling population density inside biofilm.<sup>12</sup> As a result, antibiofilm approaches based on the inhibition or disruption of the quorum sensing pathway in bacteria can be developed.<sup>13</sup> In addition to its role in forming biofilm, quorum sensing pathway also regulate the pathogenicity and virulence of pathogenic microbes at gene level. Therefore, novel quorum sensing inhibitors must be developed as anti-infective agents against pathogenic microorganisms.<sup>14</sup>

*Lagerstroemia speciosa* (locally known as Banaba or Jarul) is a small to medium sized or rarely large deciduous or semi-deciduous plant belongs to family Lythraceae, which grows in tropical and subtropical areas.<sup>15</sup> Almost every part of plant is reported to have significant biological activities. Bark, roots, and leaves have several traditional uses. Leaves are commonly used to prepare slimming tea, to treat diabetes, hypertension, renal dysfunction, and hypercholesterolemia.<sup>16</sup> Bark of the *L. speciosa* is commonly used in diarrhea. The leaf extract is reported to have antioxidant, diuretic, hepatoprotective, and

nephroprotective properties. Because of prominent biological properties of *L. speciosa* with no side effects, the current study was planned to evaluate its antibiofilm and anti-quorum sensing potential.<sup>17</sup>

## Materials and Methods

### Collection of Plant Material and Extracts Preparation

Leaves of *L. speciosa* plant were obtained from the local market of Faisalabad, Pakistan and authenticated from the Department of Botany, University of Agriculture Faisalabad, Pakistan. Leaves were washed, shade dried, and ground into fine powder. The plant powder was then extracted with different solvents, that is, methanol, distilled water, and *n*-hexane for 72 hrs. After 72 hrs the extracts were filtered and concentrated on rotary evaporator to remove excess solvent and stored at 4°C till use.<sup>18</sup>

### Sinusitis Isolates and Culture Preparation

For antimicrobial and antibiofilm activity, sinusitis isolates (*S. aureus*, *E. faecalis*, *P. mirabilis*, and *Klebsiella pneumonia*) were procured from Medicinal Biochemistry Lab, Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan. Fresh bacterial cultures were prepared by inoculating the sterilized nutrient broth (Oxford, UK) with single colony of bacterial culture from each sinusitis isolate. The cultures were kept on orbital shaker at 37°C for overnight and used for antibacterial and antibiofilm assays.<sup>19</sup>

### Antibacterial Activity

The antibacterial activity of *L. speciosa* extracts was measured by using the agar well diffusion method.<sup>17</sup> Freshly prepared sterilized agar media with different sinusitis isolates were poured into the sterile petri plates. After solidification of nutrient agar (Oxford, UK), wells (6 mm) were made with the help of sterile cork borer. Different concentrations (100 µL) of *L. speciosa* extracts were added in to each well. Plates were incubated for 24 hrs at 37°C. Ciprofloxacin (10 µg/mL) was used as positive control. Antibacterial activity was calculated by measuring the diameter (mm) of zone of inhibition after 24 hrs.<sup>20</sup>

### Minimum Inhibitory Concentration

For MIC diluted standard inoculum of each sinusitis isolate was prepared in accordance with CSLI protocol and turbidity of the overnight inoculum was adjusted of .5 McFarland standard and dilution was performed with sterile muller nutrient broth (MHB). Minimum inhibitory concentration of different extracts of *L. speciosa* was determined through broth dilution method. For assay, 100 µL of different plant extract (200 µg/mL) was poured into the each well of 1st column of

96-well microtiter plate. Then 50  $\mu\text{L}$  of sterile MHB was added into each well of columns 2–10. Columns 11 and 12 contain 100  $\mu\text{L}$  of diluted inoculum and sterile nutrient broth for growth control and sterility control of the assay. Double serial dilution of each sample was performed with multi-channel pipette by taking 50  $\mu\text{L}$  of each extract from columns 1–10 that result in 50  $\mu\text{L}$  of extract per column. After that 50  $\mu\text{L}$  of diluted inoculum was added into each well containing plant extract. Plates were incubated for 24 hrs at 37°C. After incubation, 30  $\mu\text{L}$  of resazurin dye (.015%) was added to each well, and plates were again incubated for 4 hrs at 37°C. The column before the color change were scored as above MIC value.<sup>20</sup>

### Biofilm Inhibition

Biofilm inhibition was performed through crystal violet method by following the procedure described by Qasim et al<sup>21</sup> The assay was performed in 96-well plate by incubating sinusitis isolate (10  $\mu\text{L}$ ) and 100  $\mu\text{L}$  of each plant extracts and nutrient broth. Plates were covered with aluminum foil and incubated for 24 hrs at 37°C. After 24 hrs, plates were washed three times with 220  $\mu\text{L}$  PBS (phosphate buffer saline) to remove non-adherent bacteria. After washing, the biofilm was fixed with methanol for 15 mins. After fixation biofilm was stained with a 7% of crystal violet dye (200  $\mu\text{L}$ ) for 10 mins. After 10 mins, excess dye was washed with distilled water thrice. To solubilize the biofilm, glacial acetic acid (33%) was added, and plate were read at 600 nm against negative control containing bacteria and growth media only. Ciprofloxacin was used as positive control. The results were represented in percentage (%) inhibition.

### Phase Contrast Microscopy

The microscopic slides of native and treated biofilm were prepared. Smear on the sterile glass slides were prepared by adding 25  $\mu\text{L}$  of nutrient broth, 25  $\mu\text{L}$  of *L. speciosa* extracts, and 5  $\mu\text{L}$  of bacterial strain. Slides were incubated at 37°C for 48 hours. After incubation, slides were stained using 2% crystal violet dye for 7 mins. Slides were washed three times with distilled water. The positive control slide was prepared by adding nutrient broth, ciprofloxacin, and bacterial strain and negative with nutrient broth and bacterial strain only. After drying, the slides were examined using a microscope in central Hi-tech laboratory, University of Agriculture Faisalabad.<sup>21</sup>

### Hemolytic Assay

The cytotoxic potential of *L. speciosa* extracts were evaluated by following the method described by Fatima et al<sup>22</sup> Fresh blood (5 mL) was collected from a healthy person in EDTA containing vials and then centrifuged for 10 min at 3000 r/min. Supernatant was discarded and pellet of RBCs was washed three times with PBS (phosphate buffer saline, pH 7.4). RBCs

were resuspended in 10 mL of PBS. Plant extract (20  $\mu\text{L}$ ) was mixed with RBCs suspension (180  $\mu\text{L}$ ) and incubated for 30 mins at 37°C. After incubation, samples were centrifuged for 10 min at 3000 r/min. The supernatant (100  $\mu\text{L}$ ) was diluted with chilled PBS (900  $\mu\text{L}$ ) and absorbance was taken at 576 nm. Triton-X-100 was used as positive control. The results were represented as % hemolysis calculated by following formula

$$\text{Percentage hemolysis} = \frac{\text{Absorbance of sample} - \text{Absorbance of negative control}}{\text{Absorbance of positive control}} \times 100$$

### Mutagenic Potential of *L. speciosa* Extracts

Mutagenic potential of *L. speciosa* extracts was determined through Ames fluctuation assay using two mutant strains of *Salmonella typhimurium* TA98 and TA100 by following the method of Iqbal et al.<sup>23</sup>

### Thrombolytic Activity

Plant extracts were also tested for clot lysis potential against streptokinase. For clot formation, fresh blood aliquots were collected and transferred to pre-weighed Eppendorf tubes. The tubes were incubated at 37°C for 45 min. After the clot formation, the serum was thoroughly discarded with a sterile cotton bud without damaging the clot and tubes were again weighed to calculate the weight of clot. The plant extract (100  $\mu\text{L}$ ) was added into tubes containing preformed clot and incubated at 37°C for 90 min. After incubation, the fluid separated from the clot was discarded carefully. The percentage (%) clot lysis was calculated by taking the difference in weight of clot before and after lysis.<sup>24</sup>

### Quorum Sensing Inhibition Assay

Quorum sensing inhibition potential of different *L. speciosa* extracts was determined. *C. pseudoviolaceum* was cultured in Luria-Bertani (LB) broth and incubated for 24 hrs at 27°C. Using sterile LB broth, bacterial culture was diluted 1:1000 v/v to achieve 10<sup>6</sup> CFU/mL. The *L. speciosa* extracts was then dissolved in DMSO (10%) and diluted in LB media to a final concentration of 10 mg/mL. For assay, individual plant extract (500  $\mu\text{L}$ ) was added in different columns of 48 wells microplate and serially diluted with LB broth. After dilution, 500  $\mu\text{L}$  of diluted *C. pseudoviolaceum* culture (10<sup>6</sup> CFU/mL) was added into each well and incubated at 27°C. After incubation, contents from each well was removed and centrifuged at 8000 r/min for 5 mins in Eppendorf's tubes. The supernatant was discarded, and the pellets were resuspended in DMSO (1000  $\mu\text{L}$ ) and centrifuged again for 5 mins at 8000 r/min. The optical density of obtained supernatants was measured at 595 nm using ELISA microplate reader. To confirm that different extracts of *L. speciosa* inhibited the quorum sensing without inhibiting microbial growth, the

pellet was resuspended in sterile water (1 mL) and vortex again and read at 595 nm.<sup>25</sup>

### Statistical Analysis

All obtained data were analyzed through one way analysis of variance and final results were presented as means  $\pm$  standard deviation.<sup>26</sup>

## Results and Discussion

Sinusitis is one of the most prevalent problems encountered in general medical practice.<sup>2</sup> Development of biofilm within sinusitis is believed to contribute to recurrent infections and chronic mucosal inflammation that prolong duration of disease. Due to hardy nature of biofilms, their complete eradication is almost impossible and result in multidrug resistance.<sup>13</sup> The ability of microorganisms to produce biofilm play significant role in the microbial pathogenicity and protect the microbe against antibiotics and host immune response. In biofilm, microorganisms are well known for causing chronic or recurrent infections.<sup>27</sup> Plants are important source of a wide range of secondary metabolites, which are used as pharmaceuticals, agrochemicals, flavors, fragrances, colors, bio-pesticides, and food additives.<sup>28</sup> *L. speciosa*, also known as jarul, is one of these valuable medicinal herbs of family *Lythraceae* and reported to have wide range of pharmacological activities such as anti-diabetic, hypoglycemic, antioxidant, anti-inflammatory, anti-obesity, xanthine-oxidase inhibitors, antibacterial, antiviral, cytotoxic, anti-fibrotic, antinociceptive, and antidiarrheal.<sup>29</sup> The literature on antibiofilm and quorum sensing inhibition potential of this plant is very scarce. Therefore, in this study, we particularly focus on

antibacterial and antibiofilm activities of different extracts of *L. speciosa* leaves against sinusitis isolates. The anti-quorum sensing activity of extracts was also evaluated.

### AntiBacterial and Antibiofilm Potential of *L. speciosa* Leaf Extracts

Finding novel antimicrobial compounds from natural sources is one way to combat the problem of bacterial resistance, and medicinal plants are good and effective source.<sup>30</sup> Antibiotic resistance is frequently attributed to the development of biofilms, which are intricate protective matrices that enable populations of sessile bacteria to endure harsh environmental factors like hunger, host defenses, and antibiotics.<sup>31</sup> Biofilms can be formed on both living and nonliving surfaces, and because bacteria within biofilms remain alive even after the death of planktonic bacteria, biofilm formation is a method of resistance to antibiotics.<sup>32</sup>

The antibacterial activity of *L. speciosa* leaf extracts was performed against sinusitis isolates (*S. aureus*, *P. mirabilis*, *K. pneumoniae*, and *E. faecalis*) at different concentrations, that is, .2, .4, .6, .8, and 1.0 mg/mL and results are given in Table 1. Results showed that antibacterial activity of *L. speciosa* extracts is dose-dependent. The methanolic extract of *L. speciosa* was found to be most active against all tested sinusitis isolates and showed the highest zone of inhibition with diameter of 22 mm, 21 mm, 20 mm, and 18 mm against *E. faecalis*, *P. mirabilis*, *K. pneumoniae*, and *S. aureus*, respectively. The aqueous extract of *L. speciosa* showed the antibacterial activity with inhibition zone of 17 mm, 17 mm, 16 mm, and 15 mm, against *E. faecalis*, *K. pneumoniae*, *P. mirabilis*, and *S. aureus*. Similarly, the *n*-hexane extract showed lowest

**Table 1.** Antibacterial and AntiBiofilm Activities of the Different Extracts of *L. speciosa* Leaves Extracts Against Sinusitis Isolates.

Extract	Concentration mg/mL	<i>S. aureus</i>		<i>P. mirabilis</i>		<i>K. pneumoniae</i>		<i>E. faecalis</i>	
		DZI (mm)	Biofilm inhibition	DZI (mm)	Biofilm inhibition	DZI (mm)	Biofilm inhibition	DZI (mm)	Biofilm inhibition
Methanolic	0.2	12 $\pm$ 0.43	24.06 $\pm$ 1.01	17 $\pm$ 0.48	65.87 $\pm$ 2.07	13 $\pm$ 0.29	69.06 $\pm$ 2.06	16 $\pm$ 0.54	58.71 $\pm$ 1.46
	0.4	16 $\pm$ 0.27	36.27 $\pm$ 1.03	16 $\pm$ 0.57	66.09 $\pm$ 2.00	13 $\pm$ 0.58	69.32 $\pm$ 2.96	18 $\pm$ 0.76	60.72 $\pm$ 1.98
	0.6	17 $\pm$ 0.78	38.56 $\pm$ 1.54	19 $\pm$ 0.56	66.44 $\pm$ 2.03	15 $\pm$ 0.37	71.28 $\pm$ 2.97	19 $\pm$ 0.32	61.75 $\pm$ 1.35
	0.8	15 $\pm$ 0.58	40.85 $\pm$ 1.25	17 $\pm$ 0.48	67.62 $\pm$ 2.02	17 $\pm$ 0.62	72.19 $\pm$ 2.93	19 $\pm$ 0.12	63.57 $\pm$ 1.02
	1	18 $\pm$ 0.65	57.65 $\pm$ 1.07	21 $\pm$ 0.57	69.04 $\pm$ 2.01	20 $\pm$ 0.69	77.42 $\pm$ 1.51	22 $\pm$ 0.93	64.08 $\pm$ 1.12
Aqueous	0.2	11 $\pm$ 0.43	20.23 $\pm$ 1.00	8 $\pm$ 0.48	62.42 $\pm$ 1.35	5 $\pm$ 0.29	61.66 $\pm$ 0.97	2 $\pm$ 0.43	50.60 $\pm$ 2.45
	0.4	13 $\pm$ 0.57	22.51 $\pm$ 1.03	10 $\pm$ 0.57	62.70 $\pm$ 1.20	5 $\pm$ 0.58	62.81 $\pm$ 1.45	4 $\pm$ 0.12	51.25 $\pm$ 1.85
	0.6	14 $\pm$ 0.38	22.52 $\pm$ 2.04	12 $\pm$ 0.56	63.27 $\pm$ 1.01	6 $\pm$ 0.37	67.43 $\pm$ 2.03	14 $\pm$ 0.25	52.66 $\pm$ 2.21
	0.8	15 $\pm$ 0.28	22.54 $\pm$ 1.09	14 $\pm$ 0.48	63.37 $\pm$ 2.34	16 $\pm$ 0.62	68.03 $\pm$ 2.50	15 $\pm$ 0.19	56.17 $\pm$ 1.01
	1	15 $\pm$ 0.43	23.28 $\pm$ 1.78	16 $\pm$ 0.54	64.68 $\pm$ 2.05	17 $\pm$ 0.56	68.67 $\pm$ 0.98	17 $\pm$ 0.22	57.86 $\pm$ 2.03
<i>n</i> -Hexane	0.2	2 $\pm$ 0.43	2.68 $\pm$ 1.06	3 $\pm$ 0.48	12.73 $\pm$ 1.02	7 $\pm$ 0.29	54.34 $\pm$ 1.98	2 $\pm$ 0.18	29.97 $\pm$ 2.09
	0.4	3 $\pm$ 0.57	4.19 $\pm$ 2.02	3 $\pm$ 0.57	47.07 $\pm$ 1.07	6 $\pm$ 0.58	55.48 $\pm$ 1.55	3 $\pm$ 0.37	30.37 $\pm$ 2.05
	0.6	5 $\pm$ 0.38	6.88 $\pm$ 1.04	4 $\pm$ 0.56	55.34 $\pm$ 1.21	15 $\pm$ 0.37	56.14 $\pm$ 1.35	3 $\pm$ 0.16	47.36 $\pm$ 2.01
	0.8	5 $\pm$ 0.28	12.60 $\pm$ 1.01	11 $\pm$ 0.48	56.54 $\pm$ 1.02	16 $\pm$ 0.62	57.56 $\pm$ 1.79	5 $\pm$ 0.28	48.39 $\pm$ 2.02
	1	6 $\pm$ 0.54	19.47 $\pm$ 1.21	11 $\pm$ 0.62	61.96 $\pm$ 1.08	17 $\pm$ 0.32	59.86 $\pm$ 1.88	6 $\pm$ 0.12	49.56 $\pm$ 2.00
Ciprofloxacin (1 mg/mL)		26 $\pm$ 0.74	65.89 $\pm$ 2.19	34 $\pm$ 0.47	75.67 $\pm$ 1.97	36 $\pm$ 0.51	88.56 $\pm$ 2.37	33 $\pm$ 0.91	79.66 $\pm$ 1.39



antibacterial activity among all extracts with inhibition zone of 17 mm, 11 mm, 6 mm, and 6 mm against *K. pneumoniae*, *P. mirabilis*, *S. aureus*, and *E. faecalis*. The minimum inhibitory concentration values of *L. speciosa* leaf extracts against

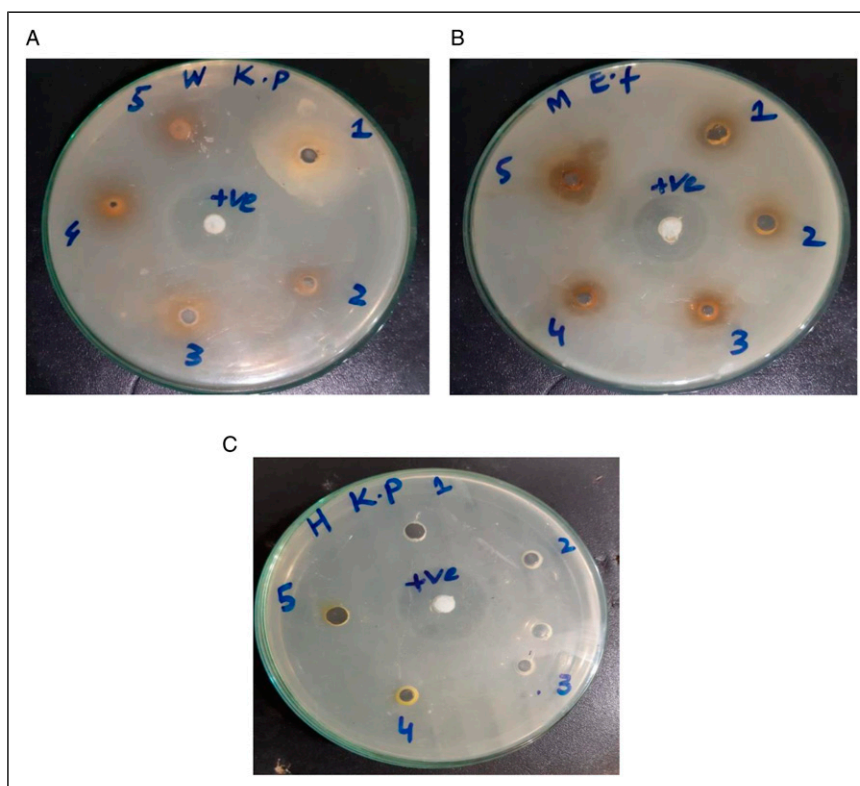
**Table 2.** Minimum Inhibitory Concentration of Different Extracts of *L. speciosa* Extracts Against Sinusitis Isolates.

Extract	MIC mg/mL			
	<i>S. aureus</i>	<i>P. mirabilis</i>	<i>K. pneumoniae</i>	<i>E. faecalis</i>
Methanolic	0.62	0.31	0.62	0.15
Aqueous	0.75	0.73	0.64	0.51
<i>n</i> -hexane	0.83	0.90	0.94	0.82
Ciprofloxacin	0.02	0.03	0.03	0.01

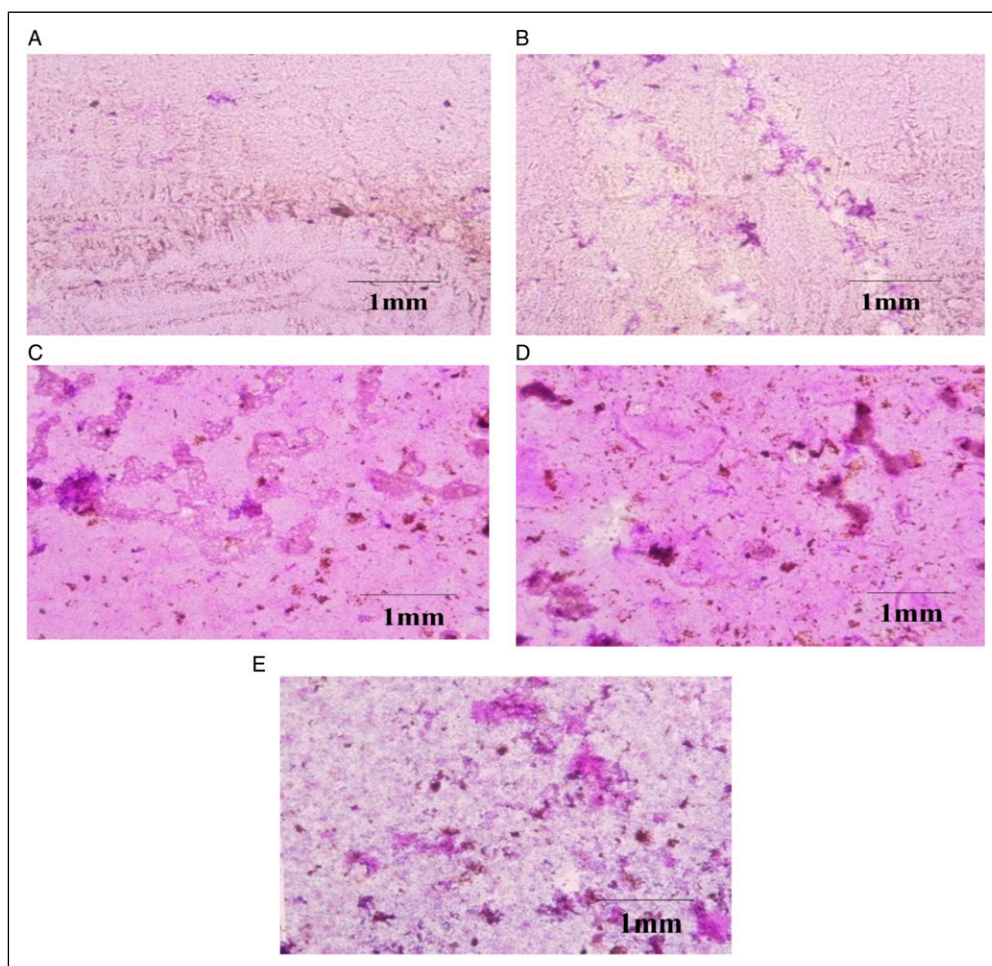
**Table 3.** Mutagenic/Non-Mutagenic Potential of Different Extracts of *L. speciosa* Extracts Through Ames Assay.

Samples	Mutagenic/non-mutagenic potential			
	TA98	Result	TA100	Result
Standard	78/96	Mutagenic	87/96	Mutagenic
Methanolic	9/96	Non-mutagenic	11/96	Non-mutagenic
Aqueous	10/96	Non-mutagenic	10/96	Non-mutagenic
<i>n</i> -Hexane	9/96	Non-mutagenic	17/96	Non-mutagenic

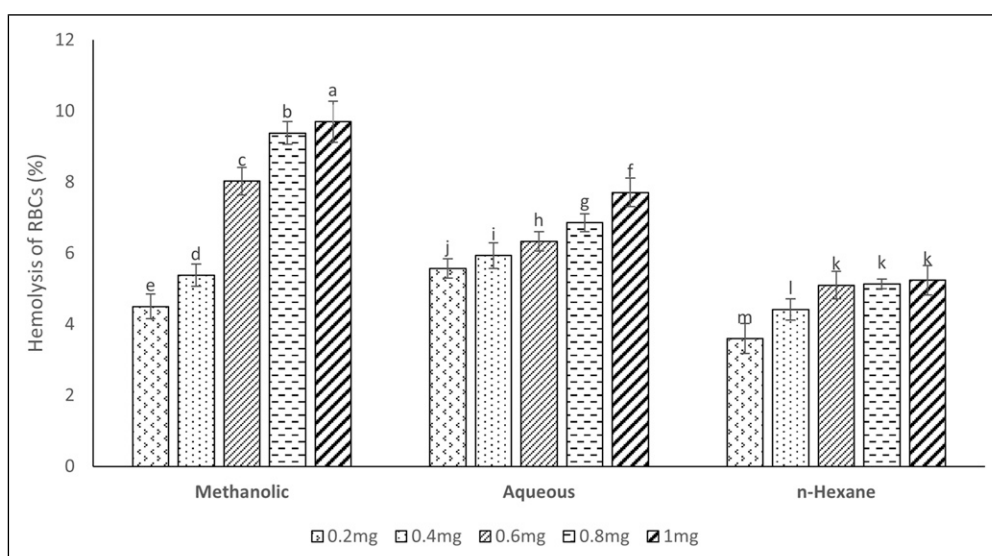
sinusitis isolates are presented in Table 2. The methanolic extracts has lowest MIC values against all bacteria while the *n*-hexane extracts has highest MIC values in comparison to methanol and aqueous extracts. Results of antibiofilm activity of *L. speciosa* leaf extracts are presented in Table 1, performed against sinusitis isolates at different concentrations and results showed dose-dependent inhibition of biofilm. Like antibacterial activity, the methanolic and aqueous extracts of *L. speciosa* showed significant biofilm inhibition against all tested sinusitis isolates. The highest biofilm inhibition was shown by methanolic extract of *L. speciosa* against *K. pneumoniae* (77.42%) while the least activity was shown by *n*-hexane extract of *L. speciosa* against *S. aureus* (19.47%). The percentage biofilm inhibition of methanolic extracts against *S. aureus*, *P. mirabilis*, *K. pneumoniae* and *E. faecalis* was 56.65%, 69.04%, 77.42%, and 64.08% while aqueous extracts exhibited 23.28%, 64.68%, 68.67%, and 57.86%. Likewise, the *n*-hexane extracts showed biofilm inhibition of 19.47%, 61.96%, 59.86%, and 49.56% against *S. aureus*, *P. mirabilis*, *K. pneumoniae*, and *E. faecalis*, respectively. The result of antibiofilm assay was also confirmed through phase contrast microscopic studies which further strengthen the antibiofilm potential of *L. speciosa* leaves extract Figure 1. The treatment of biofilm with different extracts disrupts the structure of biofilm in comparison to negative control (non-treated) (Figure 2).



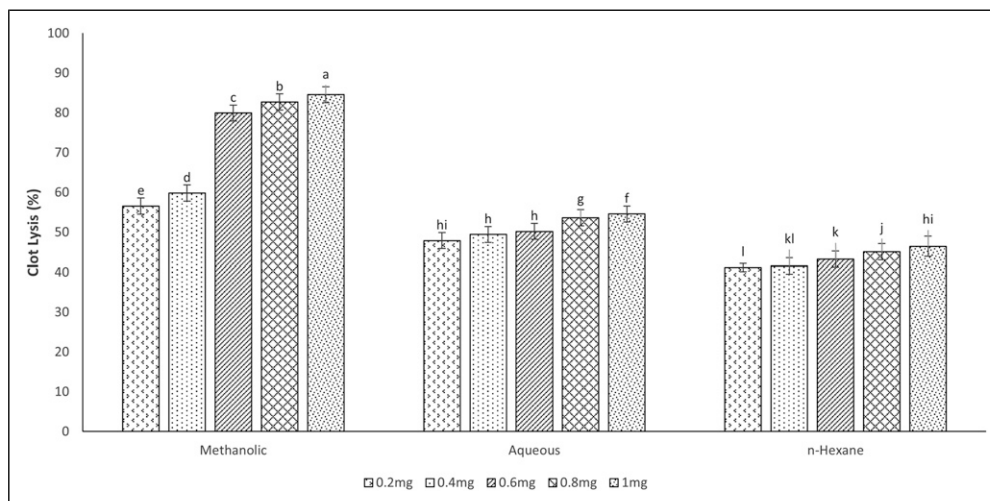
**Figure 1.** Representative pictures of antibacterial activity of different extracts of *L. speciosa* extracts against sinusitis isolates.



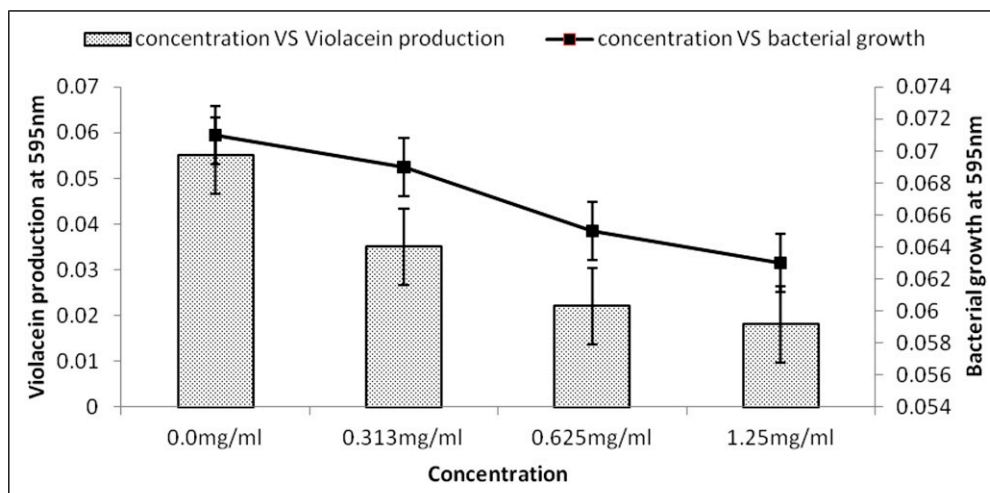
**Figure 2.** Representative pictures of phase contrast microscopic images of *L. speciosa* plant extracts (A), Positive (ciprofloxacin) (B), Negative control (C), methanolic and (D). aqueous extract against *K. pneumoniae* and E. n-hexane extract against *P. mirabilis*.



**Figure 3.** Hemolytic activity of different extracts of *L. speciosa* against human RBCs.



**Figure 4.** Percentage clot lysis potential of different concentrations of *L. speciosa* extracts.



**Figure 5.** Quorum sensing inhibition assay.

### Hemolytic and Thrombolytic Potential of *L. speciosa* Extracts

Hemolytic assay allows to assess the ability of plant extracts to damage the red blood cells membrane. Disruption of RBC's membrane by tested plant extracts caused significant increase in the absorbance and release hemoglobin which indicates plant's toxicity.<sup>33</sup> The detergent triton-X-100 was used as standard. Hemolytic activity of *L. speciosa* extracts is expressed as Mean  $\pm$  S.D. Results of hemolytic assay of different extracts of *L. speciosa* exclude cytotoxic behavior of the plant. Extracts exhibited low to mild hemolytic activities against human RBCs. Methanolic extract showed highest hemolytic activity (9.72% at 1 mg/mL *L. speciosa*) while *n*-hexane extract of *L. speciosa* exhibited lowest (5.24% at 1 mg/mL *L. speciosa*) hemolytic activity. The aqueous extract of *L. speciosa* showed moderate lysis of activity. The hemolytic

activity of different extracts of *L. speciosa* was found to be dose dependent (Figure 3). Similarly, the thrombolytic potential of different extracts of *L. speciosa* was also assessed at different concentrations against preformed clot in eppendorf's tubes and results of clot lysis potential of different extracts of *L. speciosa* are presented in Figure 4. Among different extracts of *L. speciosa*, Methanolic extract showed the highest (84.56%) clot lysis potential, whereas the *n*-hexane extract of *L. speciosa* showed lowest (46.47%) clot lysis activity in comparison to standard streptokinase (91.56%).

### Toxicological Screening of *L. speciosa* Extracts Through Ames Assay

Mutagenic potential of *L. speciosa* leaves extract was evaluated through Ames reverse mutation assay. Change in color from purple to yellow in 96 wells plate after treatment



of *S. typhimurium* indicates mutagenic behavior of tested plant extracts. The results of mutagenicity/non-mutagenicity of different extracts of *L. speciosa* extracts are presented in Table 3. In current assay, the mutagenic activity of plant extracts was calculated by comparing it with background plate. If the positive wells in test plate are more than double of the background plate the extract would be considered as mutagenic. None of the plant extracts showed mutagenic activity as total number of positive wells are less than background plate. Standards of both strains TA98 and TA100 showed potent genotoxic potential in comparison to *L. speciosa* extracts.

### Quorum Sensing Inhibition Assay

Inhibition of quorum sensing mechanism is an important factor to decrease the virulence of pathogenic microbes.<sup>34</sup> It has been proven that several plant-derived compounds disrupt the quorum sensing mechanism by preventing the synthesis of peptidoglycans, altering the composition of microbial membranes, or controlling quorum sensing, all of which could influence development of biofilms.<sup>29</sup> There are several ways to inhibit quorum sensing including inhibition of signal detection and inhibition of signaling molecules through quorum sensing inhibitors (QSI).<sup>35</sup> The compounds halogenated furanones from marine alga *Delisea pulchra* which inhibited QS through proteolytic degradation, were the first QSI to be identified but the effective dose of halogenated furanones is reported to have toxic effects.<sup>36</sup> Moreover, the inhibition of binding between receptors and their signal molecules through competitive inhibition or reducing the DNA-binding activity of receptor molecules affect downstream process of gene expression.<sup>37</sup> Several different mechanisms, including chemically (change in pH), physically (low-high temperature), enzymatically (degrading enzymes), and through microorganisms (metabolizing signaling molecules), could cause quorum quenching of signaling molecules to inactivate or degrade QS signals.<sup>38</sup>

In this assay, the effect of different concentrations of *L. speciosa* leaf extracts on violacein (a purple pigment) production through *C. pseudoviolaceum* was evaluated for its anti-quorum sensing inhibition assay. When growing the Chromobacterium produces violet color pigment violacein which has antioxidant effect and protect the membrane of bacteria against oxidative stress through a quorum sensing mediated process. The Chromobacterium is utilized for qualitative screening of violacein production, which is shown by the reduction or absence of violet coloration.<sup>36,37</sup> The anti-quorum sensing inhibition activity of *L. speciosa* extracts was determined at MIC and sub-MIC concentration. The extracts exhibited moderate to good inhibition of violacein pigment without influencing the bacterial growth. The methanolic and aqueous extracts were most active and showed significant inhibition of violacein production at

MIC concentration. The n-hexane extract showed moderate inhibition. The results of quorum sensing inhibition are regarded as positive when violacein is not produced by *C. pseudoviolaceum* and negative when violacein is produced by the bacteria after treatment with plant extracts. Leaves of *L. speciosa* were not reported for its quorum sensing inhibition potential. Singh et al<sup>39</sup> reported the anti-quorum sensing inhibition potential of *L. speciosa* fruit extracts and results showed positive correlation with our results. The results of quorum sensing inhibition activity are shown in Figure 5

### Conclusion

From current study it was concluded that *L. speciosa* leaves are good source of antibacterial, antibiofilm, and anti-quorum sensing compounds. Methanolic extracts was most active extract among all tested extracts followed by aqueous extracts. While n-hexane extract was least active extract with least bioactive compounds responsible for antibacterial and antibiofilm compounds. The extracts also not exhibited any toxicological properties against human RBCs and trough Ames assay. Further studies should be focused on isolation and identification of these compounds from *L. speciosa* leaves.

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