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In Vitro Anti-Bacterial Properties of Lagerstroemia speciosa (L.) Pers (Lythraceae) Ethanolic Extracts of Green and Red Leaf against Bacteria that Cause Urinary Tract Infection (UTI)

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ABSTRACT: Urinary tract infections (UTIs) are caused by common microorganisms like Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Staphylococcus aureus, posing a public health concern. This study aims to investigate the effectiveness of medicinal plants used to treat infections in the urinary tract. The plant known as the "Pride of India" is Lagerstroemia speciosa, a member of the Lythraceae family. The qualitative phytochemical investigation of L. speciosa leaf extracts shows carbohydrates, tannins, saponins, flavonoids, alkaloids, quinones, glycosides, triterpenoids, coumarins, and steroids. The results showed that the antibacterial action was determined by the well diffusion method, bacterial growth curve analysis, broth microdilution method, intracellular ROS measurement, live/dead cell assays, and biochemical assays. The purpose of staining techniques is to demonstrate the viability of bacterial stains. The investigation concludes that LERLEs possess strong antibacterial properties, suggesting that herbal plants, as a potential solution to antimicrobial resistance, hold great promise.

KEYWORDS: Urinary tract infections, Lagerstroemia speciosa, anti-bacterial activity, MIC, MBC and Antimicrobial Resistance.

I. INTRODUCTION

Urinary tract infections (UTIs) are common in humans, with Escherichia coli being the most common causative agent. Around 80-90% of UTIs are caused by E. coli, which lives in the intestines but can also enter the urinary tract [1]. Antibiotics are typically the first treatment, with levofloxacin being a widely studied third-generation fluoroquinolone. The anti-uropathogenic and bactericidal activity of many plant extracts has been reported by many researchers; cranberry is the best-studied home remedy for UTI [2]. Antibiotics have significantly improved human health worldwide, especially in developing nations with inadequate public health infrastructure, by lowering the morbidity and mortality rate brought on by bacterial infections [3]. The overuse and misuse of antibiotics and other antimicrobial drugs have led to the development of resistant strains of bacteria, making it increasingly difficult to treat infections [4].

Medicinal plants provide essential healthcare and nutrition to developing countries, aiding in urinary tract infections and providing essential resources. Lagerstroemia speciosa, a tropical and subtropical Asian plant, is a potential candidate [5]. The Western Ghats are one of the rich biodiversity regions of India, especially Coimbatore, Tamil Nadu. The experimental plant L. speciosa is commonly called Pride of India, or Poomaruthu in Tamil. A wide variety of phytochemical compounds, such as secondary metabolites, are synthesised by plants. The secondary metabolites of medicinal plants have very strong antioxidant properties and act as an efficient source of natural antioxidants [6]. The chemical constituents, pharmacological effects, and therapeutic effects of the selected parts of L. speciosa are discussed in this study. The sample plant we used has two different leaf types: a green leaf and a red leaf. The leaf's pigments exhibit the colour variation.

Anti-microbial resistance is a natural process that happens over time through genetic changes in pathogens [7].L. speciosa has antimicrobial properties. Antibacterial activity of ethanol and water extracts of leaves of L. speciosa was tested by the plate agar diffusion method

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against Gram positive and Gram-negative bacteria [8]. Leaf decoction or infusion was used for bladder and kidney inflammation, dysuria and other urinary dysfunctions, cholesterol deduction, hypertension, and diabetes [9]. Flavonoids provide pigments for plants, with anthocyanins and proanthocyanins being condensed tannins. Polyphenols, such as ellagitannins, are found in fruits, nuts, and seeds [10]. This study aims to investigate the effectiveness of medicinal plants used to treat infections in the urinary tract and anti-microbial activities.

II. MATERIALS AND METHODS

Collection and Authentication of plant samples

The leaves of L. speciosa were collected from the PG Girls Hostel, Government Arts College (Autonomous), Coimbatore District, Tamil Nadu, India. The identification and authentication of L.speciosa are done by the Botanical Survey of India, Coimbatore, and the voucher specimens numbered BSI/SRC/5/23/2020/Tech/50 were placed in the Department of Zoology, Government Arts College (Autonomous), Coimbatore.

Plant extracts preparation

L. speciosa leaves were collected, washed, and shade-dried for 2 weeks. The leaves were ground to powder (100g) and soaked in ethanol (1000 ml). The powder was solubilized and mixed well with intermittent stirring for 4 days. After that, the extract was filtered using Whatman No. 1 filter paper and kept in a plastic tray to dry at room temperature [11].

Quality Control Analysis

Qualitative phytochemical analysis of the green and red leaves of L. speciosa ethanolic extracts was carried out according to the methodology of [12], Trease, and Evans [13]. The GC-MS analysis at the South Indian Textile Research Association in Coimbatore identified important compounds in L. speciosa ethanolic extracts of green and red leaves. The analysis used a Thermo GC-Trace Ultra ver. 5.0, Thermo MS DSQ 11 chromatography [14].

Determination of antibacterial activity

LEGLE and LERLE's disinfection potential was assessed using the agar well diffusion method published by Bennett et al.,[15] against Gram-negative bacteria Escherichia coli, Pseudomonas aeruginosa, and Klebsiella pneumoniae, as well as Gram-positive bacteria Staphylococcus aureus. Bacterial strains were grown on Muller Hinton Agar overnight. To produce wells, a metal cork borer was employed, followed by 100 µL of LEGLE and LERLE at 1 mg/mL concentrations. Following that, the plates were incubated at 37°C. The zone of inhibition was measured in millimetres. Three copies of the experiment were carried out.

Bacterial growth curve analysis

The bacterial growth kinetics assay was used to evaluate the bactericidal activity of green and red leaves of L. speciosa ethanolic extract concentrationsaccording to the methodology of Salem et al., [16]. Various concentrations of green and red leaves of L. speciosa ethanolic extracts (25, 50, 75, and 100 µg/mL) were loaded into 96-well plates, and 10µL of overnight-grown bacterial cultures were added to each well. The plates were incubated at 37°C for 20 h. Optical density measurements from each well were taken every 2 hours using a microplate reader at 600 nm. Experiments were performed in triplicate, and growth curves were plotted between optical density and time.

Broth microdilution method

The minimum inhibitory concentration and minimum bactericidal concentration against pathogenic bacteria were evaluated by the broth microdilution method. L. speciosa ethanolic green and red leaf extract concentration solutions were prepared at a concentration of 100 µg/mL. Nutrient broth (100 µL) was added to each well of microtiter plates, followed by the addition of serially dilutedin CMC. Afterwards, 10 µL of bacterial inoculum was added to each well and incubated at 37 °C for 24 h. The absorbance was measured at 620 nm using a microplate reader. 10 µL of TTC (2,3,5-triphenyl tetrazolium chloride) was added, followed by a 30-minute incubation period. The lowest concentration of plant extract25 µL that inhibits the growth of pathogenic bacteria is taken as the MIC and compared to the control. The experiments were performed with three replications. MBC was performed by diluting the MIC culture and subculture on sterile MH agar plates. The viable colony count method was followed to calculate the MBC.

Intracellular ROS measurement

Using the methodology described by Ishwarya et al., [17] the fluorescence probe H2DCF-DA was employed to quantify ROS levels

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in bacterial cells. Bacterial cultures at a concentration of 1×10^6 CFU/mL were exposed to 100 µL of varying L. speciosa ethanolic green and red leaf extract concentrations (25, 50, 75, and 100 µg/mL) for a duration of two hours. Subsequently, the bacterial samples were washed and suspended in PBS.

Upon exposure to a 1 mM H2DCFDA solution (100 µL), the samples were incubated at 37°C for 30 minutes in the absence of light. Following bacterial lysis using a lysis buffer, the absorbance of the supernatant at 520 nm was spectroscopically measured after centrifugation at 3000 rpm for 5 minutes. The experiment was repeated three times. Fluorescence images of both the control and treated LELE extract groups were captured using a fluorescence microscope.

Live/dead cell assay

The study used the method described by Liu et al., [18], with minor adjustments, to distinguish between viable and non-viable bacteria. The microbial strain 100 µL at 1.5 × 108 CFU/mL were mixed with varying amount of L. speciosa ethanolic extract (25 to 100 µg/mL) in the second value of 50 µL. The combination was then incubated for 30 minutes at 37°C. The control was the bacterial cell that had not been treated. The reaction mixture was centrifuged at 5,000 rpm for five minutes, and the pellet was then cleaned with phosphate-buffered saline (PBS). Following a 30-minute incubation period, the pellets were dyed using a 1:1 mixture of propidium iodide (PI) and acridine orange (AO). The samples were cleaned with PBS after incubation to get rid of extra stain.

Biochemical assays

The bacterial extract was prepared by culturing E. coli, P. aeruginosa, K. pneumoniae, and S. aureus in nutrient broth. The cells were centrifuged to obtain the crude enzyme extract, which was then sonicated and combined with pyrogallol, hydrogen peroxide, and phosphate buffer for POD analysis. The purpurogallin was measured at 420 nm using a microplate reader. The experiment measured superoxide dismutase (SOD) activity using a reaction solution containing PBS, methionine, NBT, EDTA, and riboflavin. The enzyme extract and reaction solution were mixed, heated, and measured for absorbance using an Elisa plate reader. The work assessed membrane damage by assessing malondialdehyde generation using thiobarbituric acid. The enzyme extract was hydrated, centrifuged, and mixed with a thiobarbituric acid reagent. The reaction was heated, centrifuged, and the absorbance measured [19].

III. RESULTS AND DISCUSSION

Result 1. Determination of antibacterial activity

The use of L. speciosa ethanolic green and red extract remains challenging; however, developments in plant extract research have addressed various applications and improved their condition. This study looked at the high antibacterial activity of LELEs as well as their mechanisms. We investigated the bactericidal activity of LELEs against K. pneumoniae, S. aureus, E. coli, and P.aeruginosa. The investigation revealed that LERLEs have strong antibacterial activities (Table 1 and Fig. 3). At 100 µg/mL, LERLE inhibited K.pneumoniae, S. aureus, E. coli, and P. aeruginosa at 17.6 mm, 20.7 mm, 21.8 mm, and 19.7 mm, while LEGLE inhibited K. pneumoniae, S. aureus, E. coli, and P. aeruginosa at 17.4 mm, 19.6 mm, 20.2 mm, and 19.7 mm, respectively (Table 1). At 75 µg/mL, LERLE inhibited K. pneumoniae, S. aureus, E. coli, and P. aeruginosa at 14.9 mm, 15.6 mm, 17.3 mm, and 16.4 mm, while LEGLE inhibited K.pneumoniae, S. aureus, E. coli, and P. aeruginosa at 17.3 mm, 17.3 mm, 18.3 mm, and 15.6 mm (Table 1).

Result 2. Bacterial growth curve analysis

The growth kinetics of K. pneumoniae, S.aureus, E. coli, and P. aeruginosa were also examined as part of the antibacterial investigation of L. speciosa ethanolic plant extracts. With respect to increases in LELE's concentration (25, 50, 75, and 100 µg/mL), bacterial growth was inhibited. The growth of bacteria is limited by LELE's, as seen in Figs. 4 and 5. When exposed to L. speciosa ethanolic green and red plant extracts, the viability of K.pneumoniae, S. aureus, E. coli, and P.aeruginosa is more inhibited.

Result 3. Broth microdilution method

The minimum inhibitory concentration of LELEs needed to impede bacterial growth is determined. LERLE has MICs of 3µg/mL against K.pneumoniae, E. coli, and P. aeruginosa, and 4µg/mL against S. aureus. LEGLE showed MICs of 4µg/mL against K. pneumoniae, S. aureus, E. coli, and P. aeruginosa, as shown in Table 2. LERLEdemonstrated MBCs at 5µg/mL against E. coli, 6µg/mL against K. pneumoniae and P. aeruginosa, and 7µg/mL against S. aureus. Table 2

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shows that LEGLE has MBCs at 5µg/mL against K.pneumoniae, S. aureus, E. coli, and P.aeruginosa. MBC results showed that LELEs successfully suppressed the proliferation of the tested bacteria. L.speciosa green and red ethanolic leaf extracts work synergistically to inhibit bacterial viability.

Result 4. Intracellular ROS measurement

The production of ROS in bacteria using H2DCF-DA. ROS generation in bacterial cells increased when LERLE concentration increased, as shown in Figs. 6 and 7. These findings showed that LERLE might exert antibacterial properties via the generation of ROS against S. aureus, K.pneumoniae, P. aeruginosa, and E. coli. No fluorescence was seen in the control, which shows that ROS was not generated. Fluorescence images show high fluorescent intensities at all L. speciosa green and red ethanolic leaf extract concentrations tested, indicating increased ROS generation in pathogens. Additionally, when reactive oxygen species (ROS) are formed, they interact with the cytoplasmic membrane, peptidoglycan layer, DNA, lipids, proteins, and other physiological processes.

Result 5. Live/dead cell assay

Staining both living and dead cells with AO and PI allowed researchers to observe the morphology of the bacteria in response to varying concentrations of LELEs. Green fluorescence was observed in the control, showing that the bacterial cells were still viable even though PI can only penetrate cells with torn or damaged cell membranes. Strong bactericidal action of synthesised CMC was confirmed by the red coloration of dead bacterial cells at higher concentrations (75 and 100 µg/mL) (Fig. 8 and 9). These results caused a dose-dependent rise in the bactericidal activity of L. speciosa ethanolic leaf extract.

Result 6. Biochemical assays

The study evaluated the impact of ROS formation on the antioxidant system in bacterial cells exposed to LEGLE and LERLE. Results showed increased SOD and POD enzymatic activity, and elevated MDA content in bacterial pathogens, indicating the presence of ROS-scavenging enzymes.

IV. CONCLUSION

More than 60% of people worldwide utilise herbal medications because they are affordable, convenient, and safe. A priceless legacy for emerging nations is medicinal plants. Bacteria can more easily penetrate urinary tract infections due to their chronic nature. An effective way to treat recurring UTIs is to combine herbal remedies. Experimental ethanolic leaf extracts from L.speciosa exhibit potent antimicrobial properties against P.aeruginosa, K. pneumoniae, S. aureus, and E. coli. An increase in the bactericidal activity of LELE's plant extract was found to be dose-dependent, as seen by the red coloration at higher concentrations and green fluorescence indicating live cells. Phytocompounds in herbal therapy stimulate the production of urine, combat germs, and reduce pain, making it a popular treatment for UTIs.

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Fig 1. Antibacterial efficacy of L. speciosaEthanolic Green Leaf Extract against K.pneumoniae, S. aureus, E. coli and P. aeruginosa

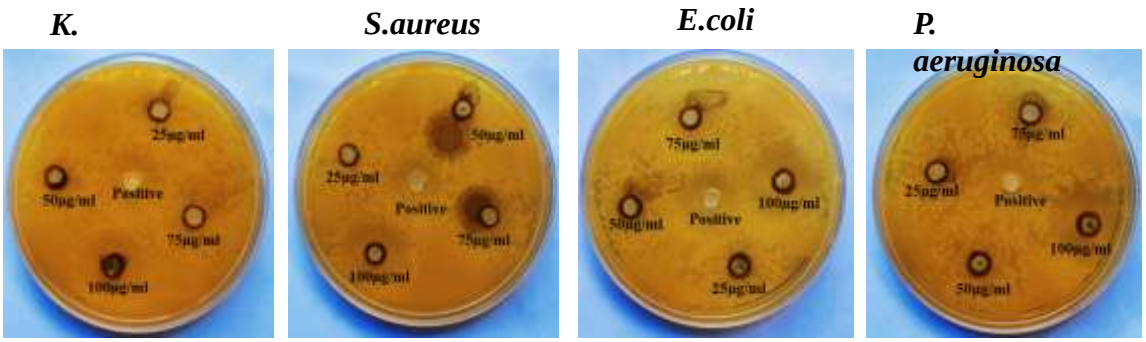

Fig 2. Antibacterial efficacy of L. speciosa Ethanolic Red Leaf Extract against K.pneumoniae, S. aureus, E. coli and P. aeruginosa.

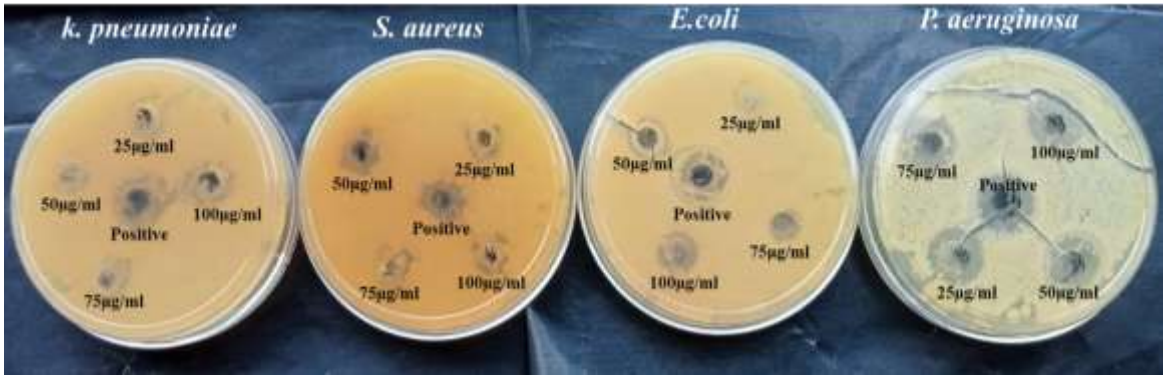

Table 1. Antimicrobial efficacy of L. speciosa ethanolic green and red extract on four microorganism using disk diffusion method

Organisms	E. coli(in mm)		S. aureus(in mm)		K. pneumoniae(in mm)		P. aeruginosa(in mm)	
	LEGLE	LERLE	LEGLE	LERLE	LEGLE	LERLE	LEGLE	LERLE
Control	14.8±1.09	14.9±0.28	14.6±1.21	15.3±0.72	13.1±1.15	13.2±0.31	14.7±0.21	14.7±0.66
25µg/ml	10.7±0.55	11.3±0.53	9.2±0.81	11.7±1.17	8.6±0.57	9.4±0.57	9.1±0.98	9.6±0.97
50µg/ml	13.4±0.77	14.4±1.16	13.8±1.39	14.8±1.15	11.8±1.12	12.3±1.15	13.6±0.57	13.2±1.12
75µg/ml	17.3±1.46	18.3±1.17	15.6±0.57	17.3±1.21	14.9±0.98	15.4±0.17	16.4±1.9	15.6±0.57
100µg/ml	20.2±0.57	21.8±1.15	19.6±1.15	20.7±1.35	17.4±1.68	17.6±1.68	20.4±1.43	19.7±1.29

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Fig 3. Bar graphs represent the zone of inhibition produced by LELE's against pathogens tested

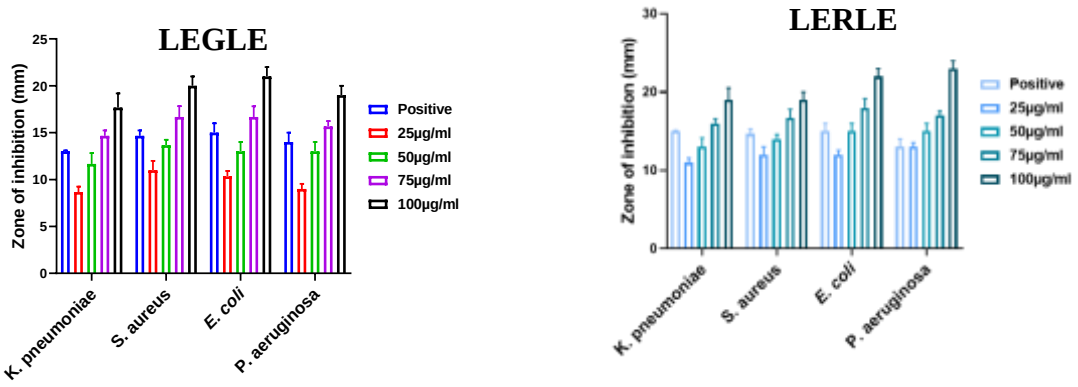

Fig 4. Effect of L. speciosaEthanolic Green Leaf Extract on growth kinetics of K.pneumoniae, S. aureus, E. coli and P. aeruginosa. Data are shown as average of three biological repeats.

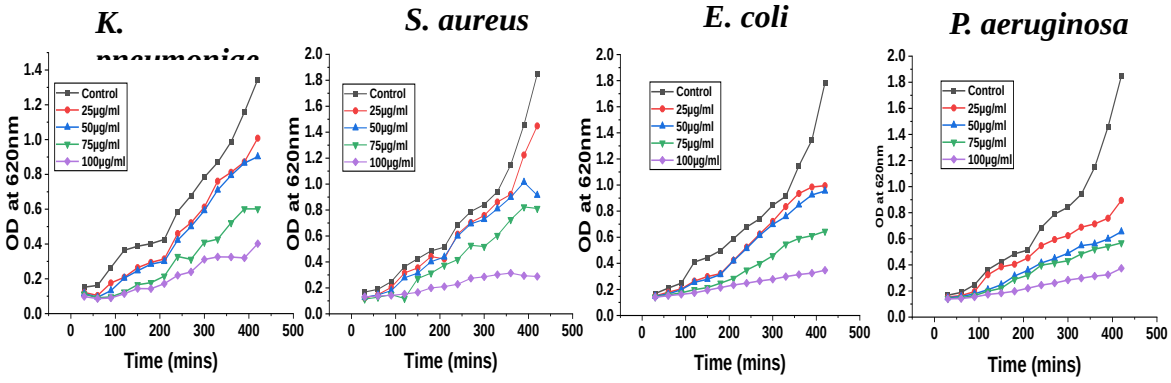

Fig 5. Effect of L. speciosaEthanolic Red Leaf Extract on growth kinetics of K.pneumoniae, S. aureus, E. coli and P. aeruginosa. Data are shown as average of three biological repeats.

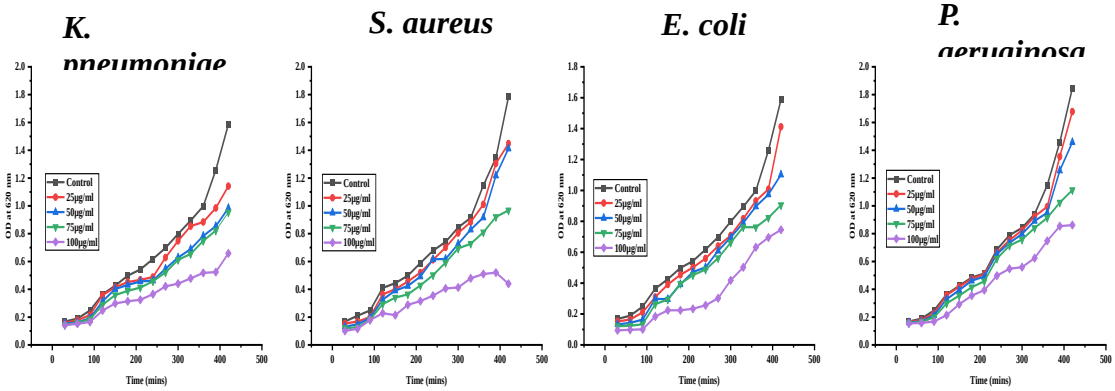

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Table 2. MIC and MBC of L. speciosaEthanolic Red Leaf Extract. Data presented are average values of three replicates.

Plant extract	K. pneumoniae		S. aureus		E. coli		P. aeruginosa	
Test	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
LEGLE	4µg	6µg	4µg	7µg	4µg	5µg	4µg	6µg
LERLE	3µg	5µg	4µg	5µg	3µg	5µg	3µg	5µg

Fig 6. L. speciosa Ethanolic Green Leaf Extractsinduced ROS production in bacterial cells K. pneumoniae, S. aureusP. aeruginosa, and E. coli in a concentration dependent fashion.

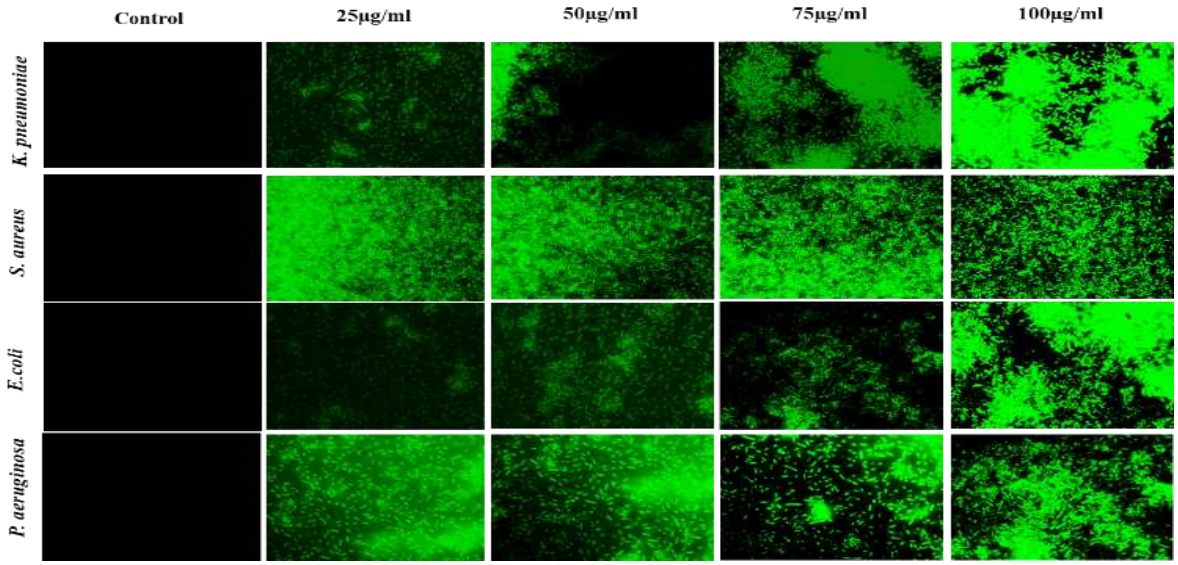

Fig 7. L. speciosa Ethanolic Red Leaf Extractsinduced ROS production in bacterial cells K. pneumoniae, S. aureusP. aeruginosa, and E. coli in a concentration dependent fashion

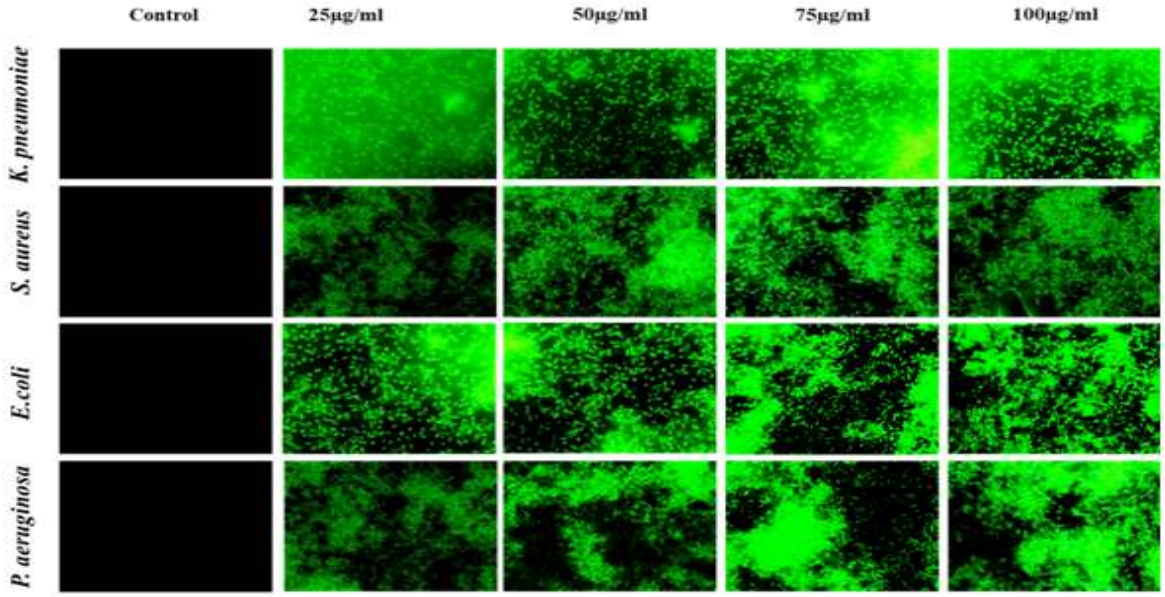

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Fig 8. Fluorescence images depicts the live (green colour) and dead (red colour) bacterial cells K.pneumoniae, S. aureus, P. aeruginosa, and E. coli against L. speciosa Ethanolic Green Leaf Extracts

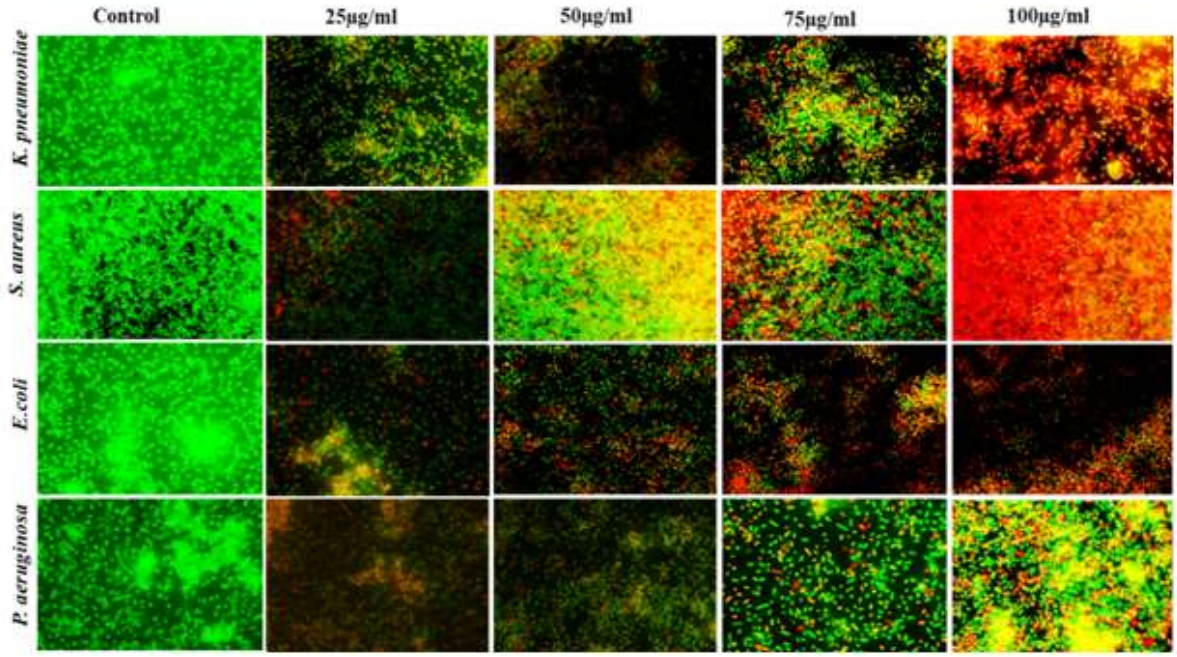

Fig 9. Fluorescence images depicts the live (green colour) and dead (red colour) bacterial cells K. pneumoniae, S. aureus, P. aeruginosa, and E. coli against L. speciosa Ethanolic Red Leaf Extracts

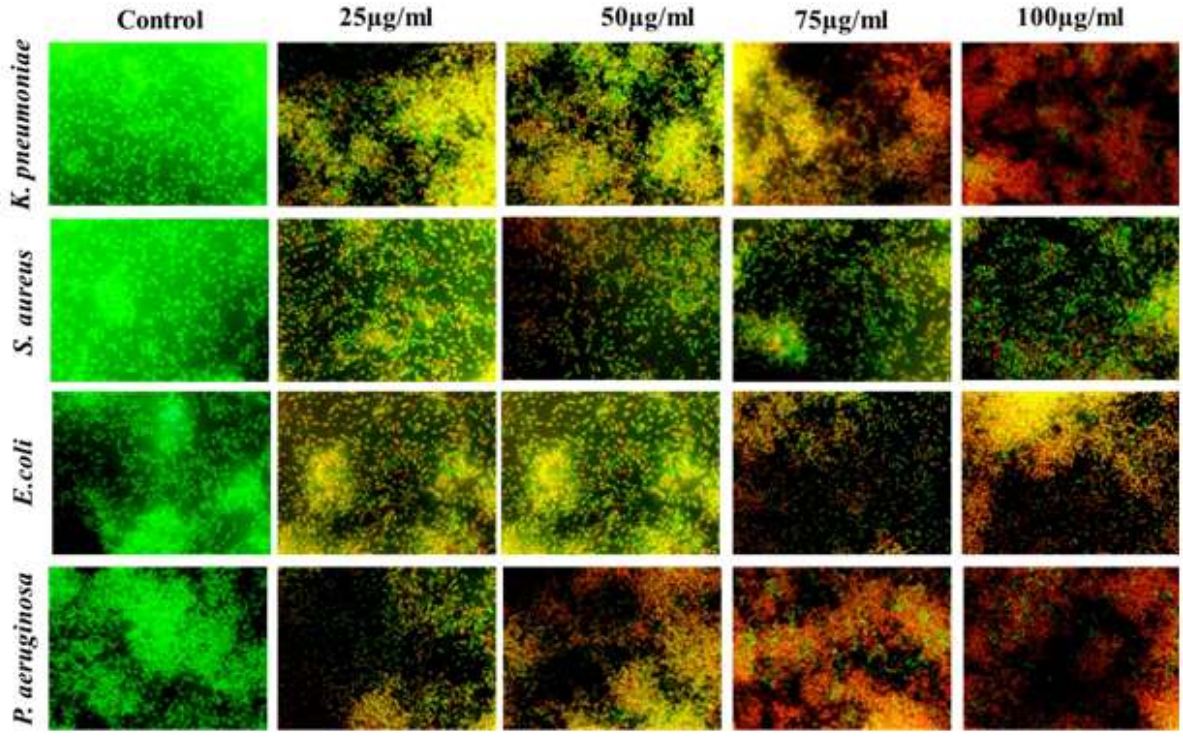

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Fig 10. L. speciosa Ethanolic Green Red Leaf Extract treatment increased the levels of A) Superoxide dismutase, B). Peroxide dismutase, C) MDA assay showed the plant extract at various concentrations cause the membrane damage

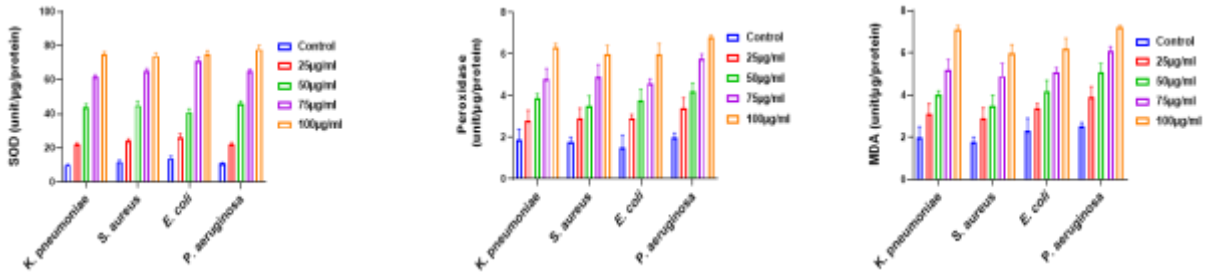

Fig 11. L. speciosa Ethanolic Green Red Leaf Extract treatment increased the levels of A) Superoxide dismutase, B). Peroxide dismutase, C) MDA assay showed the plant extract at various concentrations cause the membrane damage