

REPORT

Screening of medicinally important *Berberis lyceum* for their antimicrobial activity by disc diffusion assay

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Abstract: The present research was focused on the anti-microbial activities of different solvent extracted fractions from commercial available and fresh plants of *Berberis lyceum* against Gram positive, Gram-negative bacteria and fungi using 1 and 2 mg disc⁻¹ concentrations. Our results showed that fractions from both sources revealed different degree of antimicrobial activities. Our result indicated that *Escherichia coli*, *Citrobacter freundii* and *Candida albicans* were more susceptible to crude methanolic extract and the same microbes were resistant to water extracted fractions. Similarly, maximum reduction in the growth of *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Xanthomonas campestris* was measured by hexane-extracted fractions and minimum growth inhibition by water-extracted fractions. *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. *Staphylococcus aureus* was the most susceptible gram-positive bacteria and *Bacillus subtilis* was resistant one. Among Gram-negative bacteria, *Citrobacter freundii* showed maximum susceptibility while *Xanthomonas campestris* revealed resistivity.

Keywords: Antibacterial, antifungal activity, *Berberis lyceum*, disc diffusion assay,

INTRODUCTION

Herbal medicine obtained from different parts of various plants is the oldest method of treating different curing diseases and infections in many parts of the world (Nweze *et al.*, 2004; Vineela and Elizebath, 2005; Bako *et al.*, 2005; Parekh and Chanda, 2007; Chaun *et al.*, 2015). Medicinal plants owe their curative characteristics, due to certain bio-active substances are presents in different parts of the plants. Bioactive compounds from these diverse sources have been isolated and characterized worldwide. These bioactive compounds includes terpenes, flavonoids, bioflavonoids, benzophenones, xanthenes as well as some metabolites such as tannins, saponins, cyanates, oxalate and anthraxquinones (Iwu, 1993; Asaolu, 2003). It has been reported that more than 80% of the world's population in developing countries are relying on herbal medicines for their primary healthcare needs (WHO, 2005). Numerous studies have reported that medicinal plants produce a large number of secondary metabolites with antimicrobial effects on pathogens (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; Bilal *et al.*, 2016; Wajid *et al.*, 2016 a, b; Amjad *et al.*, 2016; Anwar *et al.*, 2016). The increasing interest in herbal medications is due to low adverse side effects compared with synthetic medicines. Coupled with reduced cost of plant preparations, makes the search for

natural therapeutics an attractive option (Chariandy *et al.*, 1999).

Berberis lyceum belongs to the family Berberidaceae, is native to Nepal and found worldwide including India and Pakistan. *Berberis lyceum* is commonly utilized for different herbal treatments including urinary tract infections, enlargement of spleen, gastric and duodenal ulcer, liver disorders, acute conjunctivitis, acne, pimples and other skin infections (Chauhan *et al.*, 1990). The principal bio-active compound found in *Berberis lyceum* is berberine (an alkaloid) (Hassan *et al.*, 2007). The dried mass of the root bark in powdered form after mixing with molten animal fat is used as bandage for bone fractures. The fruit juice is usually consumed for gums and teeth diseases. Decoction of fruit is used for the treatment of typhoid and common cold, stomach pain, diarrhea, jaundice, healing activity (Shah, *et al.*, 2006; Asif *et al.*, 2007; Ahmad *et al.*, 2009). The present research investigates the antimicrobial potential of different solvent extracted samples against different bacteria and fungi.

MATERIALS AND METHODS

Plant material

Fresh plant material (roots) was collected from different localities of Northern areas of Pakistan. Commercial samples were purchased from the local market of

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Peshawar, Pakistan. Fresh plant materials were thoroughly washed with running tap water, rinsed, chopped, shade dried and grounded with electric grinder.

Crude extract preparation

One kilogram each of powdered materials of both samples were soaked in five liters of methanol, kept at room temperature in the dark for seven days and agitated three times a day. The mixture was filtered through Whatman filter paper No.1 and the residue was mixed with three liters of fresh methanol and the whole process was repeated three times. All extracts of the filtered methanolic solution were dried at 45°C under vacuum pressure through rotary evaporator. The crude methanolic extract was divided into two portions. One portion was used as crude methanolic extract and the other portion was further fractionated with various solvents.

Fractionation of crude extract

One hundred grams of the crude extract was dissolved in 500 ml sterile distilled water, mixed with 300ml n-hexane, shaken gently and allowed to stand for 15 minutes for separation of the two phases. The upper n-hexane phase was collected and the lower aqueous phase was re-extracted thrice with fresh n-hexane. Different fractions of n-hexane were pooled together, dried at 45°C under vacuum pressure with rotary evaporator. The same process of fractionation was followed for ethyl acetate and butanol. The lower aqueous phase at the end of the procedure was dried as described previously.

Disc diffusion susceptibility assay

Nutrient broth was used for shaking incubation and standardization and nutrient agar medium for the culturing and growth of all microorganisms. The antibacterial activity of crude methanolic extract and their fractions 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 (table 1). 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 (6mm in diameter) were placed on the media in petri plates with the help of a sterile forceps. Plant extracts in concentration of 1 and 2mg in 6 and 12µl volumes were applied on the discs. Antibiotics as positive control and DMSO ($12\mu\text{l disc}^{-1}$) 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.

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 of each sample was collected in triplicate and MSTATC computer software was used for 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 antimicrobial activity of crude methanolic extract and their fraction obtained from fresh and commercially available plant samples of *Berberis lyceum* against five bacteria (gram positive and gram negative) and one fungal strains. The antibacterial activity of different extracts from fresh samples of *Berberis lyceum* against *B. subtilis* is shown in fig. 1. Our results showed that crude methanolic extract and all fractions revealed activity at both concentrations against the tested organism. *B. subtilis* showed maximum susceptibility to n-hexane (25% ZI) followed by ethyl acetate and butanol extracted samples showing 24% ZI at highest concentration. Crude methanolic extract revealed minimum reduction in the growth of the tested microbe at lower concentration.

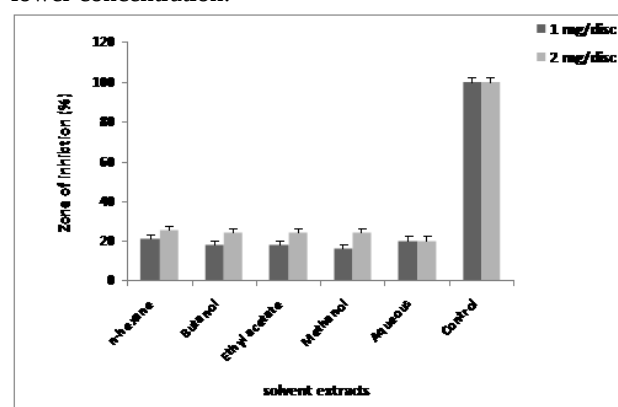


Fig. 1: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, butanol and water extracted samples from fresh samples of *Berberis lyceum* Royle against *B. subtilis* by disc diffusion assay (Bar shows LSD at $p < 0.05$).

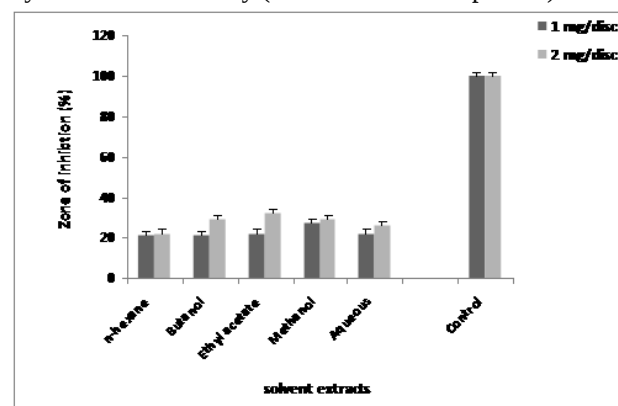


Fig. 2: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, butanol and water extracted samples from

commercially available samples of *Berberis lyceum* Royle against *B. subtilis* by disc diffusion assay (Bar shows LSD at $p < 0.05$).

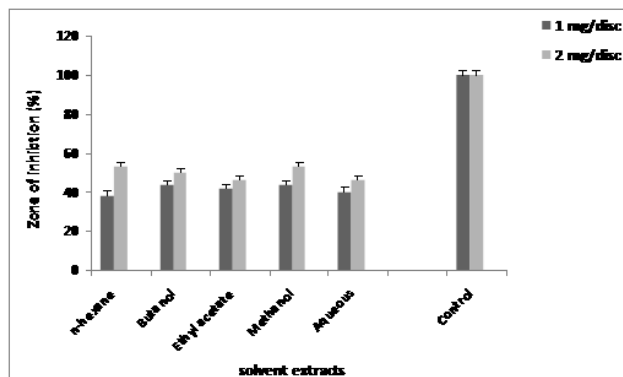


Fig. 3: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, butanol and water extracted samples from fresh samples of *Berberis lyceum* Royle against *P. aeruginosa* by disc diffusion assay (Bar shows LSD at $p < 0.05$).

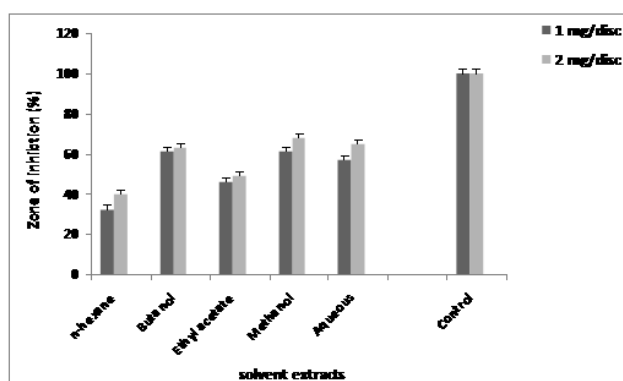


Fig. 4: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, butanol and water extracted samples from commercially available samples of *Berberis lyceum* Royle against *P. aeruginosa* by disc diffusion assay (Bar shows LSD at $p < 0.05$).

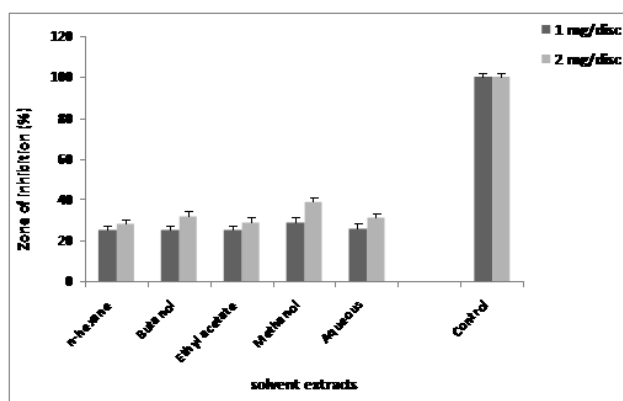


Fig. 5: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, butanol and water extracted samples from fresh samples of *Berberis lyceum* Royle against *E. coli* by disc diffusion assay (Bar shows LSD at $p < 0.05$).

The data from commercially available samples showed the highest ZI (32%) in ethyl acetate fractions followed by butanol and methanol extracts (29%) at 2 mg disc^{-1} . However, hexane and butanol at lower concentration measured minimum activity compared with other fractions and controls (fig. 2). fig. 3 shows the effect of different fractions of fresh samples of *Berberis lyceum* on the growth of *P. aeruginosa*. Among different samples, crude methanolic and hexane extracts were more effective to control the growth of the tested organisms measuring 53% of ZI each at concentrations of 2 mg disc^{-1} followed by butanol fraction (50% ZI). Hexane at lower concentration revealed minimum activity (38% ZI) against *P. aeruginosa*. Our results also revealed that crude methanolic extracts and different fractions showed activity against *P. aeruginosa* (fig. 4). Crude methanolic extract measured maximum growth inhibition of the tested organism (68% ZI) at concentrations of 2 mg disc^{-1} followed by aqueous extracted fraction with ZI of 63 at the same concentration. However, hexane extracted fraction revealed minimum activity against *P. aeruginosa* noting 32% ZI at lower concentration (fig. 4).

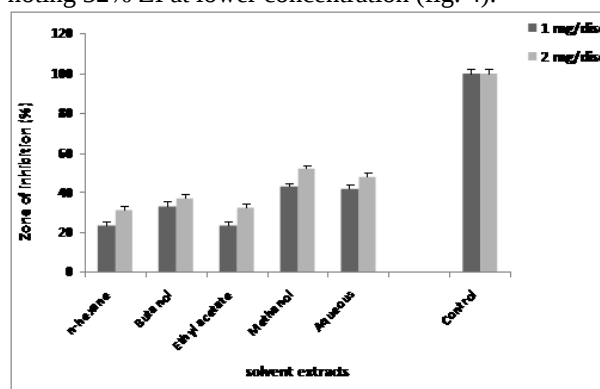


Fig. 6: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, butanol and water extracted samples from commercially available samples of *Berberis lyceum* Royle against *E. coli* by disc diffusion assay (Bar shows LSD at $p < 0.05$).

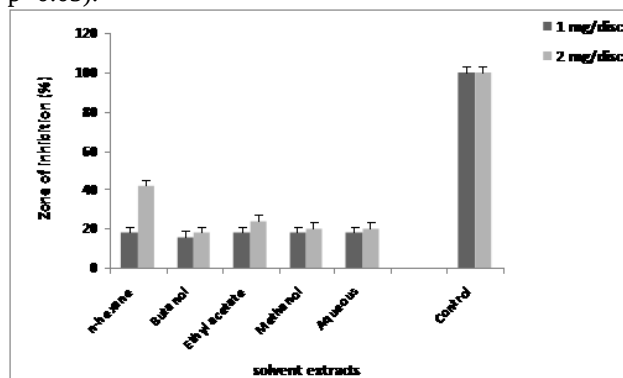


Fig. 7: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, butanol and water extracted samples from fresh samples of *Berberis lyceum* Royle against *K. pneumoniae* by disc diffusion assay (Bar shows LSD at $p < 0.05$).

Crude methanolic extract and various fractions from fresh samples inhibited the activity of *E. coli* at both concentrations (fig. 5). The data indicated that crude methanolic extract recorded maximum inhibition of growth of the tested microbe (39% ZI at 2mg disc⁻¹) followed by butanol (32% ZI) at the same concentration. Hexane, butanol and ethyl acetate on the other hand measured minimum activity of 25% against at lower concentration (2mg disc⁻¹). Our results also indicated the crude methanolic extract from commercially available samples effectively reduced the growth of *E. coli* (52% ZI) at high concentration followed by aqueous extracted fraction (fig. 6). Hexane and ethyl acetate extracted samples on the other hand showed minimum inhibition activity of 23% each at lower concentration compared with other fractions and controls. Data regarding antibacterial activity of fresh sample revealed varying degree of growth inhibition of *K. pneumoniae* (fig. 7). Maximum reduction in the growth of the tested microbes was measured by hexane fraction followed by butanol (42% and 24% respectively) at higher concentration. Butanol fraction at lower concentration recorded minimum inhibition of 18% (fig. 7). In case of commercially available samples, ethyl acetate extracted fraction measured maximum activity against *K. pneumoniae* (24% ZI at 2mg disc⁻¹) followed by butanol (21% ZI) at the same concentration (fig. 8). Methanol, butanol and ethyl acetate extracted fractions were equally effective to control the activity of the tested organisms and noted minimum growth reduction of 18% ZI each.

Crude methanolic extracted sample from the fresh samples was more effective to reduce the activity of *S. aureus* measuring 30% ZI at higher concentration followed by hexane extracted fractions (28% ZI) at the same concentration of 2 mg disc⁻¹ (fig. 9). Butanol at lower concentration was least effective to reduce the growth of *S. aureus* compared with other samples and controls. Results obtained from commercially available samples against *S. aureus* are shown in fig. 10. The data revealed that crude methanolic extract caused maximum reduction in the growth of *S. aureus* (43% ZI) followed by ethyl acetate and butanol at higher concentrations measuring 37% and 35% ZI respectively. Hexane fraction on the other hand recorded minimum zone of inhibition (27%) compared with other samples and controls (fig. 10). Fresh and commercially available samples were also screened for their antifungal activity against *C. albicans*. Butanol extracted fraction from the fresh samples revealed maximum activity (46% ZI) followed by crude methanolic extract (41% ZI) at 2mg disc⁻¹ (fig. 11). The data also suggested that aqueous extracted fraction was least effective to control the activity of *C. albicans* at lower concentration of 1mg disc⁻¹. In case of commercially available samples, maximum antifungal activity was measured by crude methanolic extracts (53% ZI) followed by butanol and aqueous fractions at higher

concentrations and minimum activity was noted by hexane fractions (30% ZI) at lower concentrations compared with other samples and controls (fig. 12).

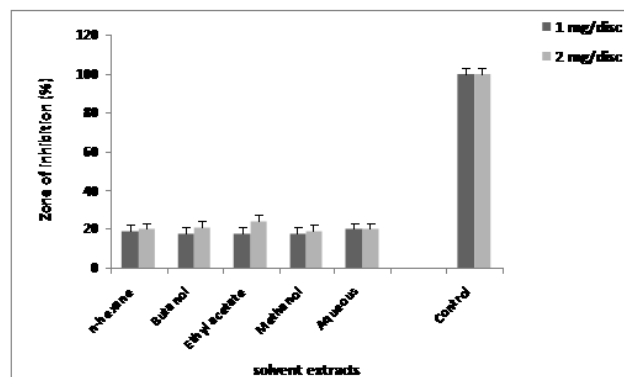


Fig. 8: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, butanol and water extracted samples from commercially available samples of *Berberis lyceum* Royle against *K. pneumoniae* by disc diffusion assay (Bar shows LSD at $p < 0.05$).

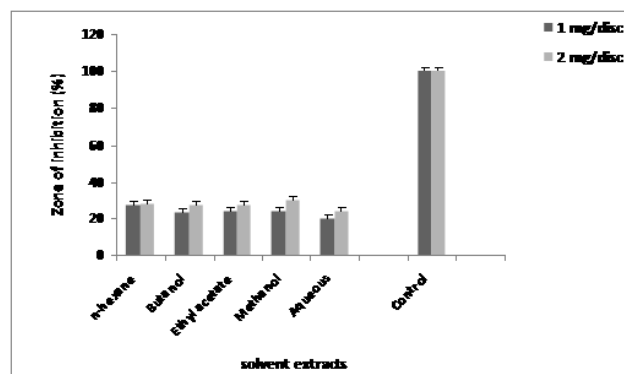


Fig. 9: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, butanol and water extracted samples from fresh samples of *Berberis lyceum* Royle against *S. aureus* by disc diffusion assay (Bar shows LSD at $p < 0.05$).

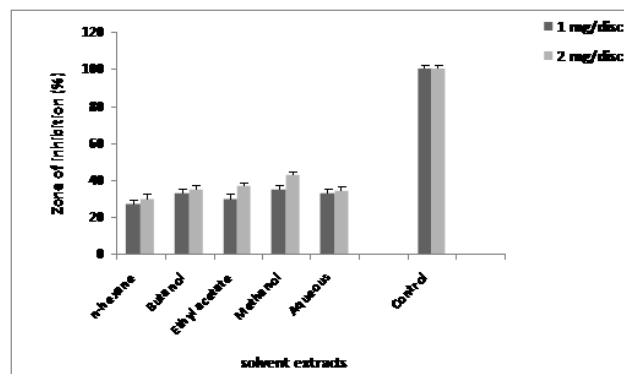
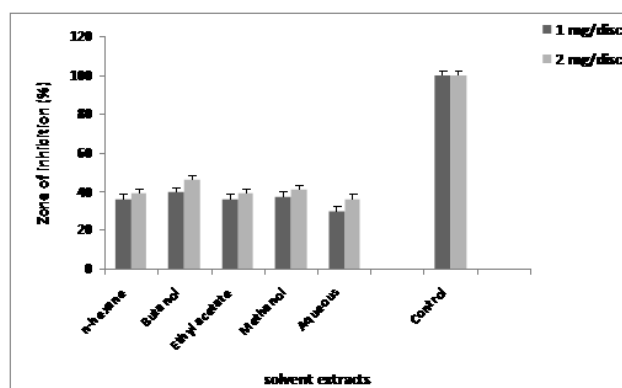
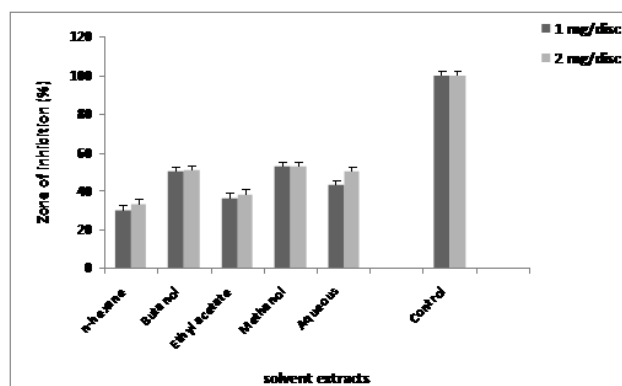


Fig. 10: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, butanol and water extracted samples from commercially available samples of *Berberis lyceum* Royle against *S. aureus* by disc diffusion assay (Bar shows LSD at $p < 0.05$).

Table 1: Microbial strains used during the experiment

Microbial Species	Gram strain type	Details of the Microbial strains used
<i>K. pneumoniae</i>	Negative	Clinical isolate obtained The Department of Microbiology, Microbiology, Quaid-I-Azam University Islamabad Pakistan
<i>P. aeruginosa</i>	Negative	ATCC # 9721
<i>S. aureus</i>	Positive	ATCC # 6538
<i>B. subtilis</i>	Positive	Clinical isolate obtained The Department of Microbiology, Microbiology, Quaid-I-Azam University Islamabad Pakistan
<i>E. coli</i>	Negative	ATCC # 25922
<i>C. albicans</i>		ATCC # 10231. Plant Pathology Department, The University of Agriculture Peshawar KPK Pakistan

**Fig. 11:** Antifungal activity of crude methanol, n-hexane, ethyl acetate, butanol and water extracted samples from fresh samples of *Berberis lyceum* Royle against *C. albicans* by disc diffusion assay (Bar shows LSD at $p < 0.05$).**Fig. 12:** Antifungal activity of crude methanol, n-hexane, ethyl acetate, butanol and water extracted samples from commercially available samples of *Berberis lyceum* Royle against *C. albicans* by disc diffusion assay (Bar shows LSD at $p < 0.05$).

DISCUSSION

Fresh and commercially available plant materials (roots) of *Berberis lyceum* were investigated for their antimicrobial potentials against five bacteria and one fungal strain through disc diffusion assay. The data

revealed that different solvent extracted samples from both sources measured varying degree of antimicrobial activity. The antimicrobial activity increased with increasing concentrations of the samples obtained from both sources. The data indicated that hexane fraction from the fresh samples effectively reduced the activity of *B. subtilis*, *P. aeruginosa* and *K. pneumoniae* when applied at higher concentration (2 mg disc^{-1}) compared with other samples and controls. Similarly, crude methanolic extract from fresh samples measured maximum activity against *E. coli* and *S. aureus* at higher concentration. In case of antifungal activity, butanol fraction from fresh samples showed good activity against *C. albicans* at 2 mg disc^{-1} . Ethyl acetate fraction from fresh samples on the other hand revealed moderate activity against the tested microbes. In case of commercially available samples, ethyl acetate recorded maximum activity against *B. subtilis* and *K. pneumoniae* when tested at higher concentrations. Crude methanolic extract was more effective among all samples and effectively reduced the growth of *P. aeruginosa*, *E. coli* and *S. aureus* at 2 mg disc^{-1} followed by butanol fractions at the same concentration. The rest of the samples revealed moderate activity against all the tested microbes by disc diffusion assay. The possible bioactive compound in the Berberidaceae family is berberine which is reported for various infectious diseases viz. Cholera (Dutta and Panse, 1962), acute diarrhea (Lahiri and Dutta, 1967), amoebiasis, latent malaria, oriental, sore and skin infections (Anonymous, 1988). In the case of tested microbes and their differences in susceptibility might be due to the differences in the cell wall composition of Gram positive and Gram-negative bacteria (Grosvenor *et al.*, 1995). Altaf *et al.* (2011) reported that the ethanolic and aqueous crud root extract of the root of *Berberis lyceum* Royle were more effective against *S. aureus*, *S. epidermidis*, *B. subtilis*, *S. typhi*, *E. coli* and a fungal strain *C. albicans*, while no significant activity was shown by the petroleum ether extract against test organisms. Similar results are also reported by Singh *et al.* (2009). Our results were innovative for two reasons when compared with the findings of other researchers. We first isolated the crude extracts and then fractionated the crude with different solvents from 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.

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