

Screening of leaves extracts from *Calamus aromaticus* for their antimicrobial activity by disc diffusion assay

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Abstract: The current research investigates the anti-microbial activities of different solvent extracted samples from the leaves of *Calamus aromaticus* against Gram positive, Gram negative bacteria and fungi using 500, 1000 and 2000 µg disc⁻¹ concentrations. *Escherichia coli*, *Citrobacter freundii* and *Candida albicans* showed resistivity to crude methanolic extract and the same microbes were more susceptible to water extracted fractions. Maximum activity was measured by hexane extracted fractions against *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Xanthomonas campestris* and minimum growth inhibition by water extracted fractions. Maximum growth of *Klebsiella pneumoniae* and *Staphylococcus aureus* was measured by ethyl acetate fraction. Majority of the tested microbes were resistant to water and butanol extracted fractions. *Staphylococcus aureus* revealed maximum susceptibility among gram positive bacteria and *Bacillus subtilis* showed minimum. Among Gram negative bacteria, *Citrobacter freundii* was more susceptible while *Xanthomonas campestris* revealed resistively.

Keywords: Antibacterial, antifungal activity, *Calamus aromaticus*, disc diffusion assay,

INTRODUCTION

According to the definition of World Health Organization (WHO), traditional medicine is "the sum total of all the knowledge and practices, whether explicable or not, used in diagnosis, prevention and elimination of physical, mental or social imbalance and based on practical experience and observation handed down from generation to generation, whether verbally or in writing" (WHO, 2005). Herbs are used as medicine in the treatment of many diseases since ancient times (Holm *et al.*, 1998). Nearly 70-80% people all over the world depend primarily on traditional, particularly herbal, medicines to meet their primary health care needs (Srivastava *et al.*, 1995). Out of total traditional medicine preparations used, roughly 85% involve the use of plants or plant extracts (Vieira and Skorupa, 1993). Moreover, medicinal plants are used as raw material in the preparation of many modern medicines because of easy and cheap availability, virtually no side effects, no environmental hazards and lasting therapeutic property. The use of medicinal plants is also rampant in the manufacture of cosmetics and as aromas in many perfumes. There are some 20,000 plant species on the WHO's list of plants used for medicinal purposes all over the world. The estimated number of plants used for medicinal purpose ranges from 35,000 to 70,000 worldwide, according to some other reports. In Pakistan, most of the people depend on medicinal plants to find treatment for their minor, even in some cases major diseases (Shinwari, 2010). Among 6000 plant species in Pakistan, 700 are considered medicinally

important (Shinwari and Qaiser, 2011; Bakht *et al.*, 2011 a, b, c and d; 2012; 2013 a,b; 2014 a, b,c; 2015; Nasir *et al.*, 2015; Ullah *et al.*, 2015; Zakir *et al.*, 2015; Chaun *et al.*, 2015; Bilal *et al.*, 2016; Wajid *et al.*, 2016 a, b; Amjad *et al.*, 2016; Anwar *et al.*, 2016). In addition to a peculiar local market system known as "Pansara" which deals specifically with the trade of medicinal plants in Pakistan, several plants are exported. Often, certain plant species have recognized activity against a particular illness but occasionally they have mixed usage.

Calamus aromaticus generally known as Sweet Flag, is a member of the family Araceae (Adoraceae). There are about 110 genera and more than 1,800 species in the family Araceae (Raja *et al.*, 2009). The plants included in this family are rhizomatous or tuberous herbs. *Calamus aromaticus* is a perennial herb commonly found on the banks of streams and in damp marshy places. The people of Asia were aware of the beneficial and medicinal virtues of *Calamus aromaticus* and the plant is grown and harvested on the commercial scale since ancient times (Singh *et al.*, 2011). In Pakistan, it is almost confined to District Swat in Khyber Pakhtunkhwa Province where it is found as a weed on the banks of streams and in marshy places. The local people identify the plant by the name of "Skha Waja". *Calamus aromaticus* is distributed both in temperate and sub-temperate zones. It is a semi aquatic plant with a distinct smell. It can attain a height of up to six feet with leaves resembling the shape of a sword, small flowers which are yellow/green in color and branched rhizomes. Several important biological activities such as antifungal (Lee *et al.*, 2004; Lee, 2007), antibacterial (McGraw *et al.*, 2002; Phongpaichit *et al.*,

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2005), allopathic (Nawamaki and Kuroyanagi; 1996), anticellular and immunosuppressive (Mehrotra *et al.*, 2003) have been attributed to the rhizomes, roots and essential oil extracted from these plant parts.

MATERIALS AND METHODS

Plant material

The present study was conducted at the Institute of Biotechnology and Genetic Engineering, The University of Agriculture, Peshawar, Pakistan. Three kg of fresh plant material was collected from different localities of District Swat, KPK Pakistan. The plant specimen was identified by plant taxonomist Prof. Furrakh Hussain, Chairman of Botany University of Peshawar. After thorough washing with running tap water, the plant materials were chopped, shade dried and grounded in electric grinder.

Crude extract preparation

One thousand grams of powdered materials were soaked in five liters of methanol, kept at 25°C in the dark for one week and agitated three times a day. The mixture was filtered through Whatman filter paper No.1. The residue was mixed with twenty five hundred ml fresh methanol and the whole procedures were repeated thrice. All fractions of the filtered methanolic solution were dried at 45°C under vacuum pressure using rotary evaporator. The crude extract was divided into two portions, one to be used as crude extract and the other part was fractionated with different solvents.

Fractionation of crude extract

Sixty grams of the crude extract was dissolved in 500ml sterile distilled water, mixed with 300ml n-hexane, shaken gently and allowed to stand for 15minutes to separate the two phases. The upper n-hexane phase was obtained the lower aqueous phase was re-extracted three times with fresh n-hexane. All fractions of n-hexane were pooled together, dried at 45°C under vacuum pressure with rotary evaporator. The same procedures of fractionation were carried out for ethyl acetate and butanol. The lower aqueous phase at the end of the procedure was dried as described previously (fig. 1).

Preparation of media

Nutrient broth was used for shaking incubation and standardization and nutrient agar medium for the culturing and growth of all microorganisms. The known quantities of nutrient agar and nutrient broth were poured into conical flasks. Twenty ml per test tube of the nutrient broth was also poured. All the media flasks and test tubes were sterilized, poured aseptically into sterilized petri plates and allowed to solidify for about an hour. After 24 hrs, uncontaminated plates were used for culturing of bacteria and fungi.

Disc diffusion susceptibility assay

The antibacterial activity of different solvent extracted samples from the leaves of *Calamus aromaticus* was

carried by disc diffusion assay as described in Bauer *et al.* (1966) and antifungal activity by Ramdas *et al.* (1998) against different bacterial and fungal strains (tables 1 and 2). Nutrient agar media plates were inoculated with 18-24 hrs cultures of microbial inoculums (a standardized inoculums $1-2 \times 10^7$ CFU/ml-1 0.5 McFarland Standard). Three discs of Whatman No. 1 filter paper (6 mm in diameter) were placed on the media in petri plates with the help of a sterile forceps. Plant extracts in concentration of 500, 1000 and 2000µg in 4, 8 and 12µl volumes were applied on the discs. Antibiotics as positive control and DMSO (12µl disc⁻¹) as negative control were also applied on the discs in separate petri plates. Inoculated plates were kept at 37°C for 18-24 hrs. The next day zones of inhibition were recorded in mm around the discs in each plate.

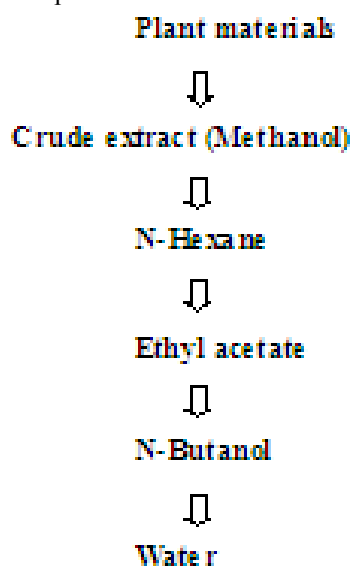


Fig. 1: Flow chart showing crude extracts preparation and different fractions by various solvents.

Positive controls

For Gram-positive bacteria; Ciprofloxacin 50µg per 12µl
 For Gram negative-bacteria; Ciprofloxacin 50µg per 12µl
 For Fungal strain; Fluconazole 50µg per 12µl

STATISTICAL ANALYSIS

Data are presented as mean values of three replicates. MSTATC computer software was used to carry out statistical analysis (Russel and Eisensmith, 1983). The significant difference among means was compared using Least Significant Difference (LSD) test (Steel *et al.*, 1997).

RESULTS

The present study investigates the antibacterial and antifungal potential of different solvent extracted samples from the leaves of *Calamus aromaticus*. Seven bacteria (gram positive and gram negative) and five fungal strains

were tested against these extracts. The antibacterial activity of different extracts from the leaves against *Klebsiella pneumoniae* is shown in fig. 2. The crude extract and all fractions exhibited activity at all concentrations against the tested organism, except water extracted fraction which showed no activity at 500 $\mu\text{g disc}^{-1}$. The tested organism showed high susceptibility to methanol, n-hexane, ethyl acetate and butanol extracted samples and moderate susceptibility to water-extracted fraction. The highest ZI (50%) was measured by ethyl acetate extracted fraction at concentration of 2000 $\mu\text{g disc}^{-1}$ and the lowest by butanol extracted samples (25.57% ZI) at concentration of 500 $\mu\text{g disc}^{-1}$. However, water-extracted fraction showed no activity at 500 $\mu\text{g disc}^{-1}$ concentration when compared with controls (fig. 2).

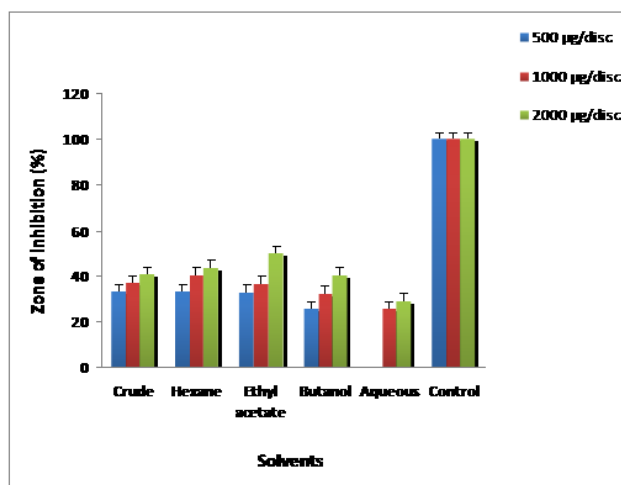


Fig. 2: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, butanol and water extracted samples from the leaves of *Acorus calamus* against *Klebsiella pneumoniae* by disc diffusion assay.

Fig. 3 shows the effect of different extracts from the leaves of *Calamus aromaticus* on the growth of *Pseudomonas aeruginosa*. Among different extracts, n-hexane-extracted fraction was more effective with 44.0%, 55.66% and 68.16% ZIs at concentrations of 500, 1000 and 2000 $\mu\text{g disc}^{-1}$ respectively followed by methanol, ethyl acetate and butanol extracted fractions. Water-extracted fraction was less effective showing no activity at concentration of 500 $\mu\text{g disc}^{-1}$ (fig. 3).

Our results also revealed that crude methanolic extracts and different fractions showed activity against *S. aureus* (fig. 4). Ethyl acetate-extracted fraction measured maximum growth inhibition of the tested organism (33.33%, 43.86% and 54.10% ZIs at concentrations of 500 $\mu\text{g disc}^{-1}$, 1000 $\mu\text{g disc}^{-1}$ and 2000 $\mu\text{g disc}^{-1}$ respectively) followed by n-hexane-extracted fraction with ZIs of 50.86% at 2000 $\mu\text{g disc}^{-1}$ and water-extracted fractions with (50.00% and 47.43% at 2000 $\mu\text{g disc}^{-1}$ respectively). Butanol extracted fraction revealed minimum activity against *S. aureus* measuring 22.03%,

37.20% and 41.86% ZIs at 500, 1000 and 2000 $\mu\text{g disc}^{-1}$ respectively (fig. 4).

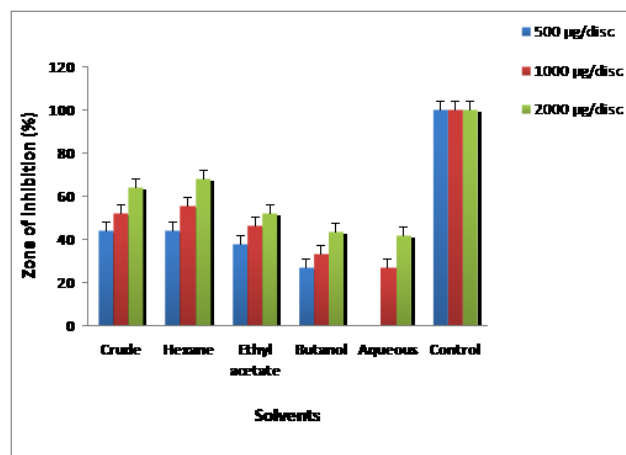


Fig. 3: Antibacterial activity of crude methanol, n-Hexane, ethyl acetate, butanol and water extracted samples from leaves of *Acorus calamus* against *Pseudomonas aeruginosa* by disc diffusion assay.

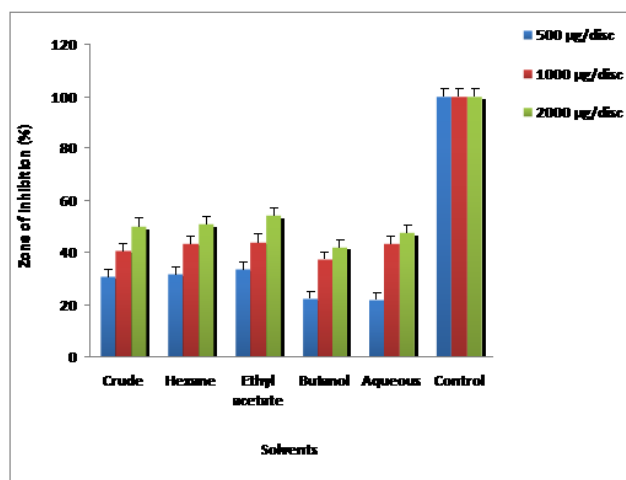


Fig. 4: Antibacterial activity of crude methanol, n-Hexane, ethyl acetate, butanol and water extracted samples from leaves of *Acorus calamus* against *Staphylococcus aureus* by disc diffusion assay.

Crude methanolic extract and different fractions reduced the growth of *Bacillus subtilis* except butanol and water-extracted fractions which showed no activity at 500 $\mu\text{g disc}^{-1}$ (fig. 5). The data indicated that n-hexane extracted fraction measured maximum reduction in the growth of the tested microbe (48.62% at 2000 $\mu\text{g disc}^{-1}$) and minimum activity (21.87% at 1000 $\mu\text{g disc}^{-1}$) was recorded by water extracted fraction when compared with controls (fig. 5). Ethyl acetate, methanol and butanol measured moderate activity against *Bacillus subtilis*. *Escherichia coli* and was susceptible to each concentration of all the tested extracts (fig. 6). The highest activity was measured by crude methanolic extract (64.38% ZI) at 2000 $\mu\text{g disc}^{-1}$ while the lowest was noted for water extracted fraction

(22.80% ZI at 500 $\mu\text{g disc}^{-1}$). Ethyl acetate, butanol and n-hexane extracted fractions revealed 45.16% and 42.03% 41.93% at 2000 $\mu\text{g disc}^{-1}$ respectively when compared with other extracts and controls.

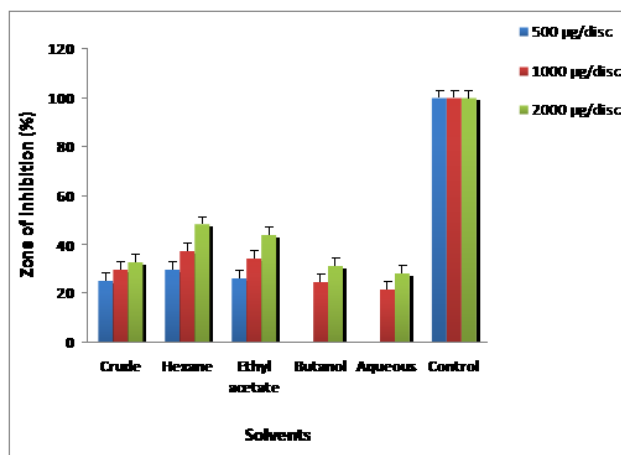


Fig. 5: Antibacterial activity of crude methanol, n-Hexane, ethyl acetate, butanol and water extracted samples from leaves of *Acorus calamus* against *Bacillus subtilis* by disc diffusion assay.

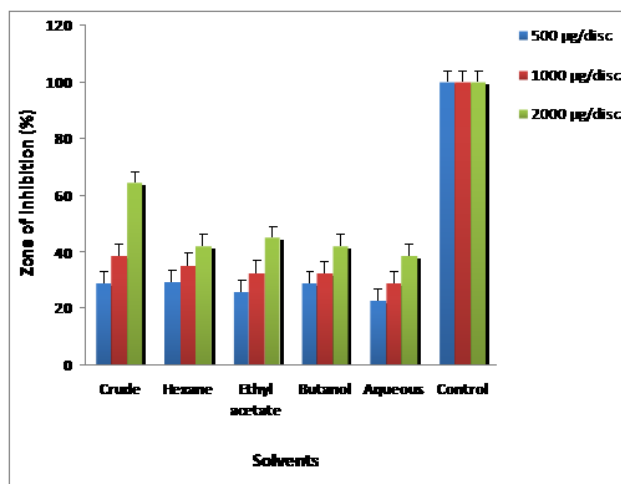


Fig. 6: Antibacterial activity of crude methanol, n-Hexane, ethyl acetate, butanol and water extracted samples from leaves of *Acorus calamus* against *Escherichia coli* by disc diffusion assay.

Xanthomonas campestris was resistant to water extracted samples showing no activity at 500 $\mu\text{g disc}^{-1}$ (fig. 7). Hexane- extracted fraction showed the highest activity (45.75% ZI) at 2000 $\mu\text{g disc}^{-1}$ while butanol extracted fraction was least effective to control the growth of *Xanthomonas campestris* (21.06%) at 500 $\mu\text{g disc}^{-1}$). Water extracted fraction measured no activity against *Xanthomonas campestris* at the same concentration. Our results showed that crude methanolic extract reduced the growth of *Xanthomonas campestris* by 30.39%, 36.36% and 45.63% at 500, 1000 and 2000 $\mu\text{g disc}^{-1}$ respectively. Similarly, ethyl acetate and butanol-extracted fractions

measured activity of 42.42% and 33.42% at 2000 $\mu\text{g disc}^{-1}$ respectively. Our result revealed that *Citrobacter freundii* was found to be highly susceptible to crude methanolic extract measuring 48.00%, 68.00% and 92.64% ZIs at concentrations of 500, 1000 and 2000 $\mu\text{g disc}^{-1}$ respectively followed by water extracted fraction (54.24%, 62.64% and 72.40% ZIs at 500, 1000 and 2000 $\mu\text{g disc}^{-1}$ respectively (fig. 8). On the other hand, ethyl acetate and butanol extracted fractions reduced the growth of *Citrobacter freundii* by 48.00% and 44.00% at 2000 $\mu\text{g disc}^{-1}$ respectively. Butanol extracted fraction, however, did not show any activity at 500 $\mu\text{g disc}^{-1}$.

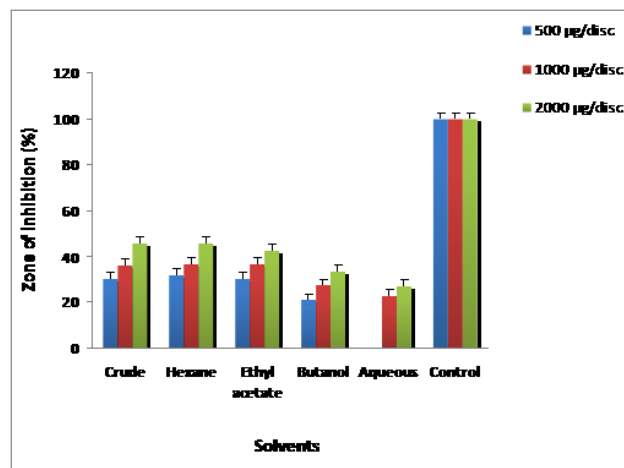


Fig. 7: Antibacterial activity of crude methanol, n-Hexane, ethyl acetate, butanol and water extracted samples from leaves of *Acorus calamus* against *Xanthomonas campestris* by disc diffusion assay.

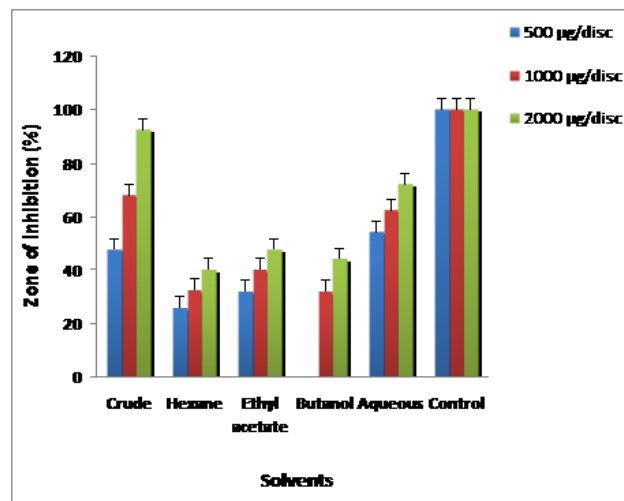


Fig. 7: Antibacterial activity of crude methanol, n-Hexane, ethyl acetate, butanol and water extracted samples from leaves of *Acorus calamus* against *Citrobacter freundii* by disc diffusion assay.

Crude methanolic extracts and its four fractions were active only against *Candida albican* out of the five tested fungal species (fig. 9). Like antibacterial activities, the

Table 1: Bacterial strains tested during the present experiment

Microbial Species	Gram strain type	Details of the Microbial strains used
<i>Klebsiella pneumoniae</i>	Negative	Clinical isolate obtained from Microbiology Laboratory, QAU, Islamabad, Pakistan.
<i>Pseudomonas aeruginosa</i>	Negative	ATCC # 9721
<i>Staphylococcus aureus</i>	Possitive	ATCC # 6538
<i>Bacillus subtilis</i>	Possitive	Clinical isolate obtained from Microbiology Laboratory, QAU, Islamabad, Pakistan.
<i>Escherichia coli</i>	Negative	ATCC # 25922
<i>Xanthomonas campestris</i>	Negative	ATCC # 33913
<i>Citrobacter freundii</i>	Negative	ATCC # 8090

Table 2: Fungal strains tested during the present experiment

Name of the specie	Details of the specie used
<i>Candida albicans</i>	ATCC # 10231. Plant Pathology Department KPK AUP Pakistan
<i>Trichoderma reesei</i>	ATCC # 26921. Plant Pathology Department KPK AUP Pakistan
<i>Acremonium alternatum</i>	ATCC # 60645. Plant Pathology Department KPK AUP Pakistan
<i>Penicillium chrysogenum</i>	ATCC # 11709. Plant Pathology Department KPK AUP Pakistan
<i>Rhizopus oryzae</i>	ATCC # 20344. Plant Pathology Department KPK AUP Pakistan

antifungal assay follows a similar dose-dependent pattern. *C. albicans* was more susceptible to crude methanolic extract reducing the growth of the tested microbe by 26.76%, 36.76% and 46.66% at 500, 1000 and 2000 μ g disc⁻¹ respectively. *Candida albicans* was less susceptible to water-extracted fraction measuring 21.76% and 30.00% ZIs at 1000 and 2000 μ g disc⁻¹ respectively, however, showed no activity at 500 μ g disc⁻¹. Similarly, n-hexane, ethyl acetate and butanol extracted fractions reduced the growth of *Candida albicans* by 40.10%, 36.76% and 33.20% at 2000 μ g disc⁻¹ respectively when compared with controls (fig. 9).

DISCUSSION

The present investigation describes the antimicrobial activity of crude methanolic extracts and its different fractions from the leaves of *Calamus aromaticus* against seven bacteria (gram positive and gram negative) and five fungal strains through disc diffusion assay. Our results indicated that different solvent extracted samples reduced the growth of *Klebsiella pneumoniae* in concentration dependent manner except water extracted fraction which did not measure any activity at lowest concentration. *Klebsiella pneumoniae* was more susceptible to methanol, n-hexane, ethyl acetate and butanol-extracted samples and moderate susceptibility to water-extracted fraction. The highest activity was noted by ethyl acetate extracted fraction at the highest concentration and lowest by butanol extracted samples at lowest concentration. The fact that water-extracted fraction showed markedly lower activity than either of the organic solvents used can be attributed to the better solubility of active compounds in organic solvents: Lin *et al.* (1999) also reported similar results. The polarity of the compounds being extracted by

different solvents is also a good measure for the rationalization of these results (Manikandan *et al.*, 2010).

Among different extracts, n-hexane-extracted fraction was more effective against *Pseudomonas aeruginosa* at highest concentration of 2000 μ g disc⁻¹ followed by methanol, ethyl acetate and butanol-extracted fractions. Again water extracted fraction was less effective showing no activity at lowest concentration of 500 μ g disc⁻¹. Here again the possible cause can be attributed to the possible better solubility of active compounds in organic solvents as reported by Lin *et al.* (1999). These results can also be interpreted in terms of the polarity of compounds being %ZI decreased with increasing polarity which suggested that the antibacterial compounds from the leaves of *Calamus aromaticus* against *P. aeruginosa* are relatively less polar. Similar results were also reported Fazal *et al.* (2012) and Manikandan *et al.* (2010). Ethyl acetate-extracted fraction measured maximum activity against *S. aureus* followed by n- hexane and water-extracted fractions. Butanol-extracted fraction measured minimum growth reduction of *S. aureus* compared with other samples and controls. Our results are in agreement with Phongpaichit *et al.* (2005) who investigated the activity of β -asarone purified from crude methanolic extract of *A. calamus* rhizome against *S. aureus*. Similarly results are also reported by Fazal *et al.* (2012) and Manikandan *et al.* (2010).

The data revealed that n-hexane extracted fraction was more effective to control the growth of *Bacillus subtilis* compared with water extracted fraction where low activity was measured. Our results agree with Fazal *et al.* (2012) Manikandan *et al.* (2010) and Rani *et al.* (2003). *Escherichia coli* were susceptible to all the tested extracts

at each concentration. The highest growth inhibition was recorded by crude methanolic extract and the lowest by water extracted fraction. Ethyl acetate, butanol and n-hexane extracted fractions measured moderate activity against the tested microbe compared with controls. These results agree with Phongpaichit *et al.* (2005), Devi and Ganjewala (2009), Manikandan *et al.* (2010) and Fazal *et al.* (2012). Our results suggested that *Xanthomonas campestris* was more resistant to water extracted samples showing no activity at lowest concentration and n-hexane-extracted fraction measured the highest activity. Butanol extracted fraction on the other hand was less effective to reduce the activity of *Xanthomonas campestris* at 500 µg disc⁻¹. Our results showed that crude methanolic extract and its fractions in ethyl acetate and butanol revealed moderate activity against *Xanthomonas campestris*. *Citrobacter freundii* was highly susceptible to crude methanolic extract at all concentrations followed by water extracted fraction. Ethyl acetate and butanol extracted fractions showed moderate activity against *Citrobacter freundii*.

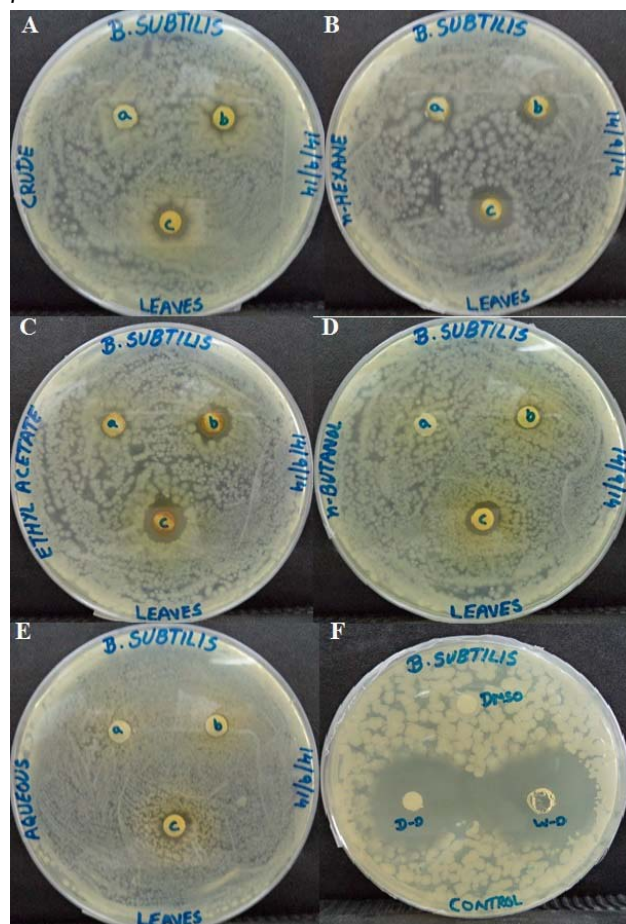


Plate 1: Antibacterial activity of samples extracted from leaves against *Bacillus subtilis*.

Among the five tested fungal species, different solvent extracted samples shows activity against *Candida albicans* in dose-dependent manner. *C. albicans* was more

susceptible to crude methanolic extract and more resistant to water-extracted fraction. Similarly, n-hexane, ethyl acetate and butanol extracted fractions revealed moderate activity against *Candida albicans* when compared with controls. Similar results are also reported by Lin *et al.* (1999), Phongpaichit *et al.* (2005), Devi and Ganjewala (2009) and Singh *et al.* (2010). The results obtained justified the use of *A. calamus* in different medicinal formulations due to its antibacterial and antifungal activity. Devi and Ganjewala (2009), Bhuvneshwari and Balasundaram (2009), Singh *et al.* (2010) and Barik *et al.* (2010) also reported the medicinal value of different plant parts of *A. calamus*. Our results were innovative for two reasons when compared with the findings of other researchers. Firstly, we first isolated the crude extracts and then fractionated the crude with different solvents in ascending polarity starting from less polar to more polar which demonstrated effective isolation of different bioactive compounds whereas other researchers used crude extract in different solvent. Secondly, some of the microbes (*Xanthomonas campestris*, *Citrobacter freundii* etc.) were tested for the first time in the present study using crude extracts and their fraction.

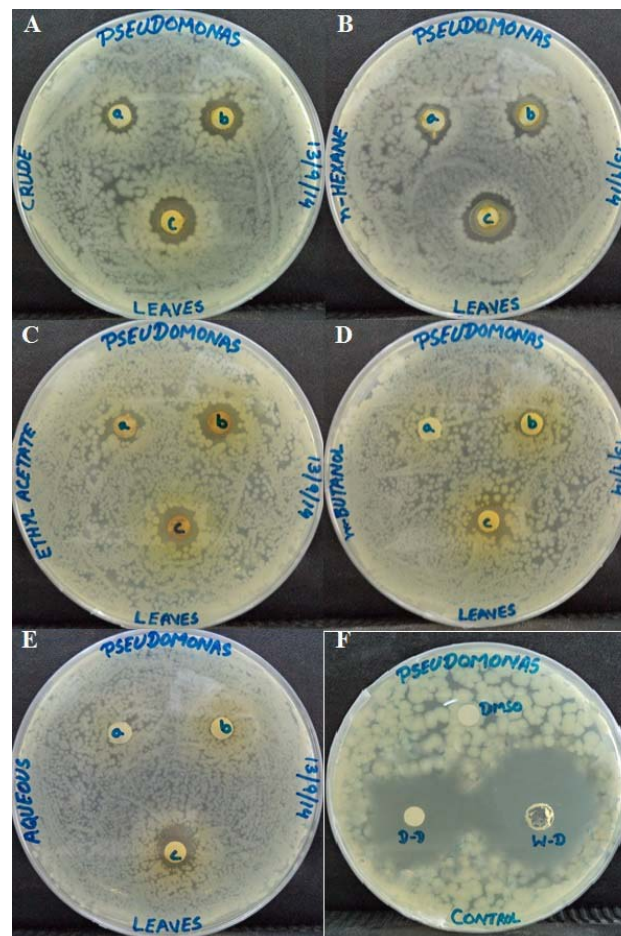


Plate 2: Antibacterial Activity of Samples Extracted from leaves against *Pseudomonas aeruginosa*.

CONCLUSION

From our results it can be concluded that crude methanolic extract was effective to control the growth of *E. coli*, *Citrobacter freundii* and *Candida albicans* while water extracted samples were ineffective inhibit the activity of the same microbes. Hexane extracted fraction caused maximum reduction in the growth of *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Xanthomonas campestris*. *Klebsiella pneumoniae* and *Staphylococcus aureus* were more susceptible to ethyl acetate fraction. Majority of the tested microbes were resistant to water and butanol extracted samples.

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