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ANTIMICROBIAL POTENTIAL AND PHYTO CHEMICAL ANALYSIS OF DIFFERENT SOLVENT EXTRACTED SAMPLES OF *VIOLA PILOSA*

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Abstract

Investigation of susceptibility of medicinal plants for bacterial pathogens is significant for suitable choice of treatment. Different solvent extracted samples of *Viola pilosa* shoots were investigated for their antibacterial and phytochemical activities using 0.5, 1 and 2 mg disc^{-1} concentrations. The antibacterial bioassay was assayed by disc diffusion method against six microbes. The studies revealed that ethyl acetate extracted fractions resulted in maximum growth inhibition of *Pseudomonas aeruginosa* and *Staphylococcus aureus* at 2000 µg disc^{-1} concentration. Similarly, *Xanthomonas campestris* and *Klebsiella pneumonia* were found more susceptible to n-butanol extract. Maximum reduction in the activity of *B. subtilis* and *E. coli* was recorded by n-hexane fractions at two mg per disc. The most susceptible microbe was *Pseudomonas aeruginosa.* The results further revealed that all the tested microbes were found completely resistant to water extracted fractions at all the tested concentrations measuring 0% ZI. Phytochemical analysis showed the presence of various bioactive compounds including flavonoids, glycosides, proteins, fats, alkaloids, steroids, saponins, carbohydrates and tannins.

Key words: Antibacterial activity, Disc diffusion assay, *Viola pilosa*, Phytochemical analysis.

Introduction

The use of herb as a medicine is as old as mankind itself (Verpoorte, 2000; Shinwari *et al*., 2006; Petrovska, 2012). About 6% have been analyzed for their biological activity and 15% for phyto-chemicals (Verpoorte, 2000). Some societies value these plants due to the prehistoric belief which states that plants are generated to supply man with food, medical treatment, and other effects (Hunt, 2000; Habiba *et al*., 2016). It is reported that medicinal plants produce a number of secondary metabolites having antimicrobial activity (Mari *et al*., 2003; Ullah *et al.*, 2015; Ahmad *et al*., 2015; Nasir *et al*., 2015; Amjad *et al*., 2016; Wajid *et al*., 2016; Bilal & Bakht, 2016; Tareen *et al*., 2016; Bilal *et al*., 2017).

Viola pilosa Blume also known as "Banafsha." belongs to the genus *Viola* of flowering plants in the violet family Violaceae or Leoniaceae which include 20 genera and nearly 800 species (Mabberley, 1987). In Pakistan the genus *Viola* (Violaceae) consists of 17 species distributed in several localities (Qaiser & Omer, 1985). *Viola pilosa* Bume is a small glabrous, perennial herb is found in moist woods and hilly districts of China, Java, Ceylon, Philippines, India and Thailand up to an altitude of 2000 m (Vishwakarma *et al.*, 2013). In Pakistan, is usually found in Siran and Swat valleys. The whole plant is therapeutically beneficial (Vishwakarma *et al.*, 2013). Abbasi *et al.* (2009) revealed presence of glycoside methyl salicylate, quercitrin, alkaloid, voiline gum, mucilage, sugar and saponins in Viola serpens Wall. Taking in account the medicinal value of *Viola pilosa*, the current investigate the phyto-chemistry and antimicrobial potential of samples extracted from the shoots of *Viola pilosa* Blume by the disc diffusion assay.

Materials and Methods

Plant materials: The current investigation was carried out at IBGE, The Univ. of Agric. Peshawar, Pakistan. The plants of *Viola pilosa* Blume were collected from Swat Valley and identified by plant taxonomists at Department of Botany, University of Peshawar.

Preparation of crude extracts: Plant materials (shoots) were carefully washed with distilled water, at room temperature under shade for two weeks and grinded into fine powder by mechanical grinder (Thomas Scientific USA). Analytical grade methanol was mixed with the milled roots and kept for 10 days with periodic shaking to dissolve the bioactive compounds. After soaking for 10 days, the solvent together with its constituents was filtered through Whatman No. 1 filter paper and methanolic solution was evaporated at 45ºC by rotary evaporator. The crude extract was dried in water bath at 35ºC, weighed and divided into two parts. Part of the extract (ten g) was assayed as crude methanolic extract and the remaining part (80 g) was partitioned with various solvents.

Fractionation of extract: The dried samples of shoots were mixed with 300 ml distilled H_2Owater and transferred to separatory funnel for further partitioning. For this purpose, 300 ml of n-hexane was mixed and the upper n-hexane portion was collected and the remaining lower aqueous phase was re-partitioned with fresh n-hexane. The whole procedure was repeated in triplicate. All the n-hexane fractions were pooled to gathered, filtered using Whatman No. 1 filter paper and dried to a semi- solid material under vacuum pressure by rotary evaporator. This semi-solid dried fraction was kept at 35°C in water bath for further drying. Similar method of partitioning was performed for other solvents. After the completion of the whole process, the lower aqueous phase was collected and dried as described earlier.

Disc diffusion susceptibility assay: Antimicrobial activity against various strains of bacteria (Table 1) was performed by disc diffusion susceptibility assay (Bauer *et al.*, 1966). Whatman No. 1 grade filter paper disks were impregnated with extract concentration of 0.5, 1 and 2 mg in 6, 12 and 18 micro litter volumes and the inoculated

plates were kept at 37 ºC for 24 hours and measured ZI as described below:

$$\text{Inhibition } \% = \frac{\text{Growth of control}}{\text{Growth of sample}} \times 100$$

Positive controls: Ciprofloxacin 50 µg per 12 µl was used for Gram-negative and Gram-positive bacteria and DMSO for negative control.

Statistical analysis: The data is presented as mean of triplicate data and LSD test was used at $p<0.05$ (Steel *et al*., 1997).

Phytochemical analysis: Crude methanolic shoots extracts of *Viola pilosa* were tested for the presence of proteins, alkaloids, carbohydrates, flavanoids, terpenoid (Siddiqui & Ali, 1997), phytosterols, oils, fats, tannins (Iyengar, 1995), saponins and glycoside (Harborne, 1988).

Results

The data indicated that the tested microbe showed maximum sensitivity to ethyl acetate, n-hexane, butanol and methanol extracts (Fig. 1). Maximum inhibitory zone of 45% was revealed by butanol samples at 2 mg $disc^{-1}$ concentration and the lowest by n-hexane fractions (22.2%) at 0.5 mg $disc^{-1}$. However, aqueous extracts presented no activity. Figure 2 illustrates the potential antibacterial activity of *Viola pilosa* shoots extract against *Pseudomonas aeruginosa.* The results indicated that inhibition in the activity of the tested microbe was dose dependent. Among different extracts, the highest activities (53.45%) were recorded by ethyl acetate fraction at 2 mg per disc and n-hexane (51.37%) at similar concentration. However, no activity was shown by water extracts against the same microbe. The antibacterial potential of fractions extracted from *Viola pilosa* against *Staphylococcus aureus* is indicated Fig. 3. The results indicated that ethyl acetate fraction was more effective to reduce the activity of the studied microbe by 45.12% at the maximum levels of 2 mg $disc^{-1}$ and hexane extracted fraction (41.38% ZI at similar concentration. The results further suggested that the butanol and water fractions did not show any zone of inhibition of the same microbe at any concentration used showing 0% ZI. Crude methanolic fraction also revealed good inhibiting activity against *S. aureus* to varying degree.

The data showed that all samples inhibited the growth of *Bacillus subtilis* except aqueous-extracted fraction which did not show activity at any concentration (Fig. 4). The highest zone of inhibition (35.94%) against *B. subtilis* was recorded by n-hexane extracts at 2 mg $discs^{-1}$ followed by ethyl acetate fraction with ZI of 32.73% at the same concentration. Crude methanolic extract and n-butanol extracted fraction showed moderate activity against *B. subtilis* while zone of inhibition was measured for water extracts at any concentration. *Escherichia coli* was more susceptible to n-hexane extracts recording maximum growth inhibition (45.12%) at of 2 mg $disc^{-1}$. *E. coli* also exhibited moderate sensitivity against ethyl acetate and crude methanolic samples i.e., 37.25% and 36.54% activity at 2 mg per disc correspondingly. These fractions were also effective at 1 and 0.5 mg $discs^{-1}$ against the same bacterium. The results also revealed that water extracts showed no activity against *E. coli* (Fig. 5). *Xanthomonas campestris* was found resistant to aqueous extracts showing no activity at any concentration. Maximum growth inhibition was showed by n-butanol extracts (48.38% ZI) at 2 mg $disc^{-1}$. The study further revealed that n-hexane and crude methanolic extracts possessed good activity of 39.77% and 33.32% ZI at 2 mg $disc^{-1}$ concentration respectively against *X. campestris* when compared to controls (Fig. 6).

In the current study various extracts from the shoots of *Viola pilosa* were subjected to phytochemical screening according to the standard procedures of analysis (Table 2). Crude methanolic extracts were found rich in tannins, carbohydrates and sterols. Phytochemical analysis of the crude extract also showed moderate quantity of alkaloids, fats, oils, flavonoids and significant traces of proteins, and saponins. The analysis further indicated that n-hexane showed negative results for alkaloids, oils, fats, saponins and tannins. Similarly ethyl acetate was found negative for alkaloids and saponins. The butanol extracted fraction showed tremendous results by exhibiting very good content of all the phytochemicals under study. The results also indicated the good content of alkaloids, tannins, carbohydrates, fats, oils, sterols and moderate content of proteins and flavonoids in the water extracted fractions.

Table 1. Microbial strains tested during the present experiment.

Microbial species	Gram strain type	Details of the microbial strains used
Klebsiella pneumoniae	Negative	Clinical isolate obtained from the Microbiology Department, Quaid-i-Azam University Islamabad, Pakistan
Pseudomonas aeruginosa	Negative	ATCC # 9721
Staphylococcus aureus	Positive	ATCC # 6538
Bacillus subtilis	Positive	Clinical isolate obtained from the Department of Microbiology, Quaid-i-Azam University Islamabad, Pakistan
Escherichia coli	Negative	ATCC # 25922
Xanthomonas campestris	Negative	ATCC # 33913

Table 2. Phytochemical profile of solvent extracted samples from shoots of *Viola pilosa*.

Extract	Alkaloids	Proteins	Tannins	Carbohydrates	Sterols	Flavonoids	Saponins	Fats and Oils
Crude	++	+	+++	+++	+++	++	+	++
N-Hexane	_	+	++	++	+	+	_	+++
Butanol	++	+	+++	+++	++	++	+++	+
E. acetate	_	+	+	+	++	++	_	_
Aqueous	++	+	+++	+++	++	+	_	+++

+++: Shows the presence in abundance, ++: Shows presence in moderate quantity, +: Shows presence but in less amount __: Shows complete absence of the compound

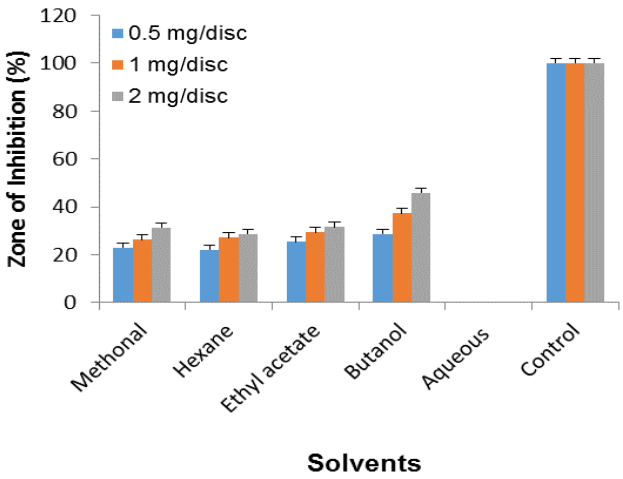

Fig. 1. Antibacterial activity of crude methanol, hexane, ethyl acetate, butanol and aqueous extracted samples from the shoot of *Viola pilosa* against *K. pneumoniae* by disc diffusion assay (Bar shows LSD at p<0.05).

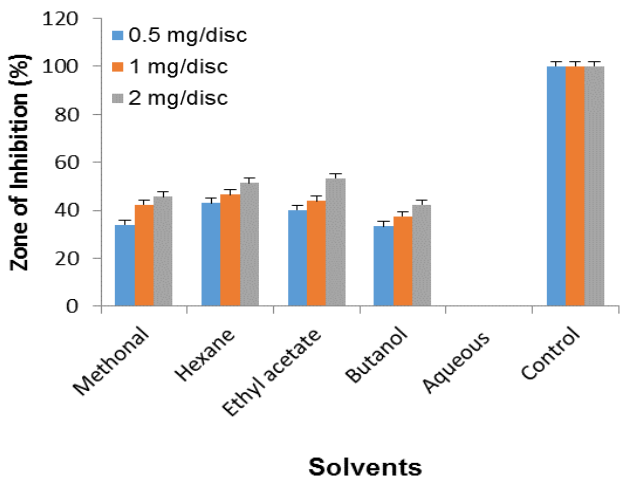

Fig. 2. Antibacterial activity of crude methanol, hexane, ethyl acetate, butanol and aqueous extracted samples from the shoot of *Viola pilosa* against *P. aeruginosa* by disc diffusion assay (Bar shows LSD at p<0.05).

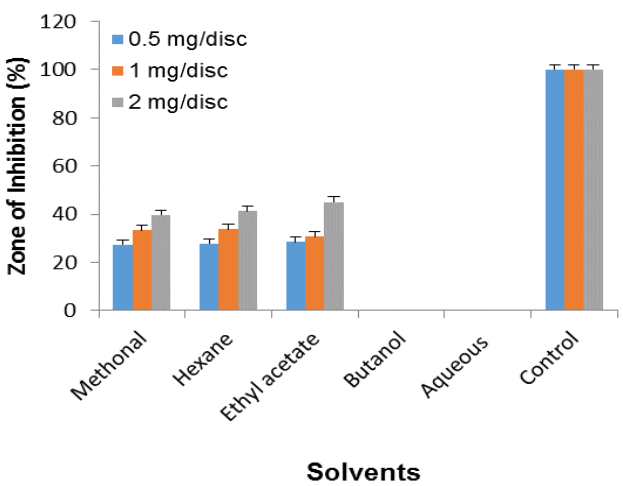

Fig. 3. Antibacterial activity of crude methanol, hexane, ethyl acetate, butanol and aqueous extracted samples from the shoot of *Viola pilosa* against *S. aureus* by disc diffusion assay (Bar shows LSD at p<0.05).

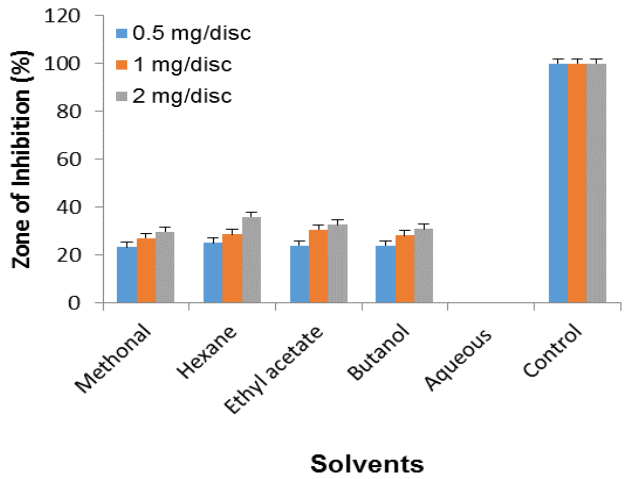

Fig. 4. Antibacterial activity of crude methanol, hexane, ethyl acetate, butanol and aqueous extracted samples from the shoot of *Viola pilosa* against *B. subtilis* by disc diffusion assay (Bar shows LSD at p<0.05).

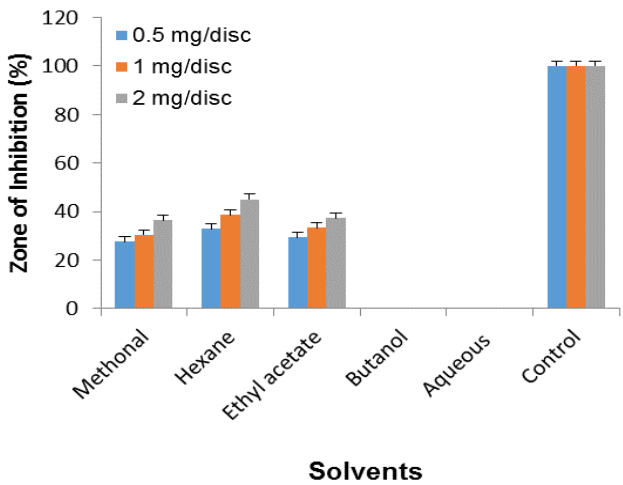

Fig. 5. Antibacterial activity of crude methanol, hexane, ethyl acetate, butanol and aqueous extracted samples from the shoot of *Viola pilosa* against *E. coli* by disc diffusion assay (Bar shows LSD at p<0.05).

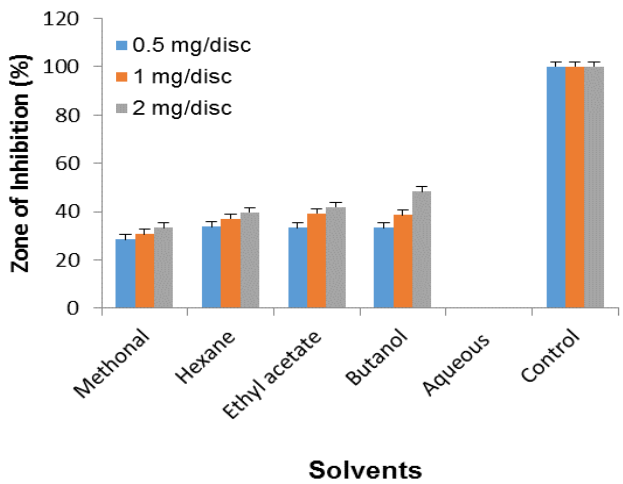

Fig. 6. Antibacterial activity of crude methanol, hexane, ethyl acetate, butanol and aqueous extracted samples from the shoot of *Viola pilosa* against *X. campestris* by disc diffusion assay (Bar shows LSD at p<0.05).

Discussion

The current investigation examines the antimicrobial activity and phytochemical analysis of various solvent extracted fractions from the shoots of *Viola pilosa*. The results showed that *Klebsiella pneumoniae* was susceptible to ethyl acetate, n-hexane, butanol and methanol extracts presenting high zone of inhibitions. Maximum activity was measured by butanol extracted fraction at the highest concentration and lowest by n-hexane extracted samples at lowest concentration. Aqueous extracted fraction was found to be in effective against the tested microbe at all concentrations. Our results agree with Daoud *et al.* (2012) who concluded that crude methanolic extracted samples and its fractions of *V. odorata* showed antibacterial activities with *K. pneumonia*, however, aqueous fraction did not exhibit any activity against *Klebsiella sp*. Among different solvent extracted fractions, ethyl acetate was more effective against *P. aeruginosa* with the highest zone of inhibition at a levels two mg per disc. Aqueous extracted fractions did not affect the activity of the tested microbe at all concentrations. Similar results were also reported by Borchardt *et al.* (2008), Pranting *et al.* (2010), Akhbari *et al.* (2012), Gautam *et al*. (2012) and Muhammad *et al*. (2013). They concluded effective control in growth of *P. aeruginosa* by the crude methanolic extracts from *V. tricolor, V. canadensis, V. betonicifolia* and *V. odorata*. Ethyl acetate extracts recorded maximum inhibiting activity against *S. aureus* followed by n- hexane extracted fraction at the same level of concentrations. Butanol and aqueous fractions were in-effective against *S. aureus*. These findings corresponds to Arora *et al*. (2007), Ahmad *et al.* (2006), Khan *et al.* (2011) Vuuren *et al.* (2008) and Tariq *et al*. (2016) who concluded that crude methanolic extracts of various medicinal plants was effective against *S. aureus*.

The data also suggested that n-hexane extract recorded highest activity against *B. subtilis* at the highest concentration as compared with other solvents extracted fractions where low activity was measured. Water extract measured 0% ZI at any concentration. Our results agree with Sahin *et al.* (2003), Xie *et al*. (2004), Simmonds (2004) Prasad (2014) and Ahmed *et al*. (2015). Shoot extracts of *Viola pilosa* showed highest inhibitory activity against *E. coli* except aqueous extracted fraction. Maximum activity was recorded by n-hexane extracted fractions and lowest by aqueous extracted fraction. Moderate antibacterial activity was measured by ethyl acetate and crude methanolic extracts against the tested microorganism. These findings are in agreement with Pranting *et al.* (2010), Sun *et al*. (2011), Daoud *et al.* (2012) and Muluye *et al*. (2014). X. *campestris* was highly resistant to aqueous extracted fractions showing no activity at any concentration and n-butanol extracted fraction showed maximum inhibiting activity. Ethyl acetate extracted sample on the other hand, was also effective to reduce the growth of *X. campestris*. Our results also indicated that n-hexane and crude methanolic extracts revealed moderate activity against *X. campestris.* These results are in agreement with Mahesh & Satish (2008) and Roshan *et al*. (2014).

Phytochemical analysis showed the occurrence of various groups of compounds. Phytochemical screening results demonstrated that methanol crude extracts of *Viola pilosa* were found rich in tannins, carbohydrates and sterols. The tested crude methanolic extract further showed moderate quantity of alkaloids, fats, oils, flavonoids and considerable traces of proteins, and saponins. The n-hexane fraction was found negative for alkaloids, oils, fats, saponins and tannins. Likewise, ethyl acetate gave negative results for alkaloids and saponins. Aqueous extracted fraction contained appreciable c ontent of alkaloids, tannins, carbohydrates, fats, oils, sterols and moderate content of proteins and flavonoids. Similar results were also reported by Vishal *et al*. (2009), Adhikary *et al*. (2011) and Muhammad *et al.* (2012).

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