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Phytochemical analysis, Antioxidant and Antimicrobial Screening of Shoot Extracts of *Viola odorata* linn. from Shikari Devi Wildlife Sanctuary, Himachal Pradesh, India

Research Article

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Abstract

The traditional healers made significant use of the *Viola odorata* Linn. of the Violaceae family for its many ethnopharmacological uses. The phytochemical composition and in vitro antioxidant activity of solvent extracts from *Viola odorata* Linn. shoots were studied, and the antibacterial potential of the optimized extract was also examined. The Soxhlet apparatus was used to extract phytochemicals from shoots in four different solvents, including n-hexane, butanol, methanol, and water. High performance liquid chromatography (HPLC) was used to analyse the stigmasterol profile of *V. odorata* shoots. The extracts were found to contain alkaloids, flavonoids, terpenoids, saponins, and phenolics, all of which were found in significant amounts in the methanolic extract. The methanolic extract also shown the highest radical scavenging activity (79.23 ± 0.44 %). TLC and HPLC profiling of methanolic extract revealed the considerable amount of stigmasterol in the shoots of *Viola odorata* Linn. HPLC analysis of methanolic shoot extract of plant revealed the presence of 0.009 µg mL-1 stigmasterol. Optimized Methanolic extract showed respectable in-vitro antimicrobial activity against *Bacillus subtilis* (2.6 ± 0.41 mm zone of inhibition), *E. coli* (3.4 ± 0.71 mm zone of inhibition) and *Candida albicans* (3.5 ± 0.48 mm zone of inhibition). The outcomes demonstrated that the methanolic shoot extract of *Viola odorata* Linn. from Shikari Devi Wildlife Sanctuary, Himachal Pradesh, India, is a valuable herb for further screening and might be prepared for in-vivo animal studies.

Keywords: Chromatography, Stigmasterol, Flavonoids, Extraction, Phytochemicals, Screening.

Introduction

A sizeable portion of the Indian population relies on plant-based natural medications. The surge in adverse pharmacological reactions and side effects is fueling a tremendous increase in interest in conventional medicine (1). Plants have been the foundation of several traditional medical systems around the world for thousands of years, and they continue to advance humankind. In the modern world, natural compounds and their derivatives account for more than 50% of all therapeutic medications (2). There are 2532 plant species in Indian ethnobotany, many of which have therapeutic characteristics (3). Plants are an abundant source of phytochemicals, which shield the body from oxidative stress and microbial infection among other maladies (4). There are about 8,000 phenolic compounds in nature, and all of them have at least one aromatic ring that is filled by one or more hydroxyl groups. These substances are the guardians of numerous biological properties, including antioxidant, antibacterial, and anticancer properties (5). The alkaloids, coumarins, terpenes, and a variety of phenolics are responsible for antibacterial activities of plants. They have also been engaged in numerous processes, such as protein synthesis, enzyme activity, structural elements, and allelopathy (6). *Viola odorata* Linn. (Violaceae) known as *Banafsha* is native to Europe and Asia, this perennial stoloniferous herb was brought to North America and Australia. In India, the plant grows in the foothills of Western Himalayas including the Shikari Devi Wildlife Sanctuary in Himachal Pradesh, which is one of the most abundant sources of biological diversity and is known as a "store house" of valuable medicinal plants (7). Plant secondary metabolites are considerable value for people and is crucial in the detection of illicit drugs (8). An alkaloid violin and stigmasterol was found in this plant (9). Every portion of the plant has therapeutic value as anti-inflammatory, diaphoretic, diuretic, expectorant and anti-hypertensive (10). This research examined the phytoconstituents in shoot extracts of *Viola odorata* Linn. from the Shikari Devi Wildlife Sanctuary in Himachal Pradesh, India, and assessed their in-vitro antioxidant and antibacterial activities.

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Materials and Methods

Collection of Plant Material

Plant fragments were collected from the shikari Devi Wildlife Sanctuary in Himachal Pradesh, India. Further Dr. Pankaj Sharma, Senior Scientist, HP State Biodiversity Board, identified and authenticated it (Authentication No. HPSBB/-4164). The material was collected, cleaned with running water, allowed to air dry at room temperature, homogenized into a fine powder, and then stored.

Preparation of shoot extracts

The pulverized aerial Parts (40g) of V. odorata was extracted with organic solvents (n-hexane, butanolic, methanolic, and aqueous (400ml)) using the Soxhlet apparatus continuously for 72 hours. The aqueous extract was evaporated in an oven at 55°C while the n-hexane, butanolic, and methanolic extracts were dried under reduced pressure. The percentage yields of the extracts in n-hexane, butanol, methanol, and water were 2.124%, 1.086%, 14.154%, and 18.517% w/w, respectively. A working solution was created by redissolving around 10 mg of each dried gummy extract in 1 mL of DMSO and 9 mL of methanol. This solution was kept at 4°C for future studies.

Qualitative estimation of phytochemicals

According to established protocols, the initial phytochemical screening was performed on all four extracts of V. odorata (n-hexane, butanolic, methanolic, and aqueous) (11, 12). (Table 1)

Table 1: Procedure for preliminary qualitative tests for Phytochemical Screening

S. No	Test Name	Brief Procedure	Observation
1	Terpenoids	In a test tube, one ml of each extract was combined with one ml of chloroform followed by 1.5 ml of conc. sulfuric acid.	The extract's reddish-brown appearance showed that terpenoids were present
2	Saponins	2.5 mL of distilled water were added to 1 mL of each extract in a test tube, which was then shaken for one to two minutes. The aforementioned mixture was then given one to two drops of olive oil.	The extract's ability to produce foam suggested the presence of saponins.
3	Flavonoids	Two to three drops of strong sulfuric acid were added to 1 mL of the extract.	The extract's yellow, red, or pink appearance suggested the presence of flavonoids.
4	Steroids	In a test tube with 1 mL of extract, 3 mL of chloroform was added. Next, 2 mL of conc. sulfuric acid was added gently from the sides of test tube.	Indication of the presence of steroids in the extract was the formation of a reddish ring at the intersection of two liquid layers.
5	Tannins	In a test tube, 5% ferric chloride was added in two to three drops to one ml of each extract.	The presence of tannins in the extract was revealed by the presence of blue/black precipitates.
6	Coumarins	One ml of 10% sodium hydroxide was added to one ml of each extract in a test tube.	The presence of coumarins in the extract was suggested by the extract's appearance of yellow colour.
7	Proteins	In a test tube, 1 mL of extract, 1 mL of 4% NaOH, and 1-2 drops of 1% CuSO4 solution were added.	The presence of proteins in the extract was revealed by the violet-pink appearance.
8	Carbohydrates	0.5 ml of Molisch's reagent was added to 1 mL of each extract in a test tube. Then, from the test tube's side, one to two drops of concentrated sulfuric acid were introduced.	The development of a violet ring at the intersection of two liquid layers suggested the extract contained carbs.
9	Phenols	One to two drops of FeCl3 were added to 1 mL of each extract in a test tube	The presence of phenols in the extract was detected by the appearance of blue or green colour.
10	Alkaloids	In a test tube, two ml of plant extract and a few drops of the Hager's reagent were added.	Yellow precipitate signals a positive alkaloids result.
11	Glycosides	In a test tube, two ml of plant extract were introduced along with two ml each of acetic acid and chloroform.	Glycosides are demonstrated by the formation of violet, blue, and green colorations.
12	Anthocyanins	Two ml of plant extract and two ml each of 2N HCl and NH3 were placed in a test tube.	Anthocyanins are indicated by the development of pinkish red to bluish violet colour.

Quantitative estimation of phytochemicals

Quantitative estimation of phytochemicals was done for determining the concentration of primary and secondary metabolites present in each of the four V. odorata extracts.

Phenolic Content

Folin-Ciocalteau reagent (FC) was used to calculate the total phenolic content of V. odorata (13). The tubes holding 100 µL of extract received around 1 mL of 1N FC reagent, and the mixture was allowed to sit at room temperature for 5 minutes. By adding 1 mL of 20% sodium carbonate solution, the reaction was stopped. The tubes were then thoroughly shaken and left in the dark for two hours leading to colour development. At 760 nm, absorbance was measured. The standard was gallic acid. The amount of total phenolic content in the plant extracts was expressed as mg of gallic acid equivalent and calculated as:

Total Phenolic Content (mg/g) = Concentration (mg/mL) * Volume of Solution (mL)/ Mass of extract (g)

Tannin Content

According to the protocol, the total tannin content was estimated (14). To 100 µL of each extract, approximately 3 mL of 4% vanillin solution in ethanol was added, followed by the addition of 2.5 mL of 72% concentrated sulphuric acid. The combination was heated to 60 °C in a water bath for 15 minutes, and it was then cooled to room temperature. At 500 nm, absorbance was measured. On a dry matter basis, tannic acid was expressed as free phenolics and used as the Standard. Total tannin content was calculated as:

Total Tannin Content (GAE mg/g) = Concentration (mg/ mL) * Volume of Solution (mL)/ Mass of extract (g)

Flavonoid Content

The calorimetric method was used to estimate the total flavonoid content (15). To the tubes holding 100 µL of extract, 150 µL of 5% sodium nitrite was added, followed by a 5-minute incubation at room temperature. The tubes were then filled with approximately 300 µL of 16% AlCl3 and left to remain at room temperature for 6 minutes, followed by addition of 1 mL of 1MNaOH (4%). At last, the tubes were filled with around 550 mL of distilled water, and then absorbance was calculated at 510 nm. Rutin served as the benchmark. The presence of flavonoids was indicated by the pink colour. Using the standard curve, the total flavonoid content was calculated as mg of rutin equivalent per gram of extract on a dry weight basis. Formula for calculating total flavonoid is as:

Total flavonoid Content (mg/g) = Concentration (mg/mL) * Volume of Solution (mL)/ Mass of extract (g)

Antioxidant Activities

DPPH Assay (2, 2-diphenyl-1-picrylhydrazyl)

The ability of the extracted sample to convert DPPH into DPPH-H (colourless compound) was used to assess the DPPH scavenging activity/ anti-oxidant activity of the plant extract. Antioxidant capacity was evaluated using a mildly modified DPPH scavenging test (16). The DPPH reagent (0.1mM) was made by combining 4 mg of DPPH with 100 mL of methanol. The mixture was given 30 minutes to stand at room temperature. Further 0.1 mL of plant extract (62.5-500 mg/mL) was mixed in 2 mL of prepared DPPH solution and kept in dark at room temperature for 1 hour. The reference utilised was BHT (butylated hydroxytoluene). Absorbance was recorded at 517 nm. The formula below was used to calculate the free radical scavenging activity (% RSA) of plant extract.

Percentage Scavenging of DPPH° = (A° - A1) ÷ A° × 100
Where A°= Initial Absorbance
A1= Final Absorbance

Thin Layer Chromatography

By combining 30 g of silica gel with 60 mL of distilled water, thin layers of silica gel were applied to glass thin-layer chromatography (TLC) plates (17). A fine capillary was used to apply stigmasterol (100 µL) as the standard, along with 100 µL of optimized extract, 2.0 cm above the bottom of the TLC plate. The mobile phase was prepared by mixing acetone and hexane in the ratio of 1:3. TLC plate was run up to 3/4th height of the plate. After that, the plate was scanned with iodine vapours and dried in a hot air oven. The plate was seen in a UV chamber, and the Rf value was determined as follows:

High Performance Liquid Chromatography

The Agilent 1220 Infinity LC system with a VWD (Variable wavelength detector), 4.6 X 250 mm, and 5 ZORBAX RX-C18 column was utilized for high-performance liquid chromatography (HPLC). The mobile phase components, acetonitrile: water (1:3), were filtered through a 0.2 M membrane filter just before use. Optimized extract (1 mg) was diluted in 1 mL of methanol. At a flow rate of 1.0 mL/min and run time of 15 minutes, mobile phase was pumped from the solvent reservoir to the column at ambient temperature. The injection volume of the sample solution was about 100 µL. Using a variable wavelength UV detector, stigmasterol was detected at 336 nm (λ max), and its concentration was quantified as a percentage of the peak area (18).

In-Vitro Anti-Microbial studies

Pathogens including Candida albicans, E. coli, and B. subtilis are utilized as test organisms. Bacterial cultures were cultivated in nutrient broth at 37°C for 12 hours. Candida albicans was first cultured and cultivated for 24 hours at 25°C in potato dextrose broth. The agar well diffusion method was used to assess the antibacterial activity of the aqueous extract of V. odorata. Prior to usage, the test strains were first cultivated for 24 hours in nutritional broth. A Mueller Hinton agar was covered with 100 µL of the standardized cell suspension. Wells were drilled into the Mueller Hinton agar using a sterile Cork borer with a 4 mm diameter. Separately, 100 µL of optimized extracts (100 mg/ml) of V. odorata, Blank solvent ((-) negative control) and standard solution ((+) Positive control) were added to the wells, allowed to stand at room temperature for about 2 hours, and then incubated for 24 hours at 37°C for bacteria and 72 hours at 25°C for Candida albicans. Following incubation, the plates were examined for zones of inhibition and contrasted with Ciprofloxacin and Fluconazole at a dosage of 100 mg/ ml (19).

Statistical analysis

For statistical analysis and data display, an open-source statistical programme was utilised. Each experiment was carried out in triplicate (n = 3) and the computed findings were provided as mean ± SEM.

Results

Phytochemical analysis:

Qualitative Phytochemical screening

The existence of primary and secondary metabolites was discovered using quantitative phytochemical screening of n-hexane, butanol,

methanol, and aqueous extracts. The aqueous extract was found to contain the highest concentrations of both primary and secondary metabolites. Only the aqueous extract included tannins (Table 2).

Table 2: Phytochemicals in solvent extracts of shoot of *Viola odorata* Linn.

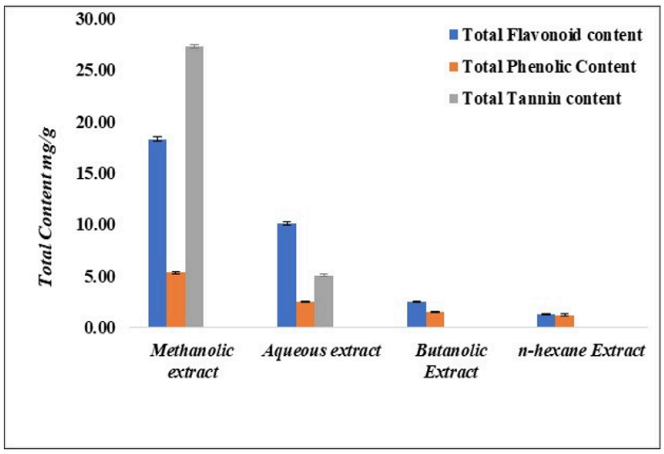
S. No	Phyto-chemical Test	Metha-nolic	Aque-ous	Buta-nolic	n-hexane
1	Terpenoids	+++	++	+	++
2	Saponins	+++	-	-	-
3	Flavonoids	+++	++	+	+
4	Steroids	+++	++	+	-
5	Tannins	+++	+	-	-
6	Coumarins	+++	++	+	-
7	Proteins	+++	+	++	+
8	Carbohydrates	++	+++	+	+
9	Phenols	+++	++	+	+
10	Alkaloids	+++	+	++	-
11	Glycosides	++	+++	+	+
12	Anthocyanins	+++	++	+	+

Quantitative Phytochemical screening

Total Flavonoid content, Phenolic content and Tannin Content

The extract had the highest total phenolic concentration (5.34 ± 0.11 mg GAE g−1), followed by the (2.53 ± 0.08 mg GAE g−1) and butanol extracts (1.53 ± 0.07 mg GAE g−1) and N-Hexane extract had the lowest total phenolic content (1.24 ± 0.07 mg GAE g−1). The range of the total flavonoid content was 18.31 to 1.33 mg QE g−1. The most flavonoid content was in the methanol extract (18.31 ± 0.23 mg QE g−1), followed by aqueous extract (10.14 ± 0.14 mg QE g−1), Butanolic extract (2.52 ± 0.08 mg QE g−1) and N-hexane extract has the lowest level of flavonoid concentration (1.33 ± 0.07 mg QE g−1). In addition to that, methanolic extract had the highest amount of tannin i.e., 27.31 ± 0.12 mg GAE g−1 followed by aqueous extract with tannin content of 5.08 ± 0.13 mg GAE g−1 and no tannin was found in Butanolic and N-hexane extract (Figure 1).

Figure 1: Total Flavonoid content, Phenolic content and Tannin Content in different shoot extracts of *Viola odorata* Linn.



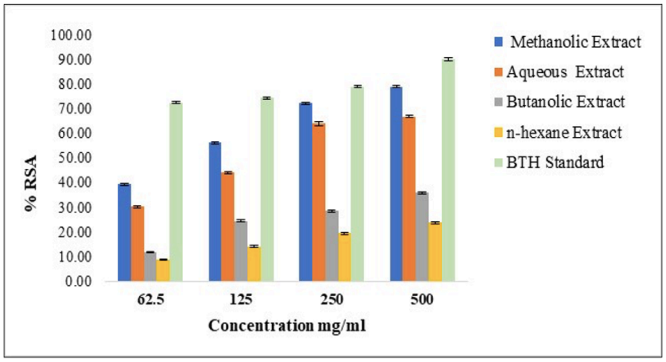
Anti-Oxidant activity

For evaluating the antioxidant activity of plant extracts, DPPH scavenging assay is regarded as highly valuable method. Obtained radical scavenging activity (RSA) was in the following order:

Methanol > Aqueous > Butanol > n-Hexane (Figure 2).

On comparison with BTH, Methanolic extract shows highest percentage of RSA (79.23 ± 0.44 % at concentration of 500 mg/ml) followed by aqueous extract (66.99 ± 0.39 % at concentration of 500 mg/ml), Butanolic (36.03 ± 0.36 % at concentration of 500 mg/ml) and least RSA activity was shown by n-Hexane extract (23.91 ± 0.39 % at concentration of 500 mg/ml). Methanolic extract was selected for further testing after being optimized based on antioxidant activity and quantitative/qualitative phytochemical screening.

Figure 2: Radical Scavenging Activity (RSA) of different shoot extracts of *Viola odorata* Linn.



Based on antioxidant activity and quantitative/ qualitative phytochemical screening, methanolic extract was optimised and selected for further testing.

TLC (Thin Layer Chromatography)

For the qualitative quantification of stigmasterol in the methanolic shoot extract of V. odorata, thin layer chromatography was used (Figure 3). Rf values of methanolic extracts was calculated as

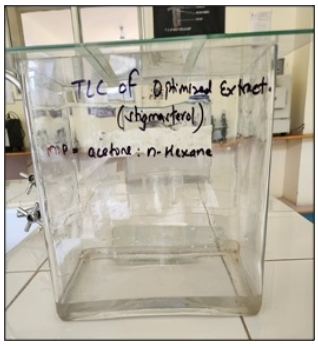
Distance travel by sample = 9.9 cm

Distance travel by sample = 14.4 cm

R_f = 9.9/14.4 = 0.7

Obtained R_f of sample was same as that of standard stigmasterol (0.9) as displayed in figure 3.

Figure 3: TLC of Methanolic extract of *Viola odorata* Linn.



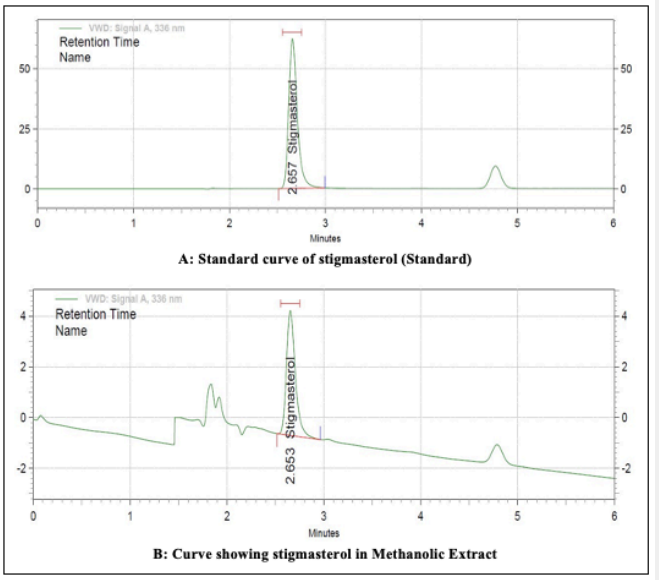
(A) (B)

(M=Methanolic extract; S= Standard stigmasterol)

HPLC High Performance Liquid Chromatography

Only the methanolic extract was quantitatively estimated by HPLC since it contained the highest quantity of phytochemicals. The presence of stigmasterol in the Methanolic extract was confirmed by TLC (Figure 3) and similar retention time of the sample with standard (2.65 min) in HPLC (Figure 4A, 4B). Using a standard curve, the amount of stigmasterol present in the Methanolic extract of V. odorata was determined. In the methanolic shoot extracts of V. odorata, stigmasterol was present at a concentration of 0.009 µg mL-1.

Figure 4: Chromatogram of standard stigmasterol and methanolic Shoot extract of *Viola odorata* Linn. presentation Stigmasterol peak



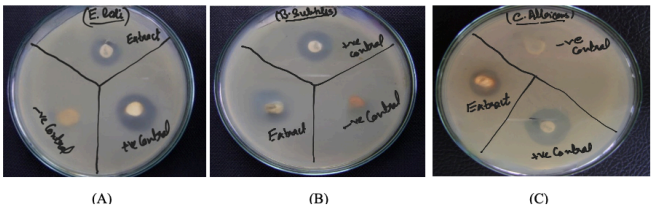
In-Vitro Anti-Microbial studies

In order to ascertain the antimicrobial characteristics of methanolic shoot extracts from *Viola odorata* Linn., its activity was demonstrated against pathogenic bacteria (E. coli (gram negative), B. subtilis (gram positive)) and fungus (Candida albicans) strains by well diffusion method. When compared with negative control methanolic extract showed a significant inhibition of bacterial and Fungus growth (Figure 5). However, zone of inhibition is less than positive control (Ciprofloxacin (bacterial study) and Fluconazole (antifungal study) (Table 3)

Table 3: Zone of inhibition of methanolic shoot extract of *Viola odorta*

S. No	Drug/ concentration	E. coli	B. subtilis	C. albicans
1	Methanolic shoot extract of *V. odorata* (100mg/ml)	3.4 ± 0.71 mm	2.6 ± 0.41 mm	3.5 ± 0.41 mm
2	Ciprofloxacin (100mg/ml)	5.5 ± 0.94 mm	4.5 ± 0.51 mm	—-
3	Fluconazole (100mg/ml)	—	—	6.5 ± 0.76 mm

Figure 5: Antimicrobial assay of methanolic shoot extract of *Viola odorata* Linn. against *E. coli* (A), *B. subtilis* (B) and *Candida albicans* (C)



Discussion

Due to abundance in secondary metabolites including phenols, tannins and flavonoids, plants are possible source of natural antioxidants and anti-microbials. These compounds produce persistent phenoxyl, hydroxyl and other radicals which are crucial in defining the antioxidant activity of plants. Both free and glycoside forms of flavonoids are found in plant tissues. In flavonoids, a propane unit sits in between the two benzene rings. As flavonoids are polyphenolic, they can scavenge free radicals, protecting against several diseases. The number of phenolic compounds and the DPPH free radical scavenging effect are positively correlated in numerous research (20, 21). The capacity of tannins to act as an antioxidant is due to the ease with which their hydroxyl groups can be oxidized (22). Beside it, phenols and flavonoids were reported to cause harm to the bacterial membrane, prevent the production of virulence factors including enzymes and toxins, and prevent the development of bacterial biofilms (23, 24). Further ability of tannins to penetrate the bacterial cell wall, interference with the metabolism and destruction of microbes account for their antimicrobial activity (23). The highest quantities of phenols, tannins, and flavonoids was found in the methanolic shoot extract of the *Viola odorata* Linn., possibly due to polarity of extracting solvent and solubility of phytoconstituent (20) and these constituents may account for strong antioxidant and antimicrobial activities of methanolic extract. An unsaturated plant sterol called stigmasterol is used to make semi-synthetic progesterone and vitamin D3. Additionally, it is also employed in the prevention of colon, breast, prostate, and ovarian cancers (24). Previous reports of stigmasterol in Viola species were confirmed by our study (25). Rf values obtained in the current study from TLC fingerprinting of Methanolic shoot extract of V. odorata were comparable to those of standard stigmasterol. Further HPLC analysis verified that the plant parts from Shikari Devi Wildlife Sanctuary in Himachal Pradesh, India, contained significant levels of stigmasterol, which is consistent with the level of stigmasterol found in plant extract of *Viola odorata* Linn. taken from Iraq (9).

Conclusion

The findings of this study demonstrate that *Viola odorata* Linn. methanolic extract has considerable antioxidant activity and antimicrobial activity. It's interesting that *Viola odorata* Linn. (Collected Shikari Devi Wildlife Sanctuary, Himachal Pradesh) shoot

extracts in methanol, butanol, aqueous, and n-hexane showed significant amounts of several phytoconstituents such phenols, flavonoids, and tannins. The presence of many phytochemicals in high concentrations and their capacity to scavenge free radicals, perhaps explains methanolic extracts' antioxidant and antibacterial properties. In conclusion, preclinical in-vitro and in-vivo models could be used to conduct additional research on *Viola odorata* Linn. shoot extracts from the Shikari Devi Wildlife Sanctuary in Himachal Pradesh.

Abbreviations:
· **DMSO:** Dimethyl sulfoxide
· **DPPH:** (2, 2-diphenyl-1-picrylhydrazyl
· **FC:** Folin-Ciocalteau reagent
· **GAE:** Gallic acid equivalents
· **RSA:** Radical scavenging activity
· **HPLC:** High-performance liquid chromatography
· **TLC:** Thin-layer chromatography

Conflict of interest
The authors declare that there is no conflict of interest**.**

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