Antimicrobial Screening of Viola betonicifolia

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Abstract: The crude methanolic extract and subsequent solvent fractions of Viola betonicifolia whole plant were tested for antibacterial and antifungal activities. In vitro antibacterial bioassay was performed against seven bacteria viz. Escherichia coli, Salmonella typhi, Staphylococcus aureus, Enterobacter aerogenes, Proteus mirabilis, Enterococcus fæcalis and Bacillus cereus. The chloroform fractions showed best activity against E. coli and S. typhi with zone of inhibition of 20 mm and 17 mm respectively. All the fractions were tested against seven fungi viz. Saccharomyces cerevisiae, Aspergillus parasiticus, Juncus effuses, Aspergillus niger, Trichophyton rubrum, Candida albicans and Fusarium solani. Crude extract, ethyl acetate and chloroform fractions exhibited reasonable activity against all tested fungi except C. albicans.

Key words: Viola betonicifolia % Antibacterial and antifungal

INTRODUCTION

Viola betonicifolia belongs to family Violaceace. Locally, it is known as banafsha. It is perennial herb of 8-20 cm in height. The stem of the plant is absent and leaves are triangular or obtuse and petiole is longer than lamina. Roots are slender, unbranched and rhizome is short. V. betonicifolia is available in various countries of the word like Pakistan, India, Nepal, Sri Lanka, China, Malaysia and Australia. In Pakistan, it is available in Swat, Hazara and Dir. Traditionally, plant has been used as antipyretic, astringent, diaphoretic, anticancer, purgative, epilepsy, nervous disorders and cough [1]. Some other uses are Sinusitis, skin, blood disorders, pharyngitis [2], kidney diseases, pneumonia, bronchitis. Flowers are used in lung troubles, cough and boil [3]. In continuation to our research work on Pakistani medicinal plants [4-7], we investigated V. betonicifolia for various biological activities. Recently we have reported various in-vitro and in-vivo pharmacological activities of this plant [8-11], however, in this piece of research work we discussed the results of antimicrobial activities of the crude extract and its various solvent fractions.

MATERIALS AND METHODS

Plant Material and Extraction: Whole plant of V. betonicifolia was collected from Swat, Khyber Pakhtoonkhwa in April 2010. Plant specimen was identified by Prof. Dr. Muhammad Ibrar, Department of Botany, University of Peshawar and specimen was deposited there in the herbarium under voucher number 6410/Bot. The collected whole plant (12 kg) was air dried and powdered. The powdered was extracted by maceration with methanol at room temperature for 14 days with occasional shaking [12]. The methanolic extract was filtered and concentrated by rotary evaporator at low temperature (45°C). The methanolic extract was dissolved in distilled water and further fractioned with chloroform, hexane, ethyl acetate, butanol and aqueous fractions. The crude extract and its subsequent fractions were screened for antimicrobial activities.

Antibacterial Activity: The crude methanolic extract as well as its subsequent solvent fractions and the sub fractions of isolated fixed oils were evaluated for their antibacterial potential, against different gram positive and
Table 1: Reference of bacterial and fungal strains.

<table>
<thead>
<tr>
<th>Bacterial</th>
<th>Reference bacterial strains</th>
<th>Fungi</th>
<th>Reference fungal strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>ATCC 25922</td>
<td>T. longifusus</td>
<td>(clinical isolate)</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>ATCC 6633</td>
<td>C. albicans</td>
<td>ATCC 2091</td>
</tr>
<tr>
<td>S. flexneri (clinical isolate)</td>
<td>ATCC 25923</td>
<td>A. flavus</td>
<td>ATCC 32611</td>
</tr>
<tr>
<td>S. aureus</td>
<td>ATCC 25923</td>
<td>M. canis</td>
<td>ATCC 11622</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>ATCC 27853</td>
<td>F. solani</td>
<td>ATCC 11712</td>
</tr>
<tr>
<td>S. typhi</td>
<td>ATCC 19430</td>
<td>C. glaberata</td>
<td>ATCC 90030</td>
</tr>
</tbody>
</table>

gram negative pathogenic bacteria as presented in Table 1, by agar well diffusion method [13]. Shortly, 3 mg/mL of either crude extract or subsequent solvent fraction was dissolved in dimethyl sulfoxide (DMSO) for the preparation of stock solution. Approximately 45 mL of molten nutrient agar was dispensed in sterilized petri-plates, and was permitted to harden. Bacterial culture was dispersed on these nutrient agar plates by preparing sterile soft agar accumulating 100 µL of bacterial culture. 6 mm long sterile metallic borer was used for well digging at suitable distance and spotted for identification. Sample (100 µL) was poured into each well, and kept in incubator for 24 h at 37°C. The antibacterial activity was observed in the form of zone of inhibition (mm). Standard antibacterial drug was Imipenem (broad spectrum antibacterial) in the assay while DMSO was used as negative control.

**Antifungal Activity:** The crude methanolic extract, it succeeding solvent fractions and the sub fractions of isolated fixed oils were evaluated for their antifungal potential. To evaluate the antifungal activity, sterile agar plates were used according to the disc diffusion assay [14]. Activated cultures of fungal strains (S. cerevisiae, A. parasiticus, A. effuses, A. niger, T. rubrum, C. albicans and F. solani) in Sabouraud’s broth were adjusted to 1 x 10⁴ CFU/mL as per Mcfarland standard. 100 mL of the inoculum was introduced to molten sabouraud dextrose agar and poured in the sterile Petri plates. Sterile filter paper discs (8 mm diameter) were impregnated with 125 mg/disc of the extract/solvent fraction which was dissolved in 100 % DMSO and dried. The discs were placed on fungal seeded plates and incubated at 28°C for 48 h. Discs supplemented with only 100 % DMSO served as control. The effect of the extract on fungal isolates was compared with amphothericin B and Itraconazole. The reference fungal strains are depicted in Table 1.

**Antibacterial Effect:** The crude methanolic extract and its subsequent solvent fractions exhibited weak to moderate antibacterial activity against various tested bacteria as shown in Table 2 and Figure 1. The maximum effect was demonstrated by crude methanolic extract and its succeeding chloroform and ethyl acetate fractions. The zone of inhibition of all the fractions was compared with broad spectrum antibacterial drug (imipenem, 10µg/disc). The maximum antibacterial effect against E.coli was exhibited by chloroform fraction followed by ethyl acetate fraction, crude methanolic extract, butanolic and aqueous fraction with zone of inhibition 20, 10, 5, 4 and 3 mm respectively and the percent antibacterial effect of these fractions were 55.54, 27.77, 13.88, 11.11 and 3.8 % respectively. The highest antibacterial effect against S.typhi was observed with chloroform fraction followed by ethyl acetate, methanolic extract and aqueous fraction with zone of inhibition 17, 15, 7 and 6 mm respectively and the percent effect of these samples against S.typhi was 42.50, 37.52, 17.50 and 15.00 respectively. The growth of P. aerogenes was inhibited most effectively by chloroform fraction followed by methanolic extract and ethyl acetate fraction with zone of inhibition 9, 8 and 5 mm respectively and the percent activity of these samples were 25.71, 22.85 and 14.28 respectively. The maximum antibacterial potential against P. mirabilis was showed by chloroform fraction followed by ethyl acetate and methanolic extract with zone of inhibition 10, 5 and 4 mm respectively, while
Fig. 1: Percent antibacterial effect of crude methanolic extract and its subsequent solvent fractions.

The percent effect of these test samples was 27.77, 13.88 and 11.11 respectively. The maximum antibacterial effect against *S. flexneri* was noticed with chloroform fraction followed by ethyl acetate fraction, crude methanolic extract, butanolic and aqueous fraction with zone of inhibition 8, 5, 5, 4 and 3 mm respectively and the percent effect was 19.51, 12.19, 12.19, 9.75 and 7.31 % respectively. All the tested samples were found with negative antibacterial activity against *S. aureus*. The antibacterial activity exhibited against *B. subtilis* was higher by chloroform followed by ethyl acetate, methanolic extract and butanolic fraction with zone of inhibition 12, 6, 5 and 4 mm respectively, while the percent activity of these samples were 33.33, 16.66, 13.88 and 11.11 respectively. No antibacterial activity was shown by *n*-hexane fraction. It is clear from our results that the crude methanolic extract, chloroform and ethyl acetate fractions are the rich source of antibacterial agents. The use of this plant or particularly the subfractions in the treatment of various ailments caused by these bacteria can provide a safe and natural remedy. As the plant is recommended in the treatment of various bacterial infections like sinusitis, pharyngitis, pneumonia, bronchitis, cough, colds and boils therefore this antibacterial study provides a scientific background to the ethnomedicinal uses of this plant.

**Antifungal Effect:** The antifungal effect of the crude methanolic extract and its various solvent fractions are presented in Table 3. The crude methanolic extract was effective against *T. longifus* and *M. canis* with 30 % and 20% inhibition, while it was not effective against
remaining fungi. The n-hexane fraction showed 17% inhibition against *M. canis* and for rest of tested fungi it was not fungicidal. The chloroform fraction showed 20 and 7% inhibition against *M. canis* and *F. solani* and rest of fungi were resistant to other tested samples. The ethyl acetate fraction exhibited similar effect like chloroform fraction and showed 30 and 10% inhibition against *M. canis* and *F. solani*. The butanolic fraction was not fungicidal against all of the tested fungi, while aqueous fraction showed 30 and 40% inhibition against *C. albicans* and *M. canis*. *M. canis* is an organism that can cause tinea capitis in humans and ringworm in pets. [15]. Its most important reservoir is pet animals like tested for their antibacterial potential against various 

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Standard</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. longius</td>
<td>Miconazole 70</td>
<td>30</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C. albicans</td>
<td>Miconazole 110.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>A. flavus</td>
<td>Amphotericin 20</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>M. canis</td>
<td>Miconazole 98.4</td>
<td>20</td>
<td>17</td>
<td>20</td>
<td>30</td>
<td>-</td>
<td>40</td>
</tr>
<tr>
<td>F. solani</td>
<td>Miconazole 73</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>10</td>
<td>-</td>
<td>-</td>
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<tr>
<td>C. glaberrata</td>
<td>Miconazole 110.8</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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A: Methanolic, B: n-hexane, C: Chloroform, D: Ethyl acetate, E: Butanolic, F: Aqueous

**DISCUSSION**

Different natural sources like microorganisms, animals and plants have been exploited for the discovery of new, safe and effective antimicrobials but irrational uses of antibiotics have led to the development of antibiotic resistance. Therefore, the discovery of new antimicrobials is the demand of present modern era to cope with the challenges of microbial resistance in life threatening infections [17]. In this connection, we tested crude methanolic extract, its various solvent fractions of the whole plant of *V. betonicifolia* for their antibacterial effect against both gram positive and negative susceptible bacteria. The present antimicrobial study is strongly supported by the reported antimicrobial literature of the related species of this valuable medicinal plant. The significant antibacterial activity of the aqueous extract of *Viola odorata* against *S. typhi* and *E. coli* has been reported, the crude ethanolic extract and its subsequent solvent fractions (petroleum ether, dichloromethane and ethyl acetate) were proved significant antibacterial against *E. coli* and *K. pneumonia* [18]. The antibacterial potential of the methanolic extract and its solvent fraction of *Viola tricolor* has been published in 2005 [19]. The methanolic and aqueous extract of the flower of *Viola odorata* showed moderate activity against salmonella typhi, salmonella typhi murium and salmonella paratyphi A the aqueous extract was more bactericidal than that of methanolic extract [20]. The methanolic and chloroform extract of the leaves of Iranian *Viola odorata* and it essential oils have been tested for their antibacterial potential against various bacteria. The methanolic extract showed antibacterial activity against *P. aeruginosa*, *E. coli*, *S. epidermidis* and *P. vulgaris*, the chloroform fraction showed activity against *S. epidermidis* and *P. vulgaris*, while essential oils showed activity against *B. subtilis*, *K. pneumonia* and *S. epidermidis* [21]. The cyclotide (vhl-1) isolated from *Viola hederacea* was tested against *E. coli* and *S. aureus* but the growth of tested bacteria was not inhibited [22]. The crude ethanolic extract and its solvent fraction (dichloromethane ethyl acetate, methanolic) of *Viola tricolor* whole plant was responsible for low to moderate antifungal activity against *C. albicans* [19]. The methanolic and chloroform extract of Iranian *viola odorata* and the essential oils of this plant showed no antifungal activity against *C. albicans* [21]. The essential oils of *Viola odorata* has exhibited moderate activity against the hypha and spores of Aspergillus niger [23]. The cyclotide (vhl-1) isolated from *Viola hederacea* was tested against *C. albicans* but the growth of the tested fungus was not inhibited [22].

**CONCLUSION**

In conclusion, the plants showed significant antimicrobial activity against both Gram negative and Gram positive bacteria as well various fungus. The plant is using in traditional system of medicines for the
treatment of various infectious disorders like throat infections, bronchitis, pneumonia and urinary infections. Therefore, the current study validated the folk uses of the plant in infectious diseases.

ACKNOWLEDGMENT

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REFERENCES