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Original Article

Estimation of bacterial growth inhibition and antibiofilm activity of Hibiscus *Sabdariffa* plant extracts against standard pathogenic bacteria

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ABSTRACT

Present study was aimed to find the bacterial growth inhibition and antibiofilm activity of different polarity extracts of *Hibiscus sabdariffa* calyx. Dried calyx of *H. sabdariffa* plant were extracted using two different solvents petroleum ether represented organic fraction (OF), furthermore the dried marc have been introduced for second extraction process using (70%) ethanol represented hydrolacholic fraction (HF). Both fraction have been estimated for their efficacy for inhibiting bacterial growth and biofilm formation using macrodilution and modified crystal violet methods respectively. The results showed significant bacterial growth inhibition percentage of *Streptococcus pneumonia* bacteria by hydroalcholic fraction about (93.423±2.913%) while lowest activity exhibited by the organic fraction against similar bacteria (9.22±1.0252 %). Generally greater activity toward gram negative bacteria have been reported for both fractions. Significant antibioflim activity have been expressed by both fraction at (50mg\ml) concentration against which increase in a dose dependent manner. Low concentrations (3.125mg/ml) of both fractions showed promoting bacterial biofilm formation. Both fractions exhibited strong antibacterial and antibiofilm activity toward tested bacterial strains.

Keywords: *Hibiscus sabdariffa*, Bacterial growth inhibition, Biofilm, *Streptococcus pneumonia*

INTRODUCTION

Most of the plants characterized by antimicrobial activity make them important in ethnomedicine [1]. An abundant source for drugs is the nature due to an elevated demand for drugs hazard free for the environments, low side effects and inexpensive [2]. Continuous screening for new antimicrobial agents from nature (plant source) to overcome life threatening problems comprise in various pathogenic bacterial strains exhibit resistance to the allopathic drugs. In view of the fact that usage of both plant derived drugs and plant extracts showed a significant antimicrobial effect playing important role in therapeutic treatment [3-5].

Hibiscus sabdariffa L., family Malvaceae, is an abundant annual crop grown in subtropical and tropical countries [6]. Commonly known as Roselle or sorrel, is an erect annual plant, herbaceous or sub shrubby with smooth typically red stem, growing about 0.5-3 m with red calyx [7-9]. Main active constituents of *Hibiscus sabdariffa* are anthocyanine, flavonoid, alkaloids, saponin, tannin and phenol [10,11]. Roselle is a medicinal plant having manyculinary, traditional and medicinal use. Culinary has been used as drink and pickle, traditionally in folk medicine used as liver diseases, hypertension and fever. Reported medical data of the roselle efficacy were antioxidant, antihypertensive, anticancer, antidiarrheal, antistress, antispasmodic, anticlastrogenic, hypolipidaemic, hepatoprotective and diuretic activities [12-15].

The present study was aimed to find the bacterial inhibition growth and biofilm inhibition percentage of different polarity solvents extracts of *Hibiscus sabdariffa* calyx against four standard bacterial species.

MATERIALS AND METHODS

Plant extracts preparation: Dried powdered *Hibiscus sabdariffa* calyx were introduced for successive extraction process by different polarity solvents using ultrasonic extractor machine following standard procedure as described by Alpuli *et al*, 2009 [16], with slight modification. Two hundred grams of dried powdered were

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extracted using petroleum ether as solvent of extraction [the yield of extraction procedure represents the organic fraction (OF) with the extractive vale 0.44% (w/w)]. The marc was dried and introduced for the second step of extraction using ethanol (70%) as solvent of extraction [the yield of extraction procedure represents the hydro-alcoholic fraction (HF) with extractive value (82% w\w)]. The extracts then concentrated using rotary vapor machine and kept under 4^0C until use for biological analysis.

Biological analysis:

1. Tested Natural products: The two fractions (OF) and (HF) were introduced for biological analysis at concentration of 50 mg/ml. Dimethyl sulfoxide (DMSO) 10% (v/v) and tween-20 20% (v/v) used as diluents for extract fractions respectively.

2. Tested microorganisms: Four standard pathogenic bacteria selected for the antibacterial evaluation: *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 35218), *Staphylococcus aureus* (ATCC 25923), *and Streptococcus pneumoniae* (ATCC 6303). All strains were maintained on nutrient and blood agar slant, then stored at 4 ^{0}C until use. The turbidity of bacterial inoculums adjusted by McFarland standards 0.5 [17].

3. Estimation of bacterial growth inhibition percentage: The percentage of bacterial growth inhibition estimated as described previously by Janssen *et al*, 1987 [18] and Vagi *et al*, 2005 [19], with slight modification. Twofold serial dilutions of plant extract were made in sterile plastic screwed tubes containing 5ml of Mueller–Hinton broth per tube. The tested concentration ranged from (3.375 up to 50) mg/ml for both fractions (OF) and (HF). A 0.25ml of fresh bacterial suspension adjusted with (0.5 McFarland) was added to each tube. Positive control antibiotics azithromycin 30 µg/ml and ampicillin 30 µg/ml and negative control (extract in broth), were included in the study. All inoculated tubes incubated for 24 h at 37 °C. The growth inhibition estimated by measuring the absorbance (A_{450}) using spectrophotometer (Jenmary 6305 UV/visible) according to the following equation:

$$\% \text{ Bacterial growth inhibition} = A_{450 \text{ control positive}} - A_{450 \text{ Extract}} / A_{450 \text{ control positive}} \times 100$$

4. Estimation of activity index: Medicinal plant extract fractions was estimated according to equation described by Singh *et al*, 2002 [20]:

Activity index (AI) = Mean of growth inhibition of extract/ Mean of growth inhibition of standard antibiotic

5. Estimation of antibiofilm activity: A modified crystal violet assay was employed to test the effect of plant extract on biofilm formation as described by O'toole and Kolter, 1998 [21]. Twofold serial dilutions of plant extract were made in sterile screwed plastic tubes containing 5ml of Mueller–Hinton broth per tube. The tested concentration ranged from (3.375 up to 50) mg/ml for (OF) and (HF). A 0.025ml of fresh bacterial suspension adjusted with (0.5 McFarland) was added to each tube. Positive control (bacterial suspension in broth) and negative control (extract in broth), were included. Following incubation at 37 °C for 24 h, the content of each well was gently removed by tapping the plates. The wells were washed with 5ml of sterile distilled water to remove free-floating bacteria. Biofilms formed by adherent cells in tube were stained with 0.1% crystal violet and incubated at the room temperature for 30 minutes. Excess stain was rinsed off thorough washing with distilled water and tubes were fixed with 5ml of ethanol 70%. Absorbance (A_{630}) of stained adherent bacteria was measured using an UV/visible spectrophotometer.

Statistical analysis: All procedures were repeated at least three times and the mean value ± standard deviations were estimated, two ways ANOVA method used for data analysis, considering p value < 0.001 statistically significant in comparison of mean exhibited by the extracts with controls using Graphpad Prism 6 program.

RESULTS

Bacterial growth inhibition: The percentage of bacterial growth inhibition estimated for both OF and HF of *H. sabdariffa* at different concentrations. The highest activity recorded for the HF against *S. pneumonia,* which shows significant activity in comparison with standard antibiotics p value < 0.001. The antibiotics (azithromycin and ampicillin) were also estimated as shown in Table1, Table 2 and Table 3. The comparison among bacterial growth inhibition of variant extract fractions of *H. sabdariffa* and standard antibiotic is shown in Figure 1 and Figure 2.

Activity index (AI): Activity index against two standard antibiotics azithromycin and ampicillin was evaluated at different concentration of both extract fractions (OF and HF). The strongest activity index expressed by the (HF) against azithromycin and ampicillin revealed at 50mg/ml, which decreased with concentrations declining as shown in Figure 3 and Figure 4.

Estimation of antibiofilm activity: Antibiofilm activity of both extract fractions (OF and HF) shown different results in comparison with control

positive. The activity of antibiofilm increased proportionally with increasing the concentrations. Significant value of antibiofilm activity was reported for HF against *S. aureus* in comparison with control positive p value < 0.001. Figure 5 and Figure 6 showed the absorbance of different extract concentrations comparing with standard bacterial suspension as positive control.

DISCUSSION

The results revealed that the two fractions of *H. sabdariffa* have antibacterial activity against various standard bacterial species. Both fractions exhibited a dose dependent manner inhibition percentage which was agreed with findings of Chau and Yin, 2008 [22]. Higher and lower inhibition percentage were recorded against same bacterial species *S. pneumoniae* (93.423±2.913%) and (9.22±1.0252 %) for HF and OF respectively. The highest inhibition percentage expressed by the HF was significantly higher ($p < 0.0001$) than the inhibition percentage, which shown by the standard antibiotics azithromycin and ampicillin against *S. pneumoniae*. These result consistent with the findings of Samuel *et al* 2014 [23] and Hatil *et al* 2006 [24], who described antibacterial activity of plant against *S. aureus, E.coli* (clinical isolated), *P. aeruginosa* (ATCC 27853) and *S. pyogenes* (ATCC 12344).

Generally the *H. sabdariffa* extract fractions showed more activity against gram positive bacterial strains than gram negative, similar results was documented by Samuel *et al*, 2014 [23]. This activity might be due to the nature of bacterial cell membrane in the two strains, which made the gram positive bacteria was more sensitive to chemicals and antibacterial reagents than gram negative bacteria [25, 26]. The activity index against two standard antibiotics confirmed that HF was stronger than OF, the plant antibacterial activity increased with increasing the solvent extraction polarity, the findings were mirror image of the recorded finding of Samuel *et al*, 2014 [23]. The hydroalcholic solvent used for extraction of plant material seemed to be an effective solvent for extraction of antibacterial phytochemicals specially phenolic and flavonoids natural products, which are known for their antibacterial activity and their presence in *H. sabdariffa* plant were recorded in phytochemical investigations from literatures [27-29].

The results of antibiofilm activity showed significant results for both HF and OF in H. *sabdariffa* plant at high concentration (50mg/ml) in comparison with control positive ($p < 0.0001$). On the other hand decreasing the concentrations exhibited decreasing the antibiofilm activity until reach to a point there were an enhancement of biofilm formation, which observed at low concentration (3.125mg/ml) [the absorbance recorded for extract and bacterial suspension was significantly higher than absorbance of control ($p < 0.0001$)]. These observations revealed that phenolic compounds at concentration that did not or weakly inhibit bacterial growth increased biofilm formation [30].

CONCLUSION

The study concluded that a strong antibacterial and antibiofilm activity of different extract fractions of plant against tested standard bacterial species, which open a venue for production of new antimicrobial agents with antibacterial property form two points inhibition of bacterial and their corresponding biofilm which is consider a first step for series infections.

Table 1: The percentage of bacterial growth inhibition of organic fraction (OF) of *H. sabdariffa* against standard bacterial strains

Plant Extract concentration (mg/ml)	Bacterial growth inhibition percentage (mean%±SD) (*n=3*)			
	P.aeruginosa (ATCC27853)	*E. coli* (ATCC 35218)	*S.aureus* (ATCC 25923)	*S.pneumoniae* (ATCC6303)
OF (50 mg/ml)	69.337±0.903	66.283±0.957	72.183±0.878	55.837±0.554
OF (25 mg/ml)	62.753±0.785	60.330±0.781	57.970±0.606	49.353±0.683
OF(12.5 mg/ml)	61.500±0.500	53.690±1.354	53.703±0.5	31.243±1.074
OF(6.25 mg/ml)	24.367±0.874	47.360±1.546	37.147±1.05	26.59±0.639
OF(3.125 mg/ml)	16.390±0.8702	12.607±1.563	29.133±1.05	9.22±1.0252

Table 2: The percentage of bacterial growth inhibition of hydroalcholic fraction (HF) of *H. sabdariffa* against standard bacterial strains

Plant Extract concentration (mg/ml)	Bacterial growth inhibition percentage (mean%±SD) (*n=3*)			
	P.aeruginosa (ATCC27853)	*E. coli* (ATCC 35218)	*S.aureus* (ATCC 25923)	*S.pneumoniae* (ATCC6303)
HF (50 mg/ml)	65.416±1.976	91.580±0.976	79.233±1.779	93.423±2.913
HF (25 mg/ml)	60.296±0.725	90.363±1.163	78.800±3.365	92.433±2.458
HF(12.5 mg/ml)	53.656±0.505	75.200±0.818	58.447±1.578	91.833±1.680
HF(6.25 mg/ml)	49.560±0.612	19.330±0.801	55.330±1.111	65.430±0.997
HF(3.125 mg/ml)	16.606±4.816	18.620±0.680	50.563±1.617	38.617±1.882

Table 3: The percentage of bacterial growth inhibition of antibiotics against standard bacterial strains:

Standard Antibioics(µg/ml)	Bacterial growth inhibition percentage (mean%±SD) (*n=3*)			
	P.aeruginosa (ATCC27853)	*E. coli* (ATCC 35218)	*S.aureus* (ATCC 25923)	*S.pneumoniae* (ATCC6303)
Azithromycin (30 µg/ml)	74.100±1.217	83.630±1.729	85.680±0.845	89.337±1.039
Ampicillin (30 µg/ml)	59.717±0.689	59.610±1.238	66.510±1.0150	78.900±1.299

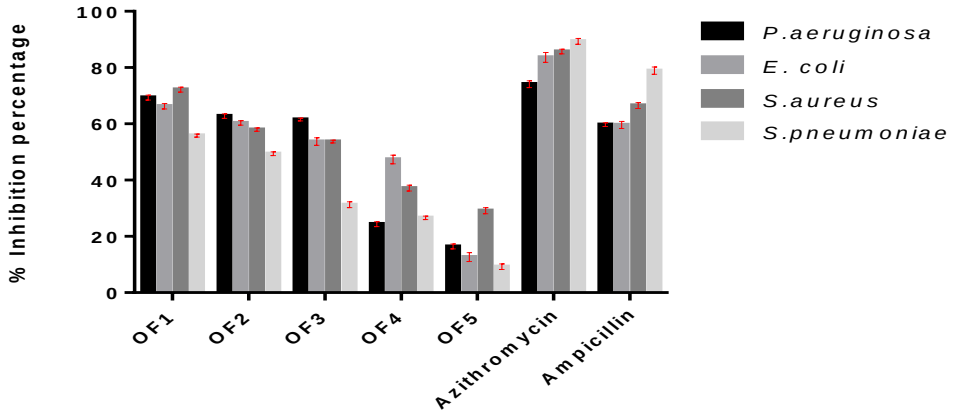

Figure 1: Comparison between inhibition percentage of OF *H. sabdariffa* and standard antibiotics, OF1 stands for (50mg/ml), OF2 stands for (25mg/ml), OF3 stands for (12.5mg/ml), OF4 stands for (6.25mg/ml) and OF5 stands for (3.125mg/ml)

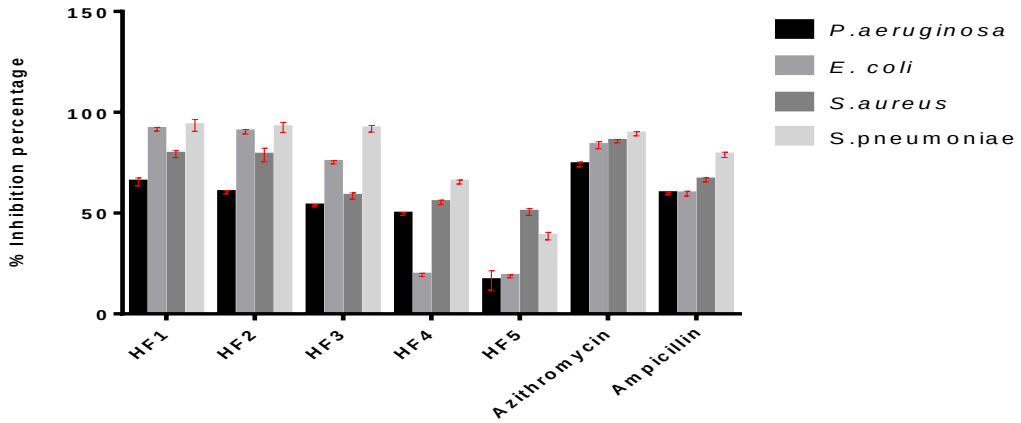

Figure 2: Comparison between inhibition percentage of HF *H. sabdariffa* and standard antibiotics, HF1 stands for (50mg/ml), HF2 stands for (25mg/ml), HF3 stands for (12.5mg/ml), HF4 stands for (6.25mg/ml) and HF5 stands for (3.125mg/ml)

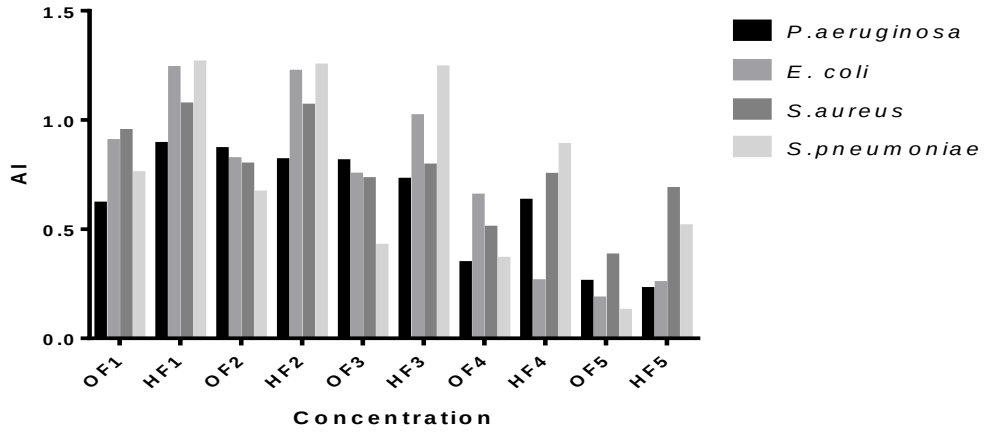

Figure 3: Activity index of different concentration of both extracts (OF and HF) against azithromycin antibiotic, OF1 & HF1 stands for (50mg/ml), OF2 & HF2 stands for (25mg/ml), OF3 & HF3 stands for (12.5mg/ml), OF4 & HF4 stands for (6.25mg/ml) and OF5 & HF5 stands for (3.125mg/ml):

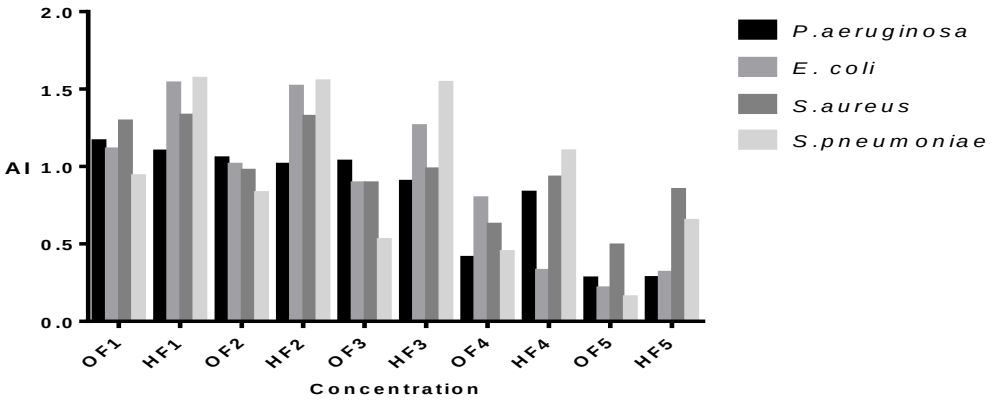

Figure 4: Activity index of different concentration of both extracts (OF and HF) against ampicillin antibiotic, OF1 & HF1 stands for (50mg/ml), OF2 & HF2 stands for (25mg/ml), OF3 & HF3 stands for (12.5mg/ml), OF4 & HF4 stands for (6.25mg/ml) and OF5 & HF5 stands for (3.125mg/ml):

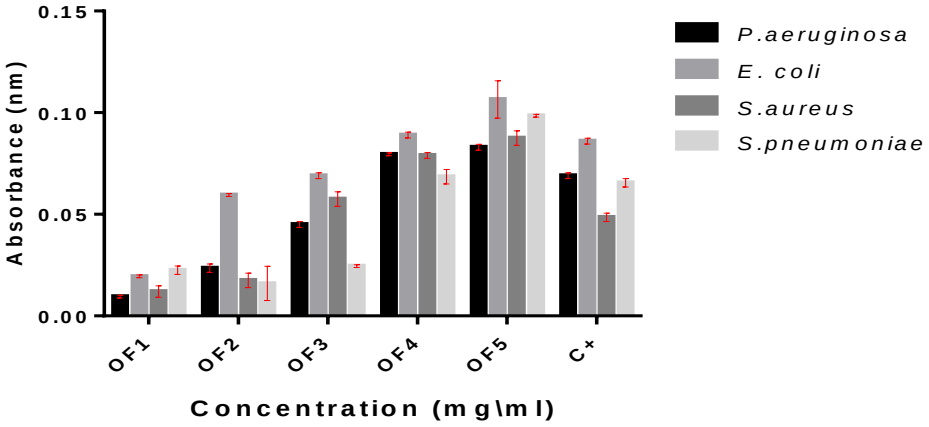

Figure 5: Antibiofilm activity of organic fraction of *H. sabdariffa* OF1 stands for (50mg/ml), OF2 stands for (25mg/ml), OF3 stands for (12.5mg/ml), OF4 stands for (6.25mg/ml), OF5 stands for (3.125mg/ml) and C+ stands for control positive[Broth + Bacterial suspension]:

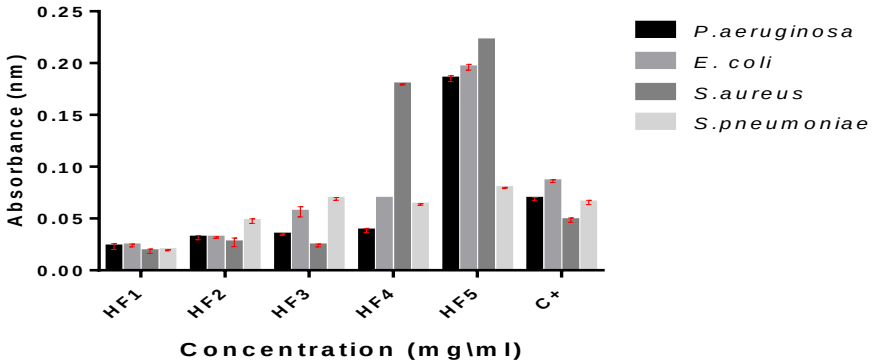

Figure 6: Antibiofilm activity of hydroalcholic fraction of *H. sabdariffa* HF1 stands for (50mg/ml), HF2 stands for (25mg/ml), HF3 stands for (12.5mg/ml), HF4 stands for (6.25mg/ml), HF5 stands for (3.125mg/ml) and C+ stands for control positive[Broth + Bacterial suspension]

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