

Antimicrobial potentials of *Catharanthus roseus* by disc diffusion assay

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Abstract: The present research work investigates the *in vitro* antimicrobial activity of different solvent extracted samples from the aerial parts (stem, leaf, fruit and flower) of *C. roseus* against different microbial species using disc diffusion assay at two different concentrations of 1 and 2 mg disc⁻¹. Hexane extracted samples inhibited the growth of all tested microbial strains except *S. typhi*. Similarly, ethyl acetate extracted samples was effective to control the activity of all the tested microbial strains. *E. coli* and *S. typhi* showed resistance to chloroform extracted samples and the remaining eight microbial strains were susceptible to the same extract. Butanol extracted samples did not inhibit the growth of *K. pneumonia* and *S. typhi* at low concentration, however, at higher concentration the same extract reduced the growth of different microbes. Methanol extracted samples effectively controlled the growth of all tested microbes at both concentrations except for *S. typhi*. Water extracted samples did not inhibit the growth at low concentration except *E. coli*, *K. pneumonia* and *S. aureus* and were ineffective against *P. aeruginosa* at both concentration. *C. albicans*, showed resistance against chloroform and water extracted samples at low concentration and susceptible to other solvent extracted samples at both concentration. All fractions were effective against plant pathogens i.e. *E. carotovora* and *A. tumefaciens*.

Keywords: Antibacterial, antifungal, disc diffusion assay, *C. roseus*.

INTRODUCTION

Infectious diseases and spread of antibiotic resistant strains of microorganisms is a serious challenge to public health. Emergence of resistance to available antibiotics resulted in the introduction of newer antibiotics of plant origin to the public domain (Russell, 2002; Gootz, 1990). Therefore, researchers around the world have been moved towards hunting for novel bio-molecules of plant origin for the development of new drugs. Despite advancement in the field of medicine and diagnosis it is estimated that 80% of the world population is still dependant on the plant derived pharmaceuticals in one or other form. Plant based products or its derivatives accounts for nearly 28% of the medicines available in the market (Newman *et al.*, 2003). Natural products and their derivatives have historically been used as a valuable source of novel therapeutic agents (Akinyemi *et al.*, 2000; Koehn and Carter, 2005). Among 2600 plant species, more than 700 are used as medicinal herbs and therapeutic compounds (Ali-Shtayeh and Abu, 1999; Kong *et al.*, 2003). Herbal-based medicines are cheap, simple, safe, effective and exhibit broad spectrum activity (Chin *et al.*, 2006). Plant based products could minimize the adverse effects of various chemotherapeutic agents as well as in prolonging longevity and attaining positive general health (Kaushik *et al.*, 2002). Plants are rich source of a variety of secondary metabolites such as tannins, terpenoides, alkaloids and flavonoid, which have been shown *in vitro* to have antimicrobial properties (Cowan, 1999; Bakht *et al.*, 2011 a, b, c and d; 2012; 2013a,b; 2014a,b,c; Nasir *et al.*, 2015).

Catharanthus roseus (Vincarosea) a traditionally used medicinal plant, belongs to the family Apocynaceae, is an erected procumbent herb or under shrub containing latex. It is widely growing to 1m tall at subtropical area (Frode *et al.*, 2008). It is short-lived perennial plant with dark green and glossy leaves. Pharmacological studies have shown that *C. roseus* contains more than 70 different types of alkaloids (indole alkaloids) and chemotherapeutic agents (Verpoorte, 1998). The anticancer drugs vincristine and vinblastine are obtained from the alkaloids of *C. roseus*. Besides anti-cancer activity, alkaloids from this plant are known for their antihypertensive and antispasmodic properties (Verpoorte *et al.*, 2002). Traditionally, *C. roseus* has been used in folk medicine to treat diabetes and high blood pressure. *C. roseus* possesses known antibacterial, antifungal, anti-diabetic, anticancer and antiviral activities. Muhammad *et al.* (2009) reported the antibacterial potential in crude extracts of different parts (*viz.*, leaves, stem, root and flower) of *C. roseus* against clinically significant bacterial strains. Perez *et al.* (1990) reported that root extracts exhibited broad-spectrum antibacterial activity against *S. typhimurium* and *S. boydii*. The flower extract showed activity against *C. diphtheriae*.

Keeping in view the importance of *C. roseus* in medicinal plants, the present experiment was carried out (a). To investigate antibacterial activity of *C. roseus* against different gram positive and gram-negative bacteria and (b) To determine antifungal potentials of *C. roseus* against *C. albicans*.

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MATERIALS AND METHODS

The present study was conducted at the Institute of Biotechnology and Genetic Engineering, The University of Agriculture Peshawar KPK Pakistan. Plant materials (aerial portion of periwinkle; *C. roseus*) were collected from the Institute of Biotechnology and Genetic Engineering, University of Agriculture Peshawar KPK Pakistan. The collected plant materials were shade dried. The dried plant materials were finely powdered by tissue homogenizer (Infinigen™ Tissue Mixer Mill).

Crude extract preparation

About 332g of dried powdered aerial part of periwinkle were macerated in 97% methanol for 6 days. During this period the solution was stirred occasionally for thorough mixing. The methanol soluble compounds were then filtered by Wattman filter paper (Whatman TM). Fresh methanol was added to the remaining plant material and filtered again and this process was repeated thrice. The filtered methanolic solution was subjected to rotary evaporator for evaporation (Rotavapor R-R 210/R215; BUCHIL Labortechnik AG). Methanol was separated at 45°C under vacuum pressure and about 80 g of the semi-solid extract was obtained (crude extract).

Crude extract fractionation

The crude extracts obtained were divided into two portions. One portion (10g) was poured into a glass vial to be tested as crude ethanol extract for antimicrobial activity. The second portion (70g) was further fractionated with different solvents. The second portion was dissolved in 200ml distilled water by glass stirrer, poured into separatory funnel and distilled hexane was added into it. Compounds soluble in upper hexane phase were collected and the lower aqueous phase was extracted three times with hexane. All fractions of hexane were combined and semisolid hexane fraction was removed through rotary evaporator. The semisolid hexane fraction was dried in water bath at 45°C and stored in glass vials until used. The same process of fractionation was carried out for chloroform, ethyl acetate and butanol. The aqueous phase at the end was taken and dried through rotary evaporator and water bath.

Culture media and its preparation

Nutrient agar media (HiMedia Laboratories Pvt. Ltd) was used for the culturing and growth and nutrient broth was used for shaking incubation and standardization of different microorganisms. Media was prepared as described in Bakht *et al.* (2011 a, b, c, d; 2012).

Microorganisms tested

Antibacterial and antifungal activity of different solvent extracted samples of periwinkle was tested against the following different bacterial and fungal strains (table 1).

Disc diffusion susceptibility method

The antibacterial activity of different solvent extracted samples of *P. minima* was tested by disc diffusion assay according to the methods of Bauer *et al.* (1966) and antifungal activity by Ramdas *et al.* (1998). Two concentrations of the extracts (1 and 2mg disc⁻¹) in volume of 6 µl were applied to the disc. Antibiotic and antifungal drugs used as positive control for Gram positive, Gram negative bacteria and fungus were Erythromycin, Ciprofloxacin and Clotrimazole respectively.

For Gram positive bacteria: Erythromycin 50 µg 6 µl⁻¹

For Gram negative bacteria: Ciprofloxacin 50 µg 6 µl⁻¹

For Fungi: Clotrimazole 50 µg 6 µl⁻¹

RESULTS

The antibacterial activities of hexane, ethyl acetate, chloroform, butanol, methanol and water extracted samples from dried periwinkle against *B. subtilis* is shown in fig. 1. Water extracted samples did not inhibit the growth of *B. subtilis* at low concentration (1mg disc⁻¹) measuring 0% zone of inhibition (ZI) when compared with their positive controls. However, at 2mg disc⁻¹, the same sample reduced the growth of *B. subtilis* by 26%. Hexane was less effective at low concentration i.e. 1mg disc⁻¹, however, at higher concentration it effectively controlled the growth of *B. subtilis*. Ethyl acetate methanol and chloroform extracted samples on the other hand were more effective against *B. subtilis* at both concentrations compared to other samples. The data further suggested that butanol extracted samples were less effective against *B. subtilis* at any concentration used.

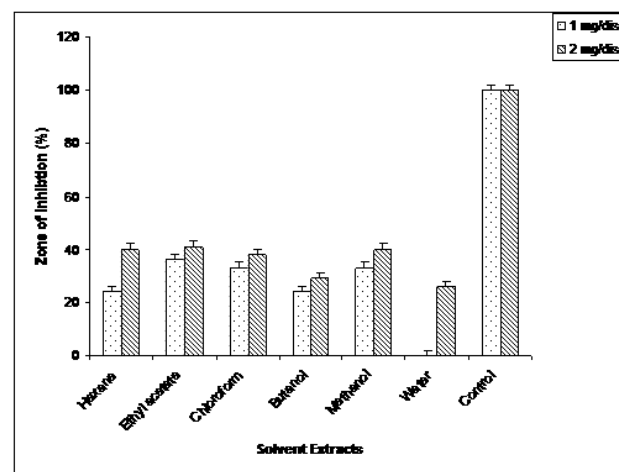


Fig. 1: Antimicrobial activity of hexane, ethyl acetate, chloroform, butanol, methanol and water extracted samples against *B. subtilis* using disc diffusion assay (Bar represent $\pm$ LSD value at $p < 0.05$).

The data also indicated that chloroform and water extracted samples of *C. roseus* were did not affect the

growth of *C. albicans* at low concentration (1mg disc^{-1}) compared with their positive controls (fig. 2). However, at high concentration (2mg disc^{-1}), these samples reduced the growth of *C. albicans* (chloroform: 29% ZI; water: 24% ZI). Hexane, butanol and methanol extracted samples were less effective against *C. albicans* at low concentration (1mg disc^{-1}) compared with high concentration (2mg disc^{-1}). Ethyl acetate extracted samples on the other hand inhibited the growth of *C. albicans* at both concentrations.

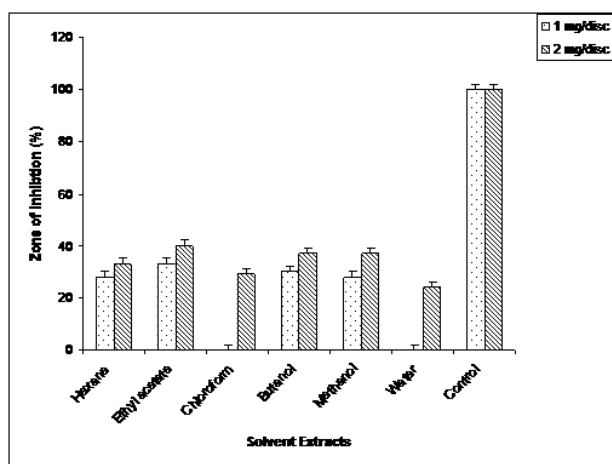


Fig. 2: Antimicrobial activity of hexane, ethyl acetate, chloroform, butanol, methanol and water extracted samples against *C. albicans* using disc diffusion assay (Bar represent $\pm\text{LSD}$ value at $p<0.05$).

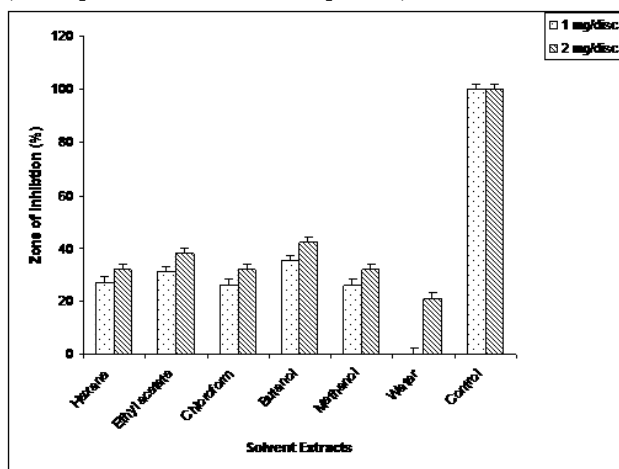


Fig. 3: Antimicrobial activity of hexane, ethyl acetate, chloroform, butanol, methanol and water extracted samples against *E. carotovora* using disc diffusion assay (Bar represent $\pm\text{LSD}$ value at $p<0.05$).

The antibacterial activities of six different solvents extracted samples from the aerial portion of dried *C. roseus* against *E. carotovora* are presented in fig. 3. Different solvent extracted samples except water extracts showed antibacterial activity against *E. carotovora* at both concentrations. Water extracted sample did not inhibit the growth at low concentration i.e. 1mg disc^{-1} , however,

minimum growth reduction was noted at 2mg disc^{-1} . Highest growth reduction was revealed by butanol extracted samples i.e. 42% ZI followed by ethyl acetate extracted samples (38% ZI) at 2mg disc^{-1} . Butanol and ethyl acetate extracted samples at low concentration was also effective to control the growth of *E. carotovora* (35% and 31% ZI respectively). Chloroform, hexane and methanol extracted samples reduced the growth of *E. carotovora* by 32% at 2mg disc^{-1} concentration.

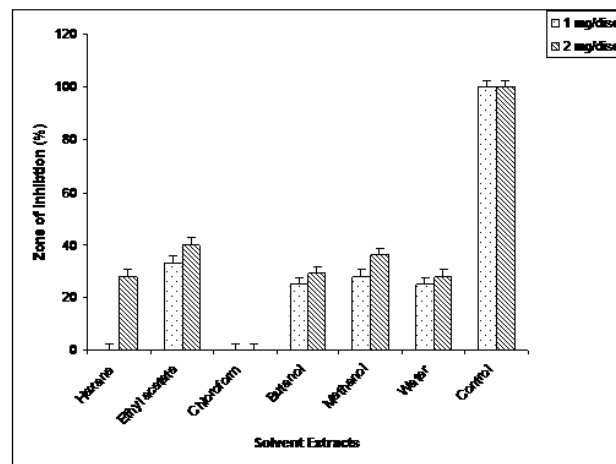


Fig. 4: Antimicrobial activity of hexane, ethyl acetate, chloroform, butanol, methanol and water extracted samples against *E. coli* using disc diffusion assay (Bar represent $\pm\text{LSD}$ value at $p<0.05$).

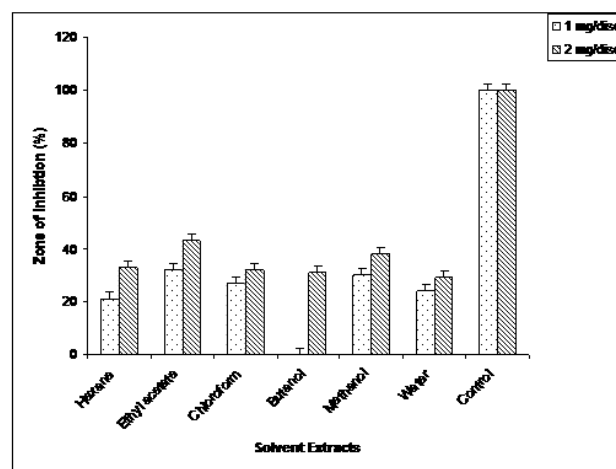


Fig. 5: Antimicrobial activity of hexane, ethyl acetate, chloroform, butanol, methanol and water extracted samples against *K. pneumoniae* using disc diffusion assay (Bar represent $\pm\text{LSD}$ value at $p<0.05$).

The data also indicated that *E. coli* was susceptible to all extracted samples at both concentrations except chloroform extracts. Methanol, ethyl acetate, butanol, hexane and water extracted samples measured maximum growth inhibition at 2mg disc^{-1} . *E. coli* was highly susceptible to ethyl acetate extracted samples (40% ZI) followed by methanol (36% ZI) at 2mg disc^{-1} when

compared with their positive controls. Minimum reduction in the growth of *E. coli* was noted by ethyl acetate and hexane extracted samples i.e. 33% ZI at 1mg disc⁻¹ concentration. Chloroform extracted samples did not inhibit the growth of *E. coli* at both concentration (fig. 4).

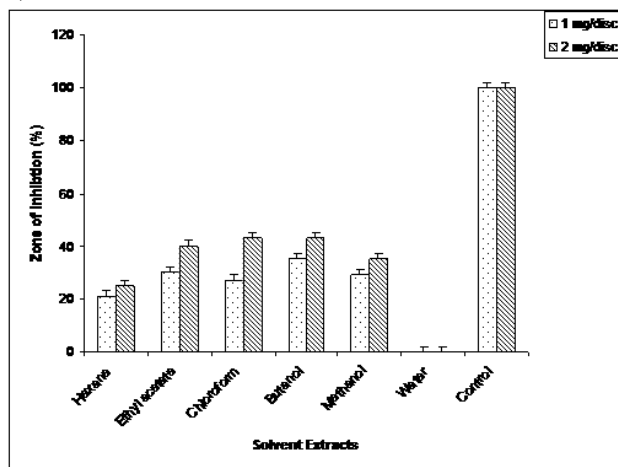


Fig. 6: Antimicrobial activity of hexane, ethyl acetate, chloroform, butanol, methanol and water extracted samples against *P. aeruginosa* using disc diffusion assay (Bar represent $\pm$ LSD value at $p < 0.05$).

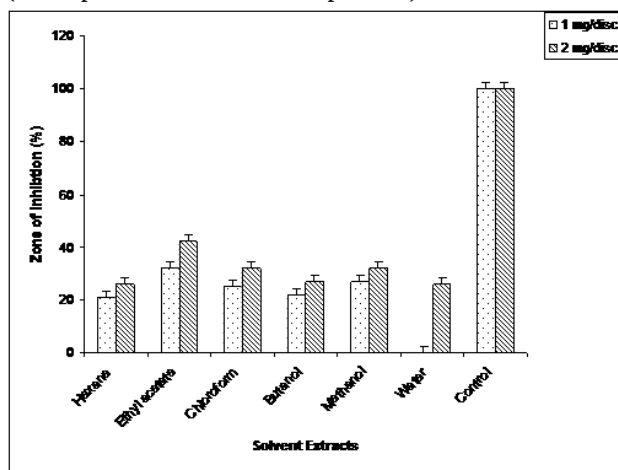


Fig. 7: Antimicrobial activity of hexane, ethyl acetate, chloroform, butanol, methanol and water extracted samples against *A. agrobacterium* using disc diffusion assay (Bar represent $\pm$ LSD value at $p < 0.05$).

Data concerning antibacterial activity of different solvents extracted samples from dried aerial parts of *C. roseus* against *K. pneumonia* is indicated in fig. 5. Butanol extracted samples did not inhibit the growth of *K. pneumonia* at low concentration and showed good activity at 2 mg disc⁻¹ (31% ZI). However, hexane, ethyl acetate, chloroform, methanol and water extracted samples measured activity against *K. pneumonia* at both concentrations. The data also suggested that maximum activity was exhibited by ethyl acetate extracted samples against *K. pneumonia* (43% ZI) at 2 mg disc⁻¹ compared

to other samples. Methanol extracted samples also reduced the growth of *K. pneumonia* by 38% at 2 mg disc⁻¹. The lowest growth inhibition of *K. pneumonia* was noted for hexane extracted samples (22% ZI) followed by water extracts (24% ZI). The results also indicated that all samples were effective in reducing the growth of *P. aeruginosa* at both concentrations when compared with their positive control. Water extracts did not control the growth of *P. aeruginosa* at any concentration. These results suggested that *P. aeruginosa* was highly resistant to water extracts at both concentrations. Hexane extracted sample as compared to other solvent, showed low growth inhibition of *P. aeruginosa* i.e. 21% ZI at 1mg disc⁻¹ and 26% ZI at 2mg disc⁻¹. Among all extracts, butanol extracted samples was more effective against *P. aeruginosa* (35% ZI at 1mg disc⁻¹ and 43% ZI at 2mg disc⁻¹). *P. aeruginosa* showed maximum susceptibility to butanol and chloroform (43% ZI) followed by ethyl acetate (40% ZI) and methanol extracts (35%) at 2mg disc⁻¹ (fig. 6).

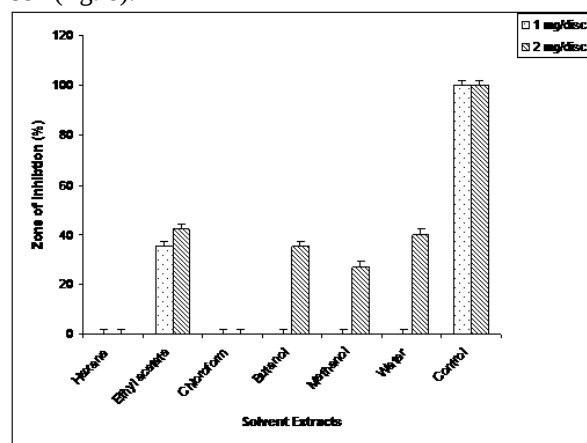


Fig. 8: Antimicrobial activity of hexane, ethyl acetate, chloroform, butanol, methanol and water extracted samples against *S. typhi* using disc diffusion assay (Bar represent $\pm$ LSD value at $p < 0.05$).

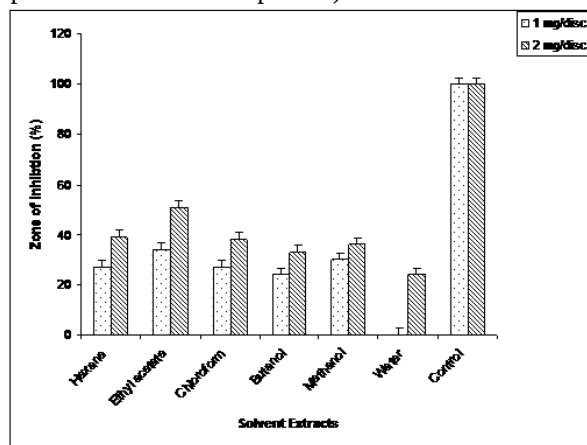


Fig. 9: Antimicrobial activity of hexane, ethyl acetate, chloroform, butanol, methanol and water extracted samples against *B. atrophaeus* using disc diffusion assay (Bar represent $\pm$ LSD value at $p < 0.05$).

Table 1: Microbial strains used in this research work

Microbial species	Gram Strain type	Details of the microbial strains used
<i>Bacillus subtilis</i>	Positive	Clinical isolate from Department of Microbiology, Quaid-e-Azam University, Islamabad, Pakistan
<i>Candida albicans</i>	Fungus	Clinical isolate obtained from Hayatabad Medical Complex Peshawar, KPK, Pakistan
<i>Erwinia carotovora</i>	Negative	Department of Plant Pathology The University of Agriculture Peshawar, KPK, Pakistan
<i>Escherichia coli</i>	Negative	ATCC # 25922
<i>Klebsiella pneumonia</i>	Negative	Clinical isolate from Department of Microbiology, Quaid-e-Azam University, Islamabad, Pakistan
<i>Pseudomonas aeruginosa</i>	Negative	ATCC # 9721
<i>Salmonella typhi</i>	Negative	Clinical isolate from Department of Microbiology, Quaid-e-Azam University, Islamabad, Pakistan
<i>Staphylococcus aureus</i>	Positive	ATCC # 6538
<i>Bacillus atrophaeus</i>	Positive	Clinical isolate obtained from PCSIR, Peshawar
<i>Agrobacterium tumefaciens</i>	Negative	Clinical isolate obtained from PCSIR, Peshawar

All the microbial stock cultures were freshened by streak method (streak 1) using sterile inoculation loop on nutrient agar media plates in a laminar flow hood and incubated at 37°C for 24hrs. The next day, the first day streak cultures were again sub cultured on media plates (streak 2) and incubated at 37°C for 24 hrs. The second streaked cultures were then inoculated into the nutrient broth in flasks and flasks were then placed in shaker incubator for 18 hrs at 37°C (200 rpm).

The data suggested that ethyl acetate extracted samples reduced the growth of *A. tumefaciens* at both concentrations showing 32% and 42% ZI respectively. Butanol and methanol extracted samples showed moderate activity against *A. tumefaciens* at both concentrations. Hexane extracted samples showed minimum antibacterial activity at both concentrations. The data further suggested that water fraction did not inhibit the growth of *A. tumefaciens* at 1mg disc⁻¹ concentration while at 2mg disc⁻¹ it reduced growth by 26% (fig. 7). *S. typhi* was resistant to hexane and chloroform extracted samples at both concentrations (fig. 8). Butanol, methanol and water fractions did not exhibit inhibition at 1 mg disc⁻¹ concentration, however, reduced the growth of *S. typhi* at 2mg disc⁻¹ (35% ZI). Methanol extracted samples reduced the growth of *S. typhi* by 27% and water extracts by 40%. Ethyl acetate on the other hand, inhibited the growth of the same bacteria at both concentrations (35% ZI at 1 mg disc⁻¹ and 42% ZI at 2 mg disc⁻¹). The data further suggested that ethyl acetate and methanol extracted samples inhibited the growth of *B. atrophaeus* at both concentrations (34% ZI at 1 mg disc⁻¹ and 51% ZI at 2mg disc⁻¹). Methanol extracted samples reduced the growth of *B. atrophaeus* by 30% at 1 mg disc⁻¹ and 36% at 2mg disc⁻¹ when compared with their positive controls. Hexane, chloroform and butanol extracted samples revealed inhibitory activity against *B. atrophaeus* at higher concentration only (39%, 38% and 33% ZI respectively at 2mg disc⁻¹). Water extracted samples on other hand, was ineffective to control the growth of *B. atrophaeus* at 1 mg disc⁻¹ concentration, while at 2 mg disc⁻¹ inhibited the growth of *B. atrophaeus* by 24% when compared to its controls (fig. 9). All extracts reduced the growth of *S. aureus* at both concentrations. Maximum antibacterial activity was shown by ethyl acetate and

butanol extracts against *B. atrophaeus* (40% ZI at 2mg disc⁻¹ respectively). Water extracted samples revealed growth inhibition of 23% at 1mg disc⁻¹ when compared with its positive control.

DISCUSSION

The antibacterial activities of different solvents extracted samples from dried periwinkle revealed that ethyl acetate, methanol and chloroform extracted samples were more effective against *B. subtilis* at both concentrations as compared to other extracts. Water extracted samples did not inhibit the growth of *B. subtilis* at low concentration when compared with their positive controls. However, at higher concentration the same extract reduced the growth of *B. subtilis*. Hexane was less effective at low concentration, however, at 2 mg disc⁻¹ hexane extracted samples effectively controlled the activity of *B. subtilis*. The results further indicated that butanol extracted samples were less effective against *B. subtilis* at both concentrations. These results agree with Ramya *et al.* (2008). The data also indicated that chloroform and water extracted samples of *C. roseus* did not inhibit the growth of *C. albicans* at 1 mg disc⁻¹. However, at higher concentration (2mg disc⁻¹), these samples reduced the growth of *C. albicans*. Hexane, butanol and methanol extracted samples were less effective against *C. albicans* at low concentration compared with high concentration (2 mg disc⁻¹). Ethyl acetate extracted sample on the other hand, inhibited the growth of *C. albicans* at both concentrations.

Different solvent extracted samples except water extracts showed activity against *E. carotovora* at both concentrations. Water extracted sample did not reduce the

growth of *E. carotovora* at low concentration, however, minimum activity was observed at 2mg disc⁻¹. Highest growth inhibition was shown by butanol extracted samples followed by ethyl acetate extracted samples. Chloroform, hexane and methanol extracted samples reduced the growth of *E. carotovora* at 2mg disc⁻¹ concentration. The results suggested that *E. coli* were susceptible to different solvent extracted samples at both concentrations except chloroform extracts. Methanol, ethyl acetate, butanol, hexane and water extracted samples showed maximum growth inhibition at 2 mg disc⁻¹ compared with 1 mg disc⁻¹ concentration. Chloroform extracted samples did not inhibit the growth of *E. carotovora* at both concentration.

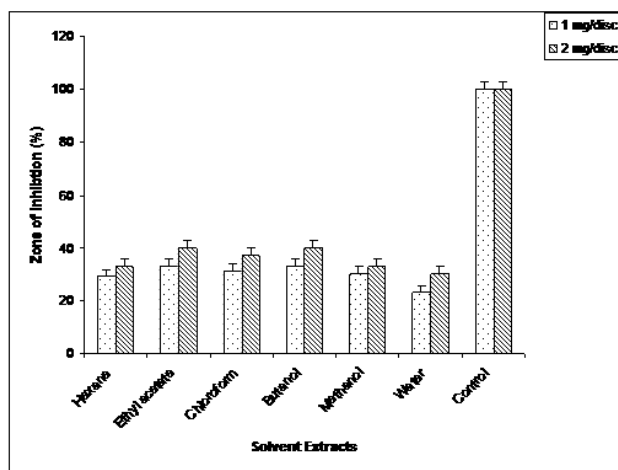


Fig. 10: Antimicrobial activity of hexane, ethyl acetate, chloroform, butanol, methanol and water extracted samples against *S. aureus* using disc diffusion assay (Bar represent $\pm$ LSD value at $p < 0.05$).

Our results also indicated that butanol extracted samples did not inhibit the growth of *K. pneumonia* at low concentration, however, was effective at 2 mg disc⁻¹. Hexane, ethyl acetate, chloroform, methanol and water extracted samples reduced the growth of *E. carotovora* at both concentrations. The data also suggested that maximum activity was noted by ethyl acetate extracted samples against *E. carotovora* compared to other samples. Methanol extracted samples also reduced the growth of *E. carotovora* at 2 mg disc⁻¹. Minimum reduction in the growth of *E. carotovora* was noted by hexane extracted samples followed by water extracts. Similar results are also reported by Ramya *et al.* (2008). Our results also indicated that all samples reduced the growth of *P. aeruginosa* at both concentrations. Water extracts did not control the growth of *P. aeruginosa* at both concentrations. These results suggested that *P. aeruginosa* was highly resistant to water extracts at these concentrations. Hexane extracted sample as compared to other samples, reduced the growth of *P. aeruginosa* at 1 and 2 mg disc⁻¹. Among all extracts, butanol extracted samples revealed more activity against *P. aeruginosa* at the tested concentrations. *P. aeruginosa* showed

maximum susceptibility to butanol and chloroform extracted samples followed by ethyl acetate and methanol extracts at 2 mg disc⁻¹. Similar results are also reported by Raza *et al.* (2009) and Prajakta *et al.* (2010).

The data further suggested that ethyl acetate extracted samples reduced the growth of *A. tumefaciens* at both concentrations. Butanol and methanol extracted samples revealed moderate activity against *P. aeruginosa* at both concentrations. Hexane extracted samples on the other hand measured minimum antibacterial activity at both concentrations. The data also indicated that water extracted samples did not inhibit the growth of *P. aeruginosa* at 1 mg disc⁻¹ concentration while at 2 mg disc⁻¹ it showed activity against the same bacteria. *S. typhi* was resistant to hexane, butanol and chloroform extracted samples at both concentrations. Butanol, methanol and water fractions did not reduce the growth of *S. typhi* at low concentration, however, showed activity at 2 mg disc⁻¹. Ethyl acetate extracted samples on the other hand, revealed activity at both concentrations. These results agree with those reported by Raza *et al.* (2009). Ethyl acetate and methanol-extracted samples reduced the growth of *B. atropheus* at both concentrations. Hexane, chloroform and butanol extracted samples showed inhibitory activity against *B. atropheus* at higher concentration only. Water extracted samples was ineffective to control the growth of *B. atropheus* at 1mg disc⁻¹ concentration, however, reduced the growth of *B. atropheus* at 2mg disc⁻¹. All extracts reduced the growth of *S. aureus* at both concentrations. The highest antibacterial activity was measured by ethyl acetate and butanol extracts against *S. aureus* at higher concentration. Water extracted samples showed minimum activity against *S. aureus* at 1 mg disc⁻¹. These results agree with Goyal *et al.* (2008), Prajakta *et al.* (2010), Verma and Singh (2010), Khalil (2012) and Govindasamy *et al.* (2012).

CONCLUSION

From the present study it can be concluded that all six different solvent extracted samples of *C. roseus* showed antimicrobial activities, thus, this plant seems to have antimicrobial bioactive compounds. Most of the antimicrobial compounds of *C. roseus* are soluble in ethyl acetate and methanol as compared to other solvents. However, different solvent extracted samples showed effective antifungal activity against *Candida albicans* suggesting a potential use of this plant as antifungal agent.

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