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Research Article

Secondary Metabolites Screening Using GC-MS and In-Vitro Biological Assessment Against Human Pathogenic Bacterial Strains of *Viola Indica*

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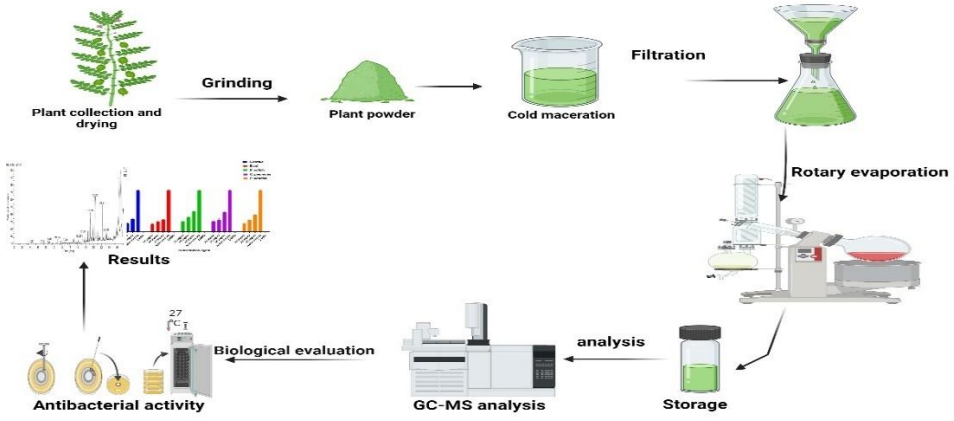
Abstract

Viola indica, a medicinal herb with a long history of traditional use in treating various diseases. In this study we analyzed methanolic extract of *Viola indica* for metabolite composition using GC-MS, and antibacterial activity through disc diffusion method. In GC-MS analysis, we found total of 25 metabolites in which the major metabolites were 9,12-Octadecadienoic acid (Z,Z)- (20%; 20.34 min), followed by Stigmasterol, 22,23-dihydro- (16%; 26 min) and α-Amyrin (12.38%; 26.65 min) . In antibacterial activity *viola indica* methanolic extract showed significant antibacterial effects against all bacterial strains. The highest antibacterial activity was recorded against *Bacillus subtilis* (8.50±0.20 mm), followed by *Klebsiella pneumoniae* (8.10±0.25 mm) zone of inhibition, while the lowest was recorded against *Staphylococcus aureus* (2.10±0.30 mm). The result of this study suggests that *viola indica* has potent antibacterial metabolites with good potential as a natural alternative to synthetic antibacterial agents. However, this study also suggest that more instrumental and biological research is required to explore it's in-depth phytochemistry, pharmacological potential and toxicological properties.

Keywords: *Viola indica*, secondary metabolites, GC-MS analysis, antimicrobial activity.

Graphical abstract



1. Introduction

Medicinal plants have been recognized for centuries as a rich source of bioactive compounds and secondary metabolites utilized in drug development for infectious diseases [1, 2]. In comparison to commercial pharmaceuticals, plant-based

medicines often exhibit greater cost-effectiveness and reduced side effects [3]. Consequently, a substantial portion of the world's population relies on locally produced herbal remedies and handcrafted medications for basic healthcare [4]. Throughout history, various civilizations have documented the efficacy of herbs in treating diverse ailments, including Mesopotamian, Ayurvedic, traditional Chinese, and Greek Unani medicine. Herbal remedies, derived from naturally occurring plants without industrial processing, play a crucial role in regional healing practices [5-7]. Herbal remedies, which are made from naturally occurring plants and require no industrial processing, are an essential component of regional healing practices [8]. In Asian countries, approximately 60% of individuals rely on herbal medications, underscoring the widespread use of local herbal remedies [9, 10]. The global market for these indigenous herbal medicines is valued at over $60 billion annually [11]. Recent years have witnessed a significant increase in the demand for herbal remedies worldwide, with projections indicating that 8.5 billion people will be utilizing herbal medicines by 2030 [12]. The World Health Organization (WHO) reports a rising trend in the regular use of therapeutic plants [13]. In developing nations, plants account for approximately 80% of total energy consumption, with this percentage varying between developed and developing countries [14]. The WHO has emphasized the importance of researching traditional herbal medicine, particularly in impoverished nations, due to scientific evidence confirming the benefits and applications of medicinal herbs [8, 15]. Scientific investigations have identified numerous plant species that produce active chemicals with distinct medicinal properties [16] The pharmaceutical industry has taken note of the *Violaceae* family, which includes several species that biosynthesize a range of beneficial compounds, such as salicylates, cyclotides, flavonoids, alkaloids, saponins, tannins, and coumarins [17]. Secondary metabolites derived from *Violaceae* plants exhibit a wide range of biological and pharmacological properties, including anticancer [18], anti-inflammatory activity[19], antiviral activity[20], antibacterial activity[21], and antioxidant properties[22], as well as antifungal, cytotoxic, and anti-bronchitis activity[23]. *Viola indica*, a member of the *Violaceae* family, is native to Pakistan and thrives in damp, cool environments, commonly found on the northern and rocky slopes of the Himalayas [24]. This perennial herb possesses slender, firmly articulated roots resembling rhizomes that can reach up to 20 cm in length. The plant either lacks a stem or is acaulescent, with simple leaves. According to local folklore, the leaf extract of *V. indica* demonstrates potential in treating internal body wounds, fevers, asthma attacks, coughing, and chest issues [24]. However, its phytochemistry has not been fully explored yet. Therefore, to gain a comprehensive understanding of the phytochemical composition and potential metabolites of *Viola indica*, this study was conducted to investigate its phytochemical profile through GC-MS and biological activity (antibacterial).

2. Materials and Methods

2.1. Plant collection and Extraction.

Specimens of *Viola indica* were collected from Pakistan's tribal region (Bajaur district). Following identification, the plant's leaves brought to the Lab at department of agricultural chemistry and biochemistry, university of agriculture Peshawar Pakistan. The leaves were washed with distilled water and subsequently air-dried at room temperature in shaded environment. The plant material was then pulverized using a grinding apparatus. The powdered sample then underwent a maceration-based extraction procedure for 48 hours. Following extraction, the resulting solutions were concentrated utilizing a rotary evaporator. The concentrated extracts were then preserved for subsequent phytochemical analysis and evaluation of antibacterial properties.

2.2. GC-MS analysis

GC-MS analysis was conducted in Centralized Resource Laboratory - University of Peshawar. The analysis was conducted using a Thermo MS DSQ II GC-MS system, which was equipped with a semi-polar DB-35 MS Capillary column [25]. The column, with specific dimensions (0.25 μm film thickness, 30 mm length, and 0.25 mm diameter), allowed for efficient separation of the extract's components. Helium was used as carrier gas and maintained at flow rate of 1.0 ml/min to

ensure optimal separation and detection of the compounds. The temperature program for the GC oven was carefully controlled to facilitate the separation of components with varying volatilities. Total running time was 35 minutes with starting at 40°C for 5 minutes allowed for the elution of highly volatile compounds, while the subsequent temperature increased to 280°C to enable the separation of less volatile components. The identification of the separated compounds was achieved by comparing their retention indices with those in the NIST library (2020) based on the search score which provided a reliable means of identifying the metabolites present in the extract of *Viola indica*.

2.3. Invitro Antibacterial activity

Antibacterial experiment was conducted in Biochemistry Lab-2 in department of agricultural chemistry and biochemistry at the university of agriculture Peshawar Pakistan. The antibacterial activity of *Viola indica* methanolic fraction was thoroughly investigated using the disc diffusion method as described [26], a widely accepted technique for assessment antimicrobial properties. Diverse human pathogenic bacterial strains, including both gram-positive and gram-negative species were used to provide a comprehensive evaluation of the extract's efficacy. The selected bacterial strains *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Bacillus subtilis and Proteus mirabilis* which represent a variety of clinically relevant pathogens. Three different concentrations of the extract were used (20 mg/ml, 25 mg/ml, and 30 mg/ml) to assess the effects of extract on bacterial growth inhibition. Nutrient Agar plates were prepared in a sterile environment by dispensing the agar into petri dishes and allowed it to solidify. To ensure an even distribution of bacterial growth, a sterile cotton swab was used to spread the bacterial suspension uniformly across the surface of the nutrient agar plates. Circular filter paper discs (6 mm in diameter) were sterilized and then immersed in the extract each concentration. Using sterile forceps, these discs were subsequently positioned on the agar plates that had been inoculated with bacteria. Azithromycin was used as a positive control for comparison, while DMSO was used as a negative control to account for any potential solvent effects. After the incubation conditions (24 hours at 27°C) were carefully controlled to ensure optimal bacterial growth and standardized results. After 24 hours, the zone of inhibition was recorded in millimeter (mm).

3. Results and discussion

3.1. GC-MS analysis

The GC-MS analysis of the menthol extract of *Viola indica* provided valuable insights into its chemical composition. The GC-MS analysis was carried out of *viola indica* methanolic extract in which total 25 bioactive compounds were identified *Viola indica* (Figure 2 and Table 1).

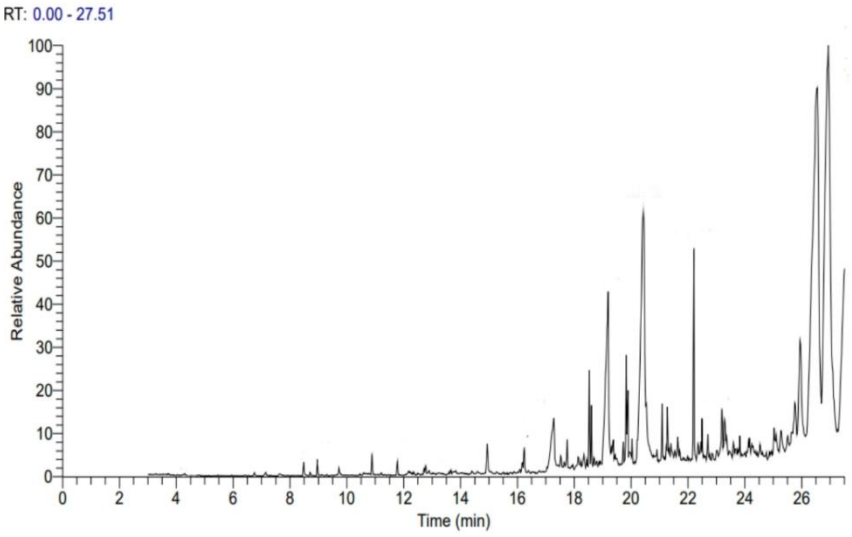


Figure 2. Chromatogram of *Viola indica* GC-MS analysis.

Table 1. GC-MS analysis results of *viola indica* methanolic fraction

S.no	Compounds	M. Formula	R. time	P. area
1	2-Cyclohexen-1-one,	C6H8O	8.42	0.10
2	4,6-Heptadienoic acid, 3,3,6-trimethyl-, methyl ester	C11H18O2	8.89	0.22
3	2-Bromo-5-trifluoromethylphenol	C17H18F3NO3	9.66	0.10
4	Tetradecane	C14H30	10.80	0.42
5	Caryophyllene	C15H24	11.13	0.24
6	1,6,10-Dodecatriene, 7,11-dimethyl-3-methylene-, (Z)-	C15H24	11.70	0.50
7	Copaene	C15H24	12.11	0.67
8	Phenol, 2,4-bis (1,1-dimethyl ethyl)-	C14H22O	12.68	1.57
9	Caryophyllene oxide	C15H24O	13.57	0.41
10	1-Naphthalenol, decahedron-1,4a-dimethyl-7-(1-methylate ethylidene)-	C15H26O	14.84	0.72
11	17-Pentatriacontene	C18H24O	17.14	0.73
12	2-Naphthalenemethanol,	C15H26O	17.46	3.30
13	Pentadecanoic acid, 14-methyl-, methyl Ester	C17H34O2	18.46	2.63
14	n-Hexadecanoic acid	C16H32O2	19.07	9.45
15	11-Octadecenoic acid, methyl ester	C19H36O2	19.81	6.93
16	9,12-Octadecadienoic acid (Z,Z)-	C18H32O2	20.34	20.93
17	1H-Cyclopropa[3,4]benz[1,2-e]azulene -4a,5,7b,9,9a(1aH)-pentol,	C28H38O10	21.21	1.39
18	Propanoic acid	C27H42O4	21.64	0.83
19	9-Octadecenoic acid, 1,2,3-propanetriyl Ester	C57H104O6	23.25	5.60
20	9,12,15-Octadecatrienoic acid	C27H52O4	23.76	1.70
21	17-Pentatriacontene	C35H70	24.10	1.02
22	á-Sitosterol trimethylsilyl ether	C32H58OSi	25.02	1.24
23	Stigmasterol	C29H48O	25.86	2.51
24	Stigmasterol, 22,23-dihydro-	C29H50O	26.26	16.20
25	α-Amyrin	C30H50O	26.65	12.38

Gas chromatography-mass spectrometry (GC-MS) analysis revealed various classes of secondary metabolites, including diverse fatty acids. 9,12-Octadecadienoic acid (Z, Z) (20.93%; 29.34 min) was the most abundant, which has reported as a potent biological control metabolite [27], followed by n-Hexadecanoic acid (9.45%; 19.07 min), which possesses antibacterial properties [28]. Similarly, 11-Octadecenoic acid, methyl ester (6.93%; 19.81 min) and 9-Octadecenoic acid, 1,2,3-propanetriyl ester (5.60%; 23.25 min) were detected. Previous studies have reported these fatty acids to exhibit various medicinal properties, such as anti-inflammatory, antioxidant, and antimicrobial effects, which contribute to the medicinal benefits of *Viola indica* [29, 30]. Furthermore, other compounds were detected, including terpenoids such as Copaene (0.67%; 12.11 min) and Caryophyllene (0.24%; 11.13 min). Studies have reported these sesquiterpenes to possess anti-inflammatory and anticancer activities [31]. Additionally, Caryophyllene oxide (0.41%; 13.57 min) and α Amyrin (12.38%; 26.65 min) were detected, which are well-known for their analgesic and anti-inflammatory properties. The presence of Stigmasterol (2.51%; 25.86) and Stigmasterol, 22,23 dihydro (16.20%; 26.26) indicates the importance of bioactive phytosterols in *Viola indica*, which are known to lower cholesterol levels and exhibit anti-inflammatory, anticancer, and antimicrobial properties [32, 33]. However, some metabolites were detected in lower concentrations, such as α Sitosterol trimethylsilyl ether (Steroidal compound) (1.24%; 25.02 min), Tetradecane (0.42%; 10.80 min), and Phenol, 2,4 bis (1,1 dimethyl ethyl) (1.57%;

16.68 min). These compounds contribute to the enhancement of the plant's pharmacological profile, augmenting its potential for medicinal purposes [34-36]. Additionally, compounds present in lower concentrations, such as 2-Bromo 5 trifluoromethylphenol (0.10%; 9.66 min), may also contribute to the plant's overall biological activity [37, 38]. The abundance of 9,12-Octadecadienoic acid Z,Z, and bioactive compounds such as terpenoids, sterols, and fatty acids in *Viola indica* demonstrated its potent anti-inflammatory, antioxidant, and antimicrobial effects [39, 40]. Various metabolites detected in *Viola indica* have been previously reported in other species, such as Caryophyllene oxide (0.6%) in *Viola dubyana* and 0.7% in *Viola calcarata*, while n-hexadecanoic acid was found at 1.6% in *Viola dubyana* and 6.1% in *Viola calcarata* [41]. Furthermore, another study reported compounds such as caryophyllene, copaene, hexadecanoic acid, octadecadienoic acid, and Neophytadiene in *Viola tricolor [42].*

Table 2. antibacterial activity results of *viola indica* against various bacterial strains.

Bacterial strains	20mg/ml	25mg/ml	30 mg/ml
Escherichia coli	3.30±0.15 mm	4.10±0.30 mm	4.75±0.25 mm
Staphylococcus aureus	2.10±0.30 mm	3.30±0.25 mm	5.10±0.20 mm
Klebsiella pneumoniae	4.30±0.10 mm	5.00±0.30 mm	8.10±0.20 mm
Bacillus subtilis	4.30±0.20 mm	6.10±0.10 mm	8.50±0.25 mm
Proteus mirabilis	3.50±0.25 mm	5.00±0.20 mm	7.00±0.30 mm
Azithromycin (standard)	17.00±.0.10 mm	17.00±.0.10 mm	17.00±.0.10 mm
DMSO (pure)	-	-	-

Sign(-) in the table shows no activity

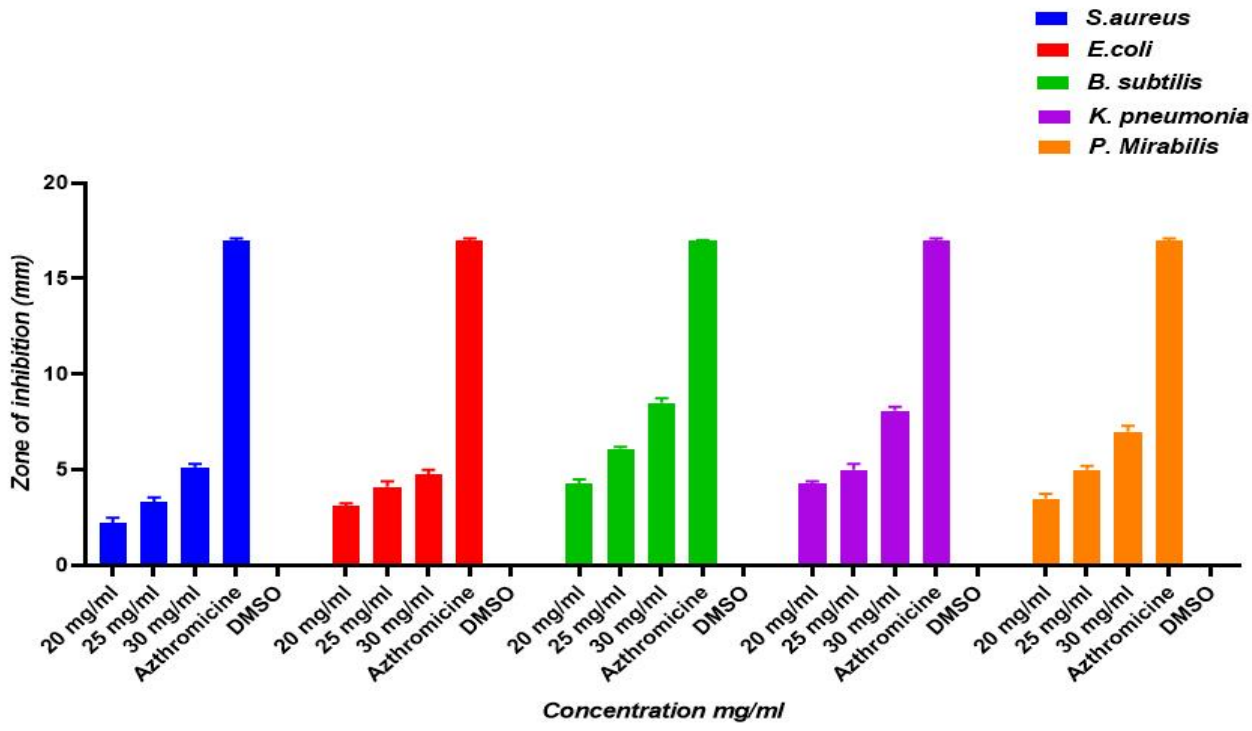


Figure 3. graphical illustration of antibacterial activity results of *viola indica*

3.2. Antibacterial activity

The antibacterial activity of *Viola indica* methanolic extract was evaluated against five distinct human pathogenic bacterial strains (gram-positive and gram-negative), specifically *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae* *Bacillus subtilis*, and *Proteus mirabilis*. The extract was assessed in triplicate at three different concentrations (20 mg/ml, 25 mg/ml, and 30 mg/ml) and compared with azithromycin as a positive control and DMSO as a negative control. The results were recorded as mean values of the zone of inhibition in millimeters (mm) and are summarized in table 2 and figure 3. The antibacterial activity results showed a dose-dependent increase in inhibition zones against bacterial strain. The highest inhibition was recorded against Bacillus subtilis 8.50 mm at concentration of 30 mg/ml, followed by *Klebsiella pneumoniae* which was 8.10 mm at 30 mg/ml. Similarly, the extract also significantly inhibited the growth of Proteus mirabilis with 7.00 mm at 30 mg/ml, while at 25 mg/ml; 5.00 mm, and 20 mg/ml; 3.50 mm. In addition, *Escherichia coli* showed low susceptibility at all three concentrations (20 mg/ml; 3.30 mm, 25 mg/ml; 4.10 mm and 30 mg/ml; 4.75 mm). Furthermore, the extract also potential inhibitory role against *Staphylococcus aureus* at 30 mg/ml with 5.10 mm zone of inhibition. Moreover, the negative control (DMSO) showed no inhibition, which gives the validation of antibacterial activity of *viola indica* which was due to the presence of active metabolites. Previously, the *Viola serpens* (ethanolic extract) reported for antibacterial activity against *E. coli, S. aureus P.aeruginosa , K.pneumoniae* and *S.typhi* [43]. This finding suggests that this *Viola* family contains potential antibacterial metabolites. Similarly, *Viola canescens*, which belongs to the same family, has also been reported to exhibit antibacterial activity against *Bacillus subtilis* (12mm) and *Pseudomonas* (3mm) strains [44], while another plant *viola odorata* also reported for antibacterial potential [45]. Furthermore, the presence of α-Amyrin in the methanolic extract of Viola indica may contribute to the significant inhibition of bacterial strains, as α-Amyrin has been identified as a potential antibacterial metabolite [46, 47]. The findings indicate that the methanolic extract of *Viola indica* exhibits notable antibacterial properties, particularly against gram-negative bacteria such as *Proteus mirabilis, Escherichia coli*, and *Klebsiella pneumoniae*. However, further research could investigate more potential application of this extract as an alternative with synthetic antibacterial agents.

4. Conclusion

Our study evaluated the metabolic composition and biological potential of *Viola indica*, In GC-MS analysis total of 25 metabolite were detected, in which with 9,12-Octadecadienoic acid (Z,Z)- (20%), and Stigmasterol, 22,23 dihydro (16.20%) were present in high percentage. Similarly, the extract showed significant antibacterial activity against the human pathogenic bacterial strains. The highest zone of inhibition observed in antibacterial activity against *Bacillus subtilis* (8.50 mm) at concentration of 30 mg/ml, followed by *Klebsiella pneumoniae* which was 8.10 mm at 30 mg/ml. These findings suggest that *Viola indica* has significant potential as a natural alternative to synthetic antibacterial medicines. However, more in-depth experimental scientific research is recommended to explore its phytochemistry, pharmacological and toxicological properties in detail.

Authors Contribution

R.U conducted the research and written the manuscript. M.S supervised the research and reviewed the manuscript. N.W, A.N and K.A helped in lab work.

Conflicts of Interest

There are no conflicts of interest reported by the writers.

Data Availability statement

The data presented in this study are available on request from the corresponding author.

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