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Antimicrobial activity of leaves extracted samples from medicinally important *Plumeria obtusa*

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The present research work was carried out to investigate the antimicrobial (8 bacteria and 1 fungus) activities of different solvent (petroleum ether, ethyl acetate, chloroform, iso-butanol and ethanol) extracted samples from leaves of *Plumeria obtusa* by disc diffusion method. The data revealed that petroleum ether, iso-butanol and ethyl acetate fractions showed inhibitory activities against all the nine microbial species except *Klebsiella pneumonia and Pseudomonas aeruginosa*, respectively. The most susceptible gram-positive bacterium was *Bacillus subtilis* while the most resistant gram-positive bacterium was *Staphylococcus aureus*. *Erwinia carotovora* was the most susceptible gram-negative bacterium while *P. aeruginosa* was highly resistant among the gram- negative bacteria.

Key words: Antimicrobial activity, solvents, fungus, disc diffusion, *Plumeria obtusa*.

INTRODUCTION

Increasing resistance of microorganisms against available antimicrobial agents is of major threat to human health and concern among scientists and clinicians world-wide. In general, it is observed that pathogenic viruses, bacteria, fungi and protozoa are becoming more difficult to treat with the available medicines (Koomen et al., 2002). To overcome the side effects of the current antimicrobial medicines, an antimicrobial drug having a novel mode of action need to be developed (Khalafi-Nezhad et al., 2005). Medicinal plant-derived phytochemicals are large group of naturally occurring antibiotics. Recently, a considerable number of medicinal plants have been studied as potential antimicrobial agents (Harborne, 1993; Middleton and Kandaswami, 1986; Cowan, 1999; Kubmarawa et al., 2007; Bakht et al., 2011 a, b, c, d, Bakht and Shafi 2012).

Presently, majority of the pharmaceutically important secondary metabolites are recovered from wild or cultivated plants as their chemical synthesis is not economically feasible (Caldentey and Inze, 2004). Major groups of antimicrobial compounds from plants include simple phenols and phenolic acids, quinones, flavones flavonoids and flavonols, tannins, coumarins, alkaloids, terpenoids and essential oils, lectins and polypeptides, and have shown *in vitro* activity (Cowan, 1999; Zahin et al., 2010). Therefore, such plants should be investigated for better understanding of their properties, safety and efficacy. The use of plant extracts and phytochemicals with known antimicrobial properties are very important in therapeutic treatments. In the last few years, a number of research studies have been conducted to prove such efficiency.

Plumeria belongs to Apocynaceae and is a large family of about 300 genera with more than 1400 species found predominantly in tropics and sub-tropics (Dassanayake and Fosberg, 1983). *Plumeria* is an introduced ornamental

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Table 1. Composition of nutrient agar used for culturing different microbes.

Nutrient agar modified QUELAB QB-39-3504	
Composition	**g l^{-1}**
Beef extract	1
Yeast extract	2
Gelatin extract	5
Sodium chloride	5
Agar	15
Total	28

Table 2. Composition of nutrient broth used for shaking incubation and standardization.

Nutrient broth modified QUELAB QB-39-3504	
Composition	**g l^{-1}**
Gelatin peptone	5
Beef extract	1
Yeast extract	2
Sodium chloride	5
Total	13

plant commonly known as 'Araliya' or temple tree. The flowers are widely used for religious purposes in Sri Lanka. The *Plumeria* plant is mainly grown for its ornamental and fragrant flowers. Species of *Plumeria* include *Plumeria rubra, Plumeria acutifolia, Plumeria obtusa, Plumeria obtusifolia, Plumeria alba, Plumeria bicolor, Plumeria tricolour* and *Plumeria jamesoni.* The bioactive compounds prepared from *P. rubra* have molluscicidal, cytotoxic and anti-bacterial activities. This plant is reported to contain amyrinacetate, mixture of amyrins, ß-sitosterol, scopotetin, the iriddoids isoplumericin, plumieride, plumieride coumerate and plumieride coumerate glucoside (Edward and Watson, 1994). Methanolic extract of its flower has shown antimicrobial activity against *Bacillus anthracis* and *Pseudomonas aeruginosa* (Amani et al., 1998). Objective of the present studies was to investigate the antimicrobial potentials of *Plumeria.*

MATERIALS AND METHODS

Plant materials

The leaves of *P. Obtusa* used in the present study were collected from Khyber Pakhtunkhwa (KPK) Agricultural University, Peshawar Campus and PCSIR Laboratory Complex Peshawar KPK.

Crude extract preparation

Leaves of *P. Obtusa* were first cut into small pieces to make the drying process faster. Cut pieces of flower were kept in a shaded room for a period of 5 to 7 days for drying followed by grinding to obtain dried powder material. The powder were placed in a tank and ethanol added to it till the powdered material was completely dipped in. Shaking of the tank for 7 to 10 days was performed twice a day. The solution (ethanol + extract) were subjected to the rotatory evaporator to separate methanol leaving only semisolid extract solution (crude extract). For further study, crude extract was fractioned with different solvents that is, petroleum ether, ethyl acetate, chloroform, iso-butanol and water.

Fractionation of crude extract

The prepared crude extract was divided into two portions; One portion (10 g) was poured into the glass vials to be tested as crude methanol extract for antimicrobial activity while the second portion (100 g) was fractionated with different solvents. The second portion was dissolved in water, poured into a separatory funnel and distilled petroleum ether was added to it. Compounds soluble in the upper petroleum ether phase were collected and the lower aqueous phase was re-extracted thrice with petroleum ether. All fractions of petroleum ether were combined and petroleum ether was isolated from the fraction leaving behind semisolid petroleum ether fraction through rotary evaporator. The semisolid petroleum ether fraction was dried at 45°C in a water bath and was stored in the glass vials until used. The same process of fractionation was carried out for petroleum ether, ethyl acetate, cholroform and iso-butanol, respectively resulting in petroleum ether, ethyl acetate, cholroform and iso-butanol. The lower aqueous phase at the end of the process was taken and dried via rotary evaporator and water bath.

Culture media

Nutrient agar media was used for the culturing and growth of all microorganisms used in the present study. Nutrient broth was used for shaking incubation and standardization of these microorganisms (Tables 1 and 2).

Preparation of media

The required quantities of nutrient agar (2.8 gl^{-1}) and nutrient broth (1.3 gl^{-1}) media were prepared in distilled water and poured into conical flasks. Some of the nutrient broth (approximately 20 ml test $tube^{-1}$) was also poured into the test tubes. All the media flasks and test tubes were plugged with cotton wool and then sterilized in an autoclave at 1.5 pounds pressure and 121°C for 15 min. After sterilization, nutrient agar media was poured aseptically into sterilized Petri plates in a laminar flow hood. A sterile environment was maintained during pouring to avoid contamination. The media was allowed to became solid in Petri plates for about 1 h and then placed in inverted position (to avoid evaporation of water from the media within the plates) in an incubator at 37°C for 24 h. After 24 h, uncontaminated plates were used for culturing of bacteria and fungi. The nutrient broth in flasks (approximately 20 ml $flask^{-1}$) were used for shaking incubation of microorganisms while nutrient broth in test tubes were used for standardization of microbial cultures.

Microorganisms tested

Antimicrobial activity of different solvent extracted samples were tested against different bacterial and fungal strains (Table 3).

All the microbial stock cultures were freshened by sterile inoculation loop on nutrient agar media plates in a laminar flow hood and incubated at 37°C for 24 h. The next day, the streaked cultures were again subcultured on media plates and incubated at 37°C for 24 h. The second streaked cultures were then inoculated into the nutrient broth in flasks and subjected to shaking incubation

Table 3. Microbial strains tested in the present study.

Bacterial strain	Gram strain type	Details of the microbial strains used
Bacillus cereus	Positive	Clinical isolate obtained from Microbiology Laboratory Quaid-I-Azam University Islamabad Pakistan
P. aeruginosa	Negative	ATCC # 9721
C. albicans	Fungus	Clinical isolate obtained from Hayatabad Medical Complex Peshawar Pakistan
E. carotovora	Negative	Plant Pathology Department of KPK Agricultural University Peshawar Pakistan
E. coli	Negative	ATCC # 25922
K. pneumoniae	Negative	Clinical isolate obtained form Microbiology Llaboratory Quaid-I-Azam University Islamabad Pakistan
S. typhi	Negative	Clinical isolate obtained from Microbiology Llaboratory Quaid-I-Azam University Islamabad Pakistan
S. aureus	Positive	ATCC # 6538

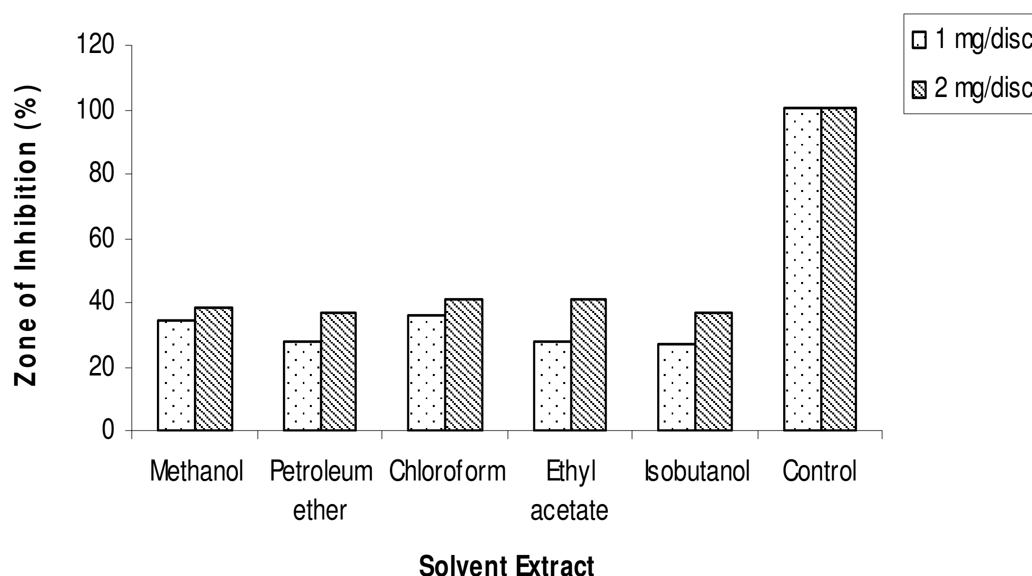

Figure 1. Antimicrobial activity of methanol, petroleum ether, chloroform, ethyl acetate and iso-butanol extracted samples from *P. obtusa* against *P. aeruginosa* by disc diffusion assay.

for 18 h at 37°C (200 rpm).

Disc diffusion susceptibility method

Nutrient agar media plates were seeded with 18 to 24 h cultures of microbial inoculums (a standardized inoculums 1 to 2×10^7 CFU ml^{-1} 0.5 McFarland Standard). Whatman No. 1 filter paper discs (6 mm in diameter) were placed with the help of a sterile forceps on the media and then plant extracts in concentrations of 1 and 2 mg $disc^{-1}$ in 6, and 12 µl volumes were applied on the discs. Antibiotics (6 µl $disc^{-1}$) as positive control and dimethyl sulfoxide (DMSO; 6 µl $disc^{-1}$) as negative control were also applied on the discs. Inoculated plates were then incubated at 37°C for 18 to 24 h. After 24 h, zones of inhibition were recorded in mm around the discs in each plate.

Positive controls

For gram-positive bacteria, Azithromycin 50 µg 6 $µl^{-1}$; For gram-negative bacteria, Ciprofloxacin 30 µg 6 $µl^{-1}$; for *Candida albicans*, Clotrimazole 50 µg 6 $µl^{-1}$.

RESULTS AND DISCUSSION

Figure 1 shows the antibacterial activities of ethanol, petroleum ether, chloroform, ethyl acetate and iso-butanol extracted samples from leaves of *Plumeria obtusa* against *P. aeuriginosa* using the disc diffusion susceptibility assay. It is clear from the data that iso-butanol, ethanol and petroleum ether extracted samples inhibited the growth of *P. aeuriginosa* at both concentrations. However, chloroform and ethyl acetate extracted samples were more effective in inhibiting the growth of *P. aeuriginosa* at high concentration (that is, 41% at 2 mg $disc^{-1}$) when compared with iso-butanol and petroleum ether extracted sample where inhibition was 37% at the same concentration (2 mg $disc^{-1}$). The data further indicated that *Bacillus subtilis* was susceptible to

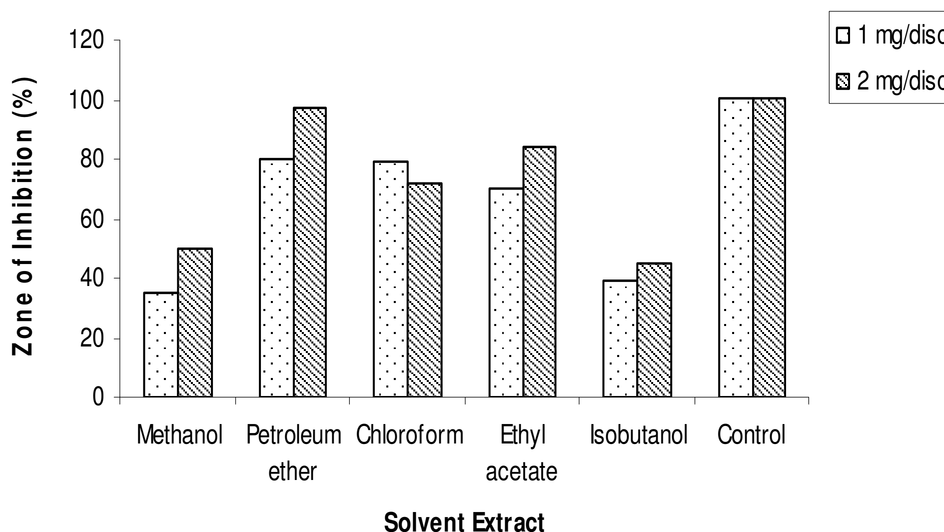

Figure 2. Antimicrobial activity of methanol, petroleum ether, chloroform, ethyl acetate and iso-butanol extracted samples from *P. obtusa* against *B. subtilis* by disc diffusion assay.

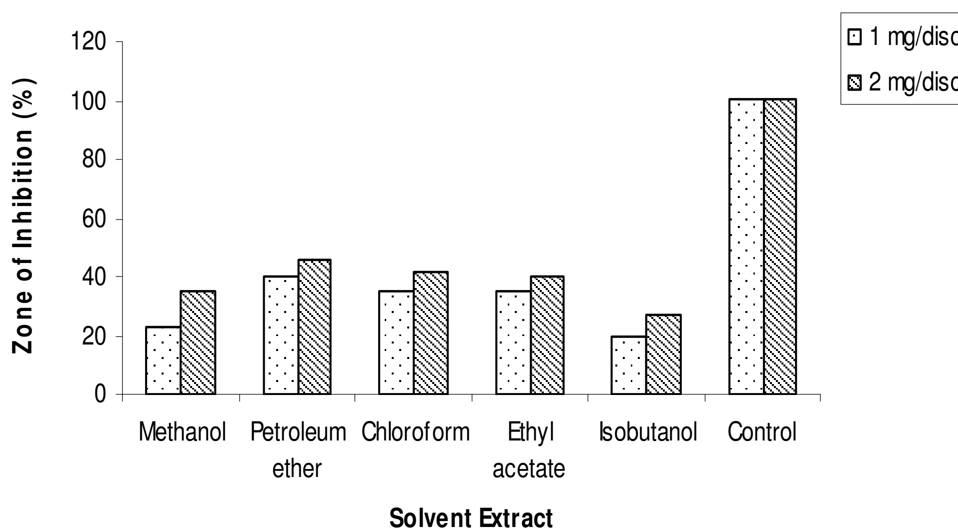

Figure 3. Antimicrobial activity of methanol, petroleum ether, chloroform, ethyl acetate and iso-butanol extracted samples from *P. obtusa* against *C. albicans* by disc diffusion assay.

all solvents extracted samples from *P. obtusa*. Chloroform and ethyl acetate extracted samples inhibited the growth of *B. subtilis* at both concentrations. Petroleum ether recorded maximum inhibitions of 80 and 97% at 1 and 2 mg disc^{-1}, respectively. Minimum reduction in the growth of *B. subtilis* was noted for ethanol and iso-butanol extracted samples when compared with other samples activity (Figure 2). These results agree with those reported by Bhagel et al. (2010).

Figure 3 shows the antifungal activity of ethanol, petroleum ether, chloroform, ethyl acetate and isobutanol extracted samples against *C. albicans*. Petroleum ether, ethyl acetate and chloroform extracted samples were more effective in controlling the growth of *C. albicans* when compared with positive (clotrimazole) and negative (DMSO) controls. Petroleum ether, ethyl acetate and chloroform recorded inhibitions of (40 and 46%), (35 and 40%) and (35 and 42%) at 1 and 2 mg disc^{-1}, respectively compared with other solvents. *Erwinia carotovora* was susceptible to all solvents extracted samples from leaves of *P. obtusa* measured by disc diffusion method. Ethanol, chloroform, ethyl acetate and iso-butanol extracted samples were effective in inhibiting the growth of *E. carotovora* at both concentrations (Figure 4). The degree

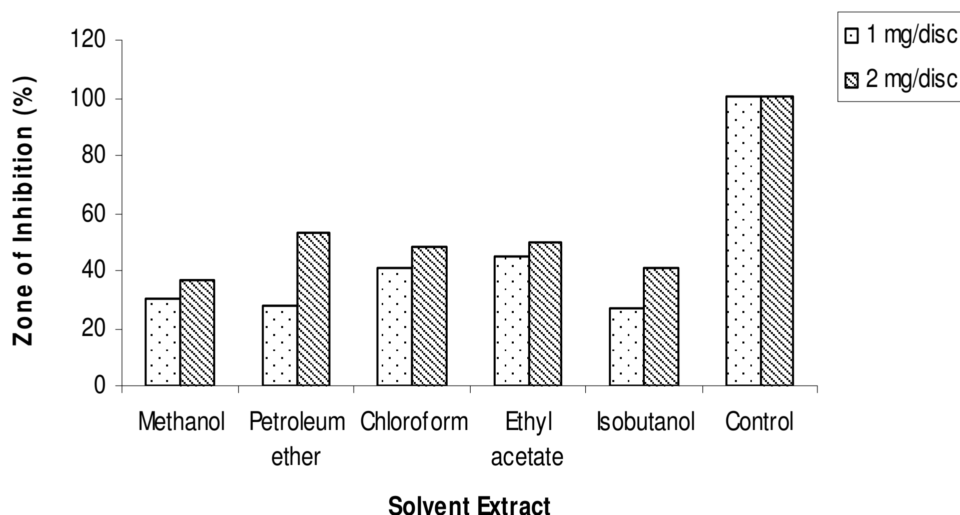

Figure 4. Antimicrobial activity of methanol, petroleum ether, chloroform, ethyl acetate and iso-butanol extracted samples from *P. obtusa* against *E. carotovora* by disc diffusion assay.

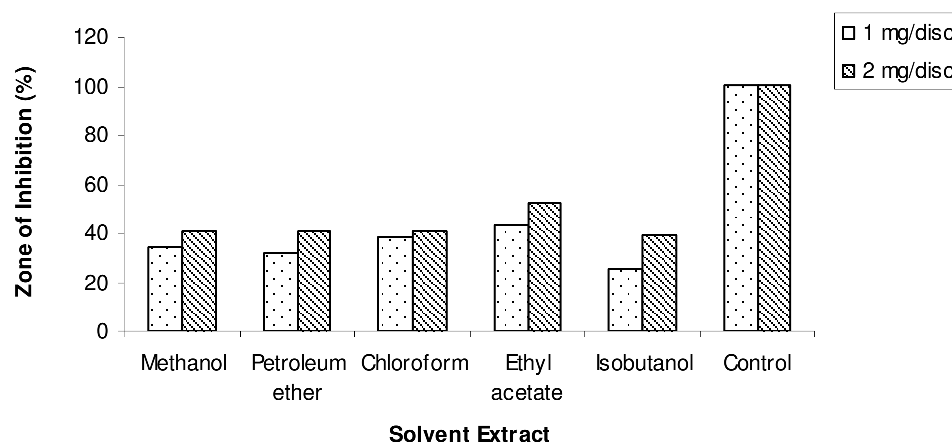

Figure 5. Antimicrobial activity of methanol, petroleum ether, chloroform, ethyl acetate and iso-butanol extracted samples from *P. obtusa* against *E. coli* by disc diffusion assay.

of inhibition was higher at increased concentrations of the samples. Petroleum ether extracted sample showed highest inhibition in the growth of *E. carotovora* that is, 53% at 2 mg disc^{-1} concentration. These results agree with those reported by Sulaiman et al. (2010).

The data shown in Figure 5 suggested that ethyl acetate extracted samples showed maximum inhibition of *Escherichia coli* growth compared with ethanol, petroleum ether, iso-butanol and chloroform. The percent zones of inhibition by ethyl acetate fraction against *E. coli* were 43 and 52% at 1 and 2 mg disc^{-1} concentrations respectively. Minimum reduction in the growth of *E. coli* was noted for iso-butanol extracted samples at 1 mg disc^{-1} concentration. *Klebsiella pneumoniae* was susceptible to ethanol, ethyl acetate, iso-butanol and chloroform extracted samples. These four extracts effectively inhibited the growth of *K. pneumoniae*. Percent inhibition of these samples increased with increasing concentration. The data suggested that inhibitions by ethyl acetate extracted sample were 37 and 47%, while for ethanol extracted samples, the percentages of inhibition were 37 and 50% at 1 and 2 mg disc^{-1} concentration, respectively. Petroleum ether did not inhibit the growth of *K. pneumoniae* at any concentration used and recorded 0% inhibition (Figure 6). Similar results were also reported by Sulaiman et al. (2010).

Analysis of the data also showed that all the solvents extracted samples had inhibitory activities against *Bacillus atrophaeus*. Ethyl acetate and petroleum ether extracted samples had the highest inhibitory activities

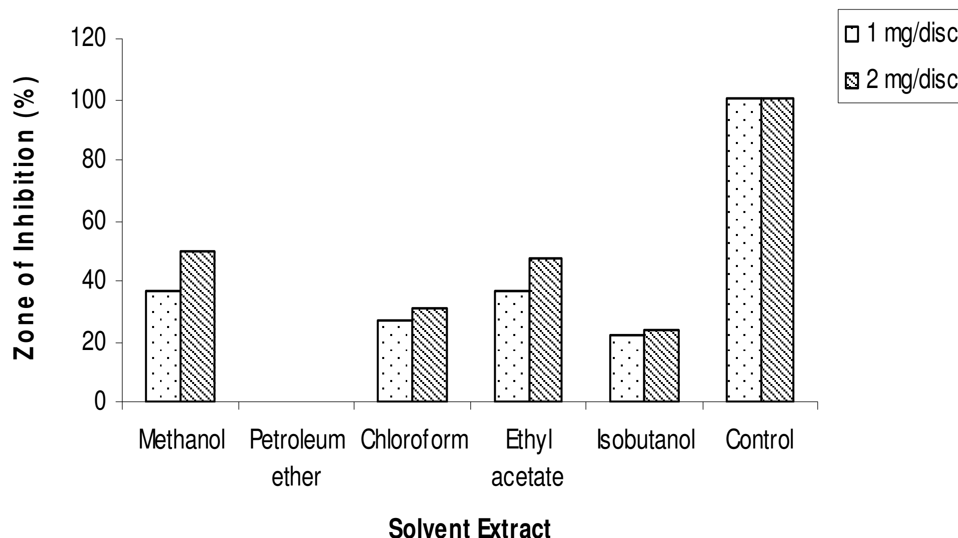

Figure 6. Antimicrobial activity of methanol, petroleum ether, chloroform, ethyl acetate and iso-butanol extracted samples from *P. obtusa* against *K. pneumoniae* by disc diffusion assay.

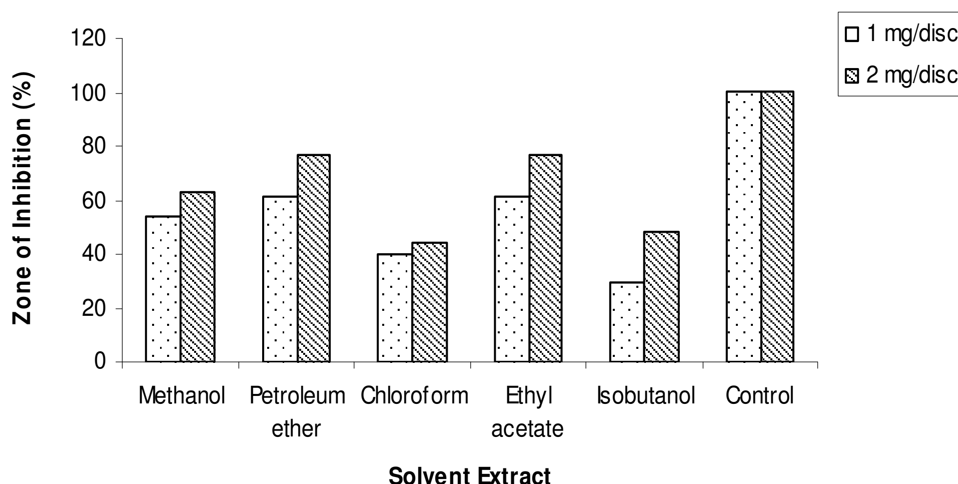

Figure 7. Antimicrobial activity of methanol, petroleum ether, chloroform, ethyl acetate and iso-butanol extracted samples from *P. obtusa* against *B. atrophaeus* by disc diffusion assay.

causing 61% of inhibition at 1 mg disc^{-1} and 77% at 2 mg disc^{-1} concentration followed by ethanol extracted samples that is, 54 and 63% inhibitions at 1 and 2 mg disc^{-1} concentrations, respectively. Lowest inhibitory activity against this bacterium was shown by chloroform extracted sample (Figure 7). Chloroform and petroleum ether extracted samples were highly effective to control the growth of *Salmonella typhi* when compared to the rest of the extracts (Figure 8). Chloroform and petroleum ether extracted samples recorded inhibitions of 42, 70% and 36, 60% at 1 and 2 mg disc^{-1}, respectively. The data further showed that ethanol, ethyl acetate and iso-butanol extracts inhibited the growth of *S.* Typhi to a limited extent that is, iso-butanol extracted samples inhibited the growth of *S.* Typhi by 32 and 42% at 1 and 2 mg disc^{-1}, respectively. It is clear from the data shown in Figure 9 that iso-butanol extracted samples was less effective in inhibiting the growth of *Staphylococcus aureus* when compared with their controls. Petroleum ether and ethyl acetate extracted samples showed varying degree of inhibitory activity. Petroleum ether extracted samples inhibited the growth of *S. aureus* by 26 and 33% at 1 and 2 mg disc^{-1} concentration, respectively. Chloroform extracted samples were more effective compared to the other extracts in inhibiting the growth of *S. aureus* that is, 36 and 45% at 1 and 2 mg disc^{-1} concentration, respectively.

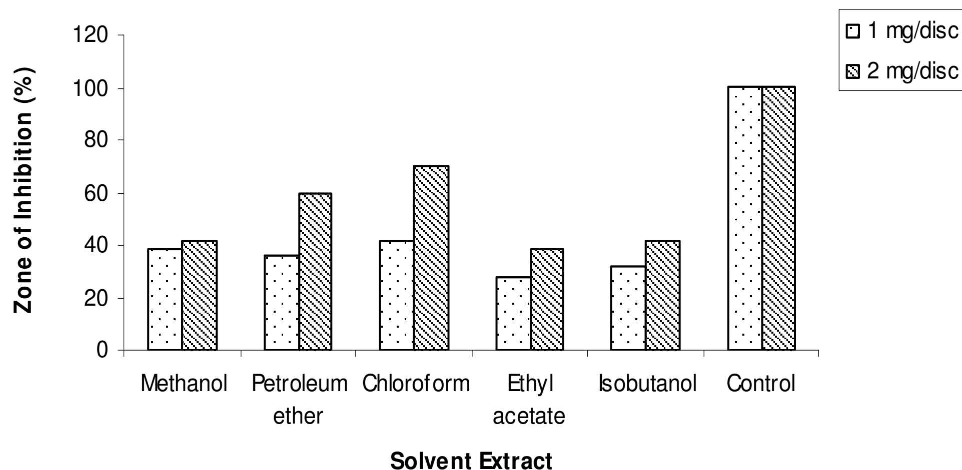

Figure 8. Antimicrobial activity of methanol, petroleum ether, chloroform, ethyl acetate and iso-butanol extracted samples from *P. obtusa* against *S. typhi* by disc diffusion assay.

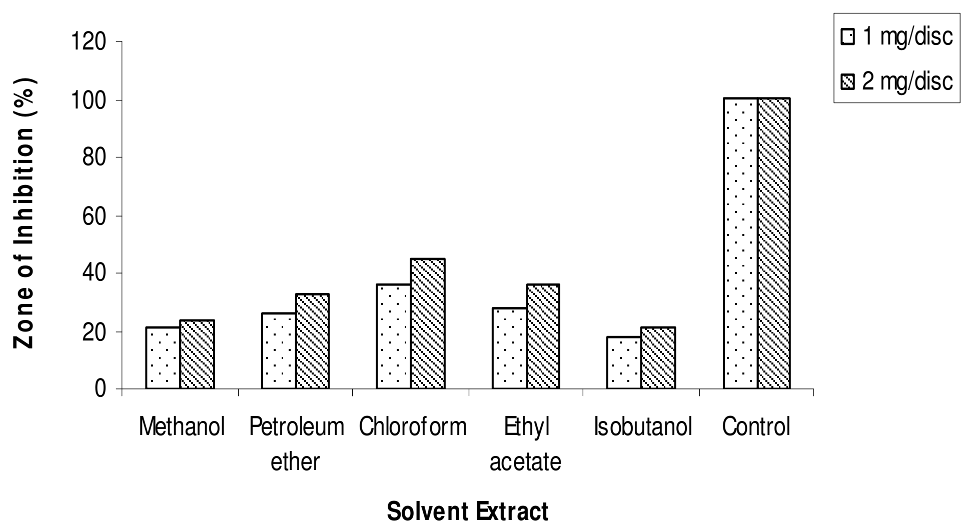

Figure 9. Antimicrobial activity of methanol, petroleum ether, chloroform, ethyl acetate and iso-butanol extracted samples from *P. obtusa* against *S. aureus* by disc diffusion assay.

respectively. Analysis of the data further revealed that crude extracts inhibited the growth of *S. aureus* by 21 and 24% at 1 and 2 mg $disc^{-1}$ concentration, respectively.

Conclusion

Different extracts from the leaves of *P. obtusa* showed antimicrobial activities against different microbes. Most of the antimicrobial compounds of *P. obtusa* were soluble in iso-butanol and ethyl acetate when compared with other solvents. Besides, chloroform and petroleum ether extracted samples showed effective antibacterial and antifungal activity suggesting a potential use of this plant. The antimicrobial compounds present in *P. obtusa* may serve as an affordable and newer source for the treatment of infectious diseases.

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