EVALUATION OF IN VITRO ANTIMICROBIAL ACTIVITY AND ESSENTIAL OIL COMPOSITION OF ETHANOL EXTRACT OF VIOLA ODORATA L. LEAVES

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ABSTRACT

Viola odorata L. belongs to the Violeaceae family. It was used as a medicine to treat bronchitis, cystitis and tonsillitis, nevertheless, exploration of scientific justifications behind its use can lead into developing new applications. Leaves of V. odorata L. was extracted using methanol and ethanol. Consequent extracts were tested for the antimicrobial activity and the minimum inhibitory concentration (MIC) using the agar dilution method and the broth microdilution method respectively. Phytoconstituents isolates were also subjected to ensure their antimicrobial activity. Essential oil composition of methanol fraction of ethanol extract was analysed by GC-MS technique.

Methanol and ethanol extracts of the leaves of V. odorata L. were found effective against all tested strains of bacteria whereas fungi showed resistance to all extracts. Ethanol extract exhibited higher inhibition against E. coli (10 mg/ml), Bacillus subtilis (20 mg/ml), Staphylococcus aureus (20 mg/ml) and Pseudomonas aeruginosa (40 mg/ml). Isolated phytoconstituents also showed significant inhibition against all bacterial strains. Methanol and ethanol extract showed considerable inhibition against all tested bacteria. The GC-MS analysis of active fraction revealed the presence of Pentane 2,3,4- Trimethyl (45%), N-Hexadecanoic acid (28.85%), 10- Undecyn-1-ol (14.43%) and Pentadecanoic acid (8.14%). These results provide scientific justification for traditional use of V. odorata L. for the
treatment of bronchitis, cystitis and tonsillitis and data that could lead into new biotechnological applications.

**KEY WORDS:** Antimicrobial activity; *Viola odorata* L.; Phytoconstituents; Bronchitis; Cystitis; Tonsillitis.

**INTRODUCTION**

Plants having medicinal importance have been used for years in daily life to treat ailments all over the world. Plants produce many natural compounds that can be used as antimicrobial agents.\(^1\) *Viola odorata* L. (Violaceae), also known as Sweet violet, is distributed in various countries including India, Pakistan, Iran, Iraq and Afghanistan. It is an ornamental plant having violet flower.

*Viola odorata* L. are traditionally used to treat skin conditions, bronchitis, cystitis and rheumatism.\(^2\) Flowers of *Viola odorata* L. are used by tribal people of India for treatment of throat infection and tonsillitis.\(^3\) Tribal healers use whole plant to treat diaphoretic, febrifuge, infantile disorder and lung ailments.\(^4\)

*Viola odorata* L. produces cyclotides, small peptides containing 28-37 amino acids. Cyclotides possess various important biological activity including uterotonic, insecticidal, anti-HIV, antimicrobial, antineurotensive, cytotoxic and haemolytic activity.\(^5\) Hence, exploring the scientific justifications behind its traditional use could support to find the probability to develop further new biotechnological applications, therefore, the present study was planned to evaluate antimicrobial activity of *V. odorata* L. leaves extracts. Along this principal objective, the essential oil composition of ethanol extract of *Viola odorata* L. leaves was also evaluated.

**MATERIALS AND METHODS**

**Plant material**

Leaves of *Viola odorata* L. were collected from Baisaran, Jammu and Kashmir, India. Plant was authenticated by Dr. Padamnabhi S. Nagar, Department of Botany, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. Voucher specimens (VS1, VS2, VS3 and VS4) were deposited at Department of Botany, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. Plant leaves were cleaned with deionized water and
dried at shade for a week. Shade dried leaves were grounded in powder form by using electrical grinder and stored in air tight container and maintained at 4°C until use.

**Preparation of extract**

Solvent systems used for the extractions were water, methanol and ethanol. Twenty grams of powdered samples were packed in muslin cloth and used for extraction by soxhlet apparatus at 100°C, 64°C and 78°C for obtaining water, methanol and ethanol extracts, respectively. After 38 hours, extracts were filtered using Whatman filter paper No. 1. Solvent of each extract was evaporated by vacuum dryer and residue was dissolved in 5% dimethylsulfoxide (DMSO) in 100 mg/ml concentration and stored at 4°C for further antibacterial activity study.

**Microbial culture**

Reference bacterial strains viz. *Escherichia coli* (MTCC 119), *Pseudomonas aeruginosa* (MTCC 424), *Bacillus subtilis* (MTCC 121), *Aspergillus niger* (MTCC282) and *Candida albicans* (MTCC 3017) were purchased from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh. Clinically isolated *Staphylococcus aureus* was obtained from Dr. Krunal Prajapati pathological laboratories, Anand, Gujarat, India. They were maintained in their respective culture conditions.

**Agar diffusion method**

Sensitivity of different bacterial strains to aqueous/organic extracts was measured in terms of zone of inhibition using agar diffusion method. Inoculum was prepared by inoculating a loopful isolated colony into 50 ml of sterile nutrient broth (Hi- Media, India) and incubated at 37°C for overnight. Culture density was adjusted at 1.5 x 10^8 cells/ml by taking optical density at 600nm. Muller Hinton agar plates (Hi- Media, India) were spread with 100 μl inoculum. Wells (8 mm Diameter) were made in agar plates using a sterilized stainless steel borer and filled with 100 μl of extract. Five percent DMSO (SRL, India) was used as negative control. Ampicillin (Hi-Media, India) (500 μg/ml) and Amphotericin- β (Hi-Media, India) (250 μg/ml) were used as positive control for bacteria and fungi, respectively. Cefoperazone (Hi-Media, India) (75 μg/ml) was used as positive control for *S. aureus*. Plates were incubated overnight at 37°C. Diameter of zone of inhibition was measured by using scale. The experiment was performed in triplicate.

**Minimum Inhibitory Concentration**

Minimum inhibitory concentration (MIC) of an effective extract was determined using broth microdilution method using 96 well plates. A series of dilutions of each extract in the
concentration of 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 15, 10, 5 and 1 mg/ml were prepared in sterile Muller Hinton broth (Hi-media, India). The solution of 5% DMSO was used as a negative control. Each well was inoculated with 50 μl of bacterial suspension at a density of $10^7$ CFU/ml. The MICs of samples were detected after 24 hours incubation at 37°C. It was also confirmed the inhibition of bacterial growth by inoculating on sterile nutrient agar plate. The MIC was determined as the lowest dilution which could completely prevent the bacterial growth. All samples were tested in triplicate.

**Phytoconstituents isolation**

Alkaloids were isolated according to the method of Harbone.\cite{9} 2g powdered sample was mixed with 10 % acetic acid in ethanol and allowed to stand for 4 hours. Mixture was then filtered by using Whatman filter paper No: 1. and filtrate were concentrated at 40°C to 1/4th of its original volume. Concentrated ammonium hydroxide solution was added drop-wise to the filtrate until precipitation was complete. Precipitates were collected and washed with diluted ammonium hydroxide. Residue was dried and used for antibacterial activity testing.

For flavonoid isolation \cite{10}, 2g of sample was dissolved in methanol and concentrated over waterbath at 40°C to 1/4th of its original volume. Concentrated extract was used to test antibacterial activity.

Isolation of saponins \cite{11} was accomplished by extracting 2 g sample with methanol on waterbath at 40°C for 10 min. The extract was filtered and concentrated to 1 ml, mixed with 1 ml water and then extracted with n- butanol three times. The n-butanol layer was collected and evaporated approximately 1 ml. Concentrated extract was subjected to test antibacterial activity.

**GC-MS analysis**

Ethanol extract was subjected for subsequent solvent partition with hexane and methanol. Oily material was found after vacuum drying and were analysed using GC-MS on Perkin-Elmer autosystem XL with turbomass. Mass spectra were obtained by electron ionisation at 70 eV. Initially, oven temperature was maintained at 60°C and was then raised to 260°C at rate of 5°C per minute. Helium gas was used as carrier at 1 mL/min. Sample (1 μL) was injected at 250°C injection temperature. Identification of components was based on computer matching with NIST (National Institute of Standards and Technology) library.
RESULTS AND DISCUSSION

In spite of the current interest in drug discovery by molecular modelling, combinatorial chemistry, and other synthetic chemistry methods, plant-derived compounds are still substantiating to be an important source of medicines for human being. The significance and uses of plants in modern drug discovery has been recounted in recent reports.\textsuperscript{[3, 12]} Plants produce wide array of phytochemicals which can be grouped into alkaloids, flavonoids, terpenoids, saponins, lectins and polypeptides. Alkaloids, flavonoids and saponins are most active compounds presenting wide variety of biological activity.\textsuperscript{[1]} We have isolated alkaloids, flavonoids and saponins as major phytoconstituents to explore their antibacterial activity in this study. Isolated groups of phytoconstituents showed significant antibacterial activity against all the tested bacteria (Table 1). Methanol and ethanol extract of \textit{Viola odorata} L. leaves showed significant zone of inhibition against two Gram positive bacteria, \textit{Bacillus subtilis}, \textit{Staphylococcus aureus} and two Gram negative bacteria \textit{Escherichia coli}, \textit{Pseudomonas aeruginosa} (Table 1). Whereas aqueous extract of \textit{V. odorata} L. leaves showed no zone of inhibition against all the tested organisms. This might be due to loss of active ingredient(s) during boiling or the antibacterial phytoconstituents may not be water soluble.\textsuperscript{[13]} Fungi were resistant to all tested extract which revealed that phytoconstituents of \textit{V. odorata} L. may not affect the eukaryotic cell. Ethanol extract showed MIC value of 20 mg/ml against both Gram positive bacteria, \textit{B. subtilis} and \textit{S. aureus} whereas methanol extract showed MIC value of 40 mg/ml and 20 mg/ml against \textit{B. subtilis} and \textit{S. aureus}, respectively. Ethanol extract showed MIC value of 10 mg/ml and 20 mg/ml against \textit{E. coli} and \textit{P. aeruginosa}, respectively whereas methanol extract showed MIC value of 20 mg/ml and 55 mg/ml against Gram negative bacteria, \textit{E. coli} and \textit{P. aeruginosa}, respectively. The result of MIC (Table 2) indicated that \textit{E. coli} is more sensitive to both extract methanol and ethanol extract of \textit{V. odorata} L. leaves compared to Gram negative bacteria.

Among the studied bacterial isolates the \textit{B. subtilis} was most sensitive organism to alkaloid as well as flavonoid fraction of ethanol extract of \textit{V. odorata} L. leaves followed by \textit{P. aeruginosa}, \textit{S. aureus} and \textit{E. coli}. Similarly, flavonoids and, secondary metabolite isolated from plant \textit{Calotropis procera} also had antimicrobial activity.\textsuperscript{[12]} Likewise alkaloid extract of \textit{Mahonia manipurensis} stem bark showed significant antibacterial activity.\textsuperscript{[14]} In present studies, qualitatively isolated alkaloids and flavonoids showed significant antibacterial activity against all studied bacterial strains but saponins fraction of ethanol extract did not displayed much impact on studied bacterial strain.
Table 1. Antimicrobial activity of *Viola odorata* leaves extract and isolated group of phytoconstituents.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Control</th>
<th><em>V. odorata</em> leaves extract</th>
<th>Standard antibiotics</th>
<th>Isolated groups of phytoconstituents from ethanol extract of <em>V. odorata</em> leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5% DMSO</td>
<td>Water extract</td>
<td>Methanol extract</td>
<td>Ethanol extract</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>-</td>
<td>-</td>
<td>15.00±0.5774</td>
<td>11.33±0.6667</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>-</td>
<td>-</td>
<td>14.67±0.8819</td>
<td>10.67±0.3333</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>-</td>
<td>-</td>
<td>11.67±0.3333</td>
<td>10.67±0.3333</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>-</td>
<td>-</td>
<td>15.67±0.3333</td>
<td>10.67±0.3333</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

-: no zone of inhibition; ± Mean standard deviation of triplicate

Table 2. Minimum inhibitory concentration of *Viola odorata* leaves extracts

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Methanol extract (mg/ml)</th>
<th>Ethanol extract (mg/ml)</th>
<th>Ampicillin (μg/ml)</th>
<th>Cefoperazone (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>20</td>
<td>10</td>
<td>70</td>
<td>-</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>55</td>
<td>40</td>
<td>70</td>
<td>-</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>50</td>
<td>20</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>40</td>
<td>20</td>
<td>-</td>
<td>75</td>
</tr>
</tbody>
</table>
GC-MS analysis revealed the presence of Pentane 2,3,4-Trimethyl (45%), N-Hexadecanoic acid (28.85%), 10-Undecyn-1-ol (14.43%) and Pentadecanoic acid (8.14%) (Table 3). N-Hexadecanoic acid was specific to *V. odorata* L. [6] Pentane 2,3,4-Trimethyl, 10-Undecyn-1-ol and Pentadecanoic acid were reported first time. N-Hexadecanoic acid are widely used in cosmetic as bath oil, hair conditioner and creams [15] N-Hexadecanoic acid, pentadecanoic acid and 10-Undecyn-1-ol reported to have antimicrobial activity [16,17].

Table 3. Chemical composition of methanol fraction of *V. odorata* L. leaves

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of compound</th>
<th>Retention Time</th>
<th>Area %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pentane 2,3,4-Trimethyl</td>
<td>16.093</td>
<td>45.02</td>
</tr>
<tr>
<td>2</td>
<td>N-Hexadecanoic acid</td>
<td>20.105</td>
<td>28.85</td>
</tr>
<tr>
<td>3</td>
<td>10-Undecyn-1-ol</td>
<td>21.781</td>
<td>14.43</td>
</tr>
<tr>
<td>4</td>
<td>Pentadecanoic acid</td>
<td>21.981</td>
<td>8.14</td>
</tr>
</tbody>
</table>

CONCLUSION

The present work confirmed *in vitro* antibacterial potential of *Viola odorata* L. The isolated group of phytoconstituents showed significant antibacterial activity. Thus, this study succeeded to demonstrate scientific justifications for its use in traditional medicine for the treatment of sore throat and tonsillitis. Hence it could be concluded that this plant may be a good source of plant antimicrobials that could be alternative of antibiotics. Fractionation and characterization of active molecules will be the future work to investigate.

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Authors’ contributions: VVS design and performed experiments. VVS, RPS, GKS and AKS, were involved in data analysis and writing the manuscript. All the authors read and approved the final manuscript.

Competing interests: The authors declare that they have no competing interests.

4. REFERENCES


