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Screening of *Viola patrinii* for Antioxidant potential and presence of Phytochemicals

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Abstract: *Viola patrinii* DC. (Violaceae), commonly known as China violet, is an important medicinal herb, mentioned in traditional medicine for a variety of therapeutic applications including the purification of blood and the treatment of bruises and ulcers in the Chinese system of medicine it is recommended for use against cancer disorders. The dried flowers are used as a purgative and for cough and cold. It is also used in Unani recipes, such as Joshanda and Rogan Banafshah. *Viola* spp. are also used for ornamental purposes. In the present investigation, the antioxidant activities of different polar and non polar solvent extracts viz. hexane (H), petroleum ether (PE), acetone (AC), choloroform (C), ethanolic (E) and water (W) extracts of whole plant (1 mg/ml) of *Viola patrinii* were determined by standard and routine in vitro antioxidant procedures. The results confirmed that ethanol and water extract of whole plant of *Viola patrinii* exhibited potent antioxidant activity in comparison to that of acetone, chloroform, hexane and petroleum ether extracts. The results thus concluded that *Viola patrinii* acts as a potent antioxidant. Further studies are however needed to investigate the potent molecule (s) responsible for antioxidant behaviour in the plant.
Keywords: *Viola patrinii*, polar and non polar solvent extracts, antioxidant activity, potent molecules.

Introduction

Plants are the best friends of human being dedicating to humanity without selfishness. They are the good source of medicines. The natural plant products could be a potential alternative for controlling the pathogen associated with diseases. Natural products and their derivatives represent more than 50 % of the drugs in clinical use in the world[1]. One of the paramount reasons for pursuing natural products chemistry resides in the actual or potential pharmacological activity to be found in alkaloids, terpenoids, coumarins, flavanoids, lignans, glycosides etc. Antimicrobial, antioxidant and anti-inflammatory activities of some potential plants of Uttarakhand were investigated[2]. *Viola patrinii* is a perennial herb without an elongated stem and it lacks signifi cant thickening by secondary woody growth. Leaves are glabrous, triangular, usually narrowly elongated, and not deeply chordate. Flowers are usually lilac in colour. In India, *V. patrinii* is distributed in the temperate Himalayan region, extending from the hills of Arunachal Pradesh, Meghalaya, and Manipur in the east southwards to the hills of the Eastern and Western Ghats, at an altitude of 900-2400 m^3. The plants do not survive in all seasons, requiring cool, moist, well-drained humus-rich soil with partial or dappled shade. Free radicals are unstable molecules formed when the body uses oxygen for energy. The instability of these molecules can damage tissues, alter DNA and change cell structure. Ultimately, free radicals start a chain reaction resulting in the reproduction of even more free radicals. Antioxidants interact with and stabilize free radicals and may prevent some of the damage, free radicals cause to the body. The role antioxidants have in free radical stabilization involves the

antioxidants donating one of their own electrons to the free radical. Numerous studies have been carried out on some plants, vegetables and fruits because they are rich sources of antioxidants, such as vitamin A, vitamin C, Vitamin E, carotenoids, polyphenolic compounds and flavanoids[4] which prevents free radical damage, reducing risk of chronic diseases. Antioxidants are substances, usually of plant origin that reduce and neutralize free radicals and play a vital role in the prevention of cancer, cardiovascular diseases and neurodegenerative diseases including Alzheimer and Parkinson diseases[5]. Two isoflavonoids, tectorigenin-7-O-β-D-glucoside (**1**) and luteolin-7-O-β-D-glucuronopyranoside(**2**), were isolated from ethyl acetate fraction of *Viola patrinii* fermentation extracts (VPFE). Of these, compounds **1** and **2** exhibited collagenase inhibitory activity (IC_{50}) at a concentration of less than 1.5 μM, and compound **2** showed gelatinases A and B inhibitory activity (IC_{50}) at 0.3 μM and 0.8 μM, respectively[6]. A number of diseases that lead to injury of the central nervous system are caused by oxidative stress and inflammation in the brain. In this study, NNMBS275, consisting of the ethanol extract of *Viola patrinii*, showed potent antioxidative and anti-inflammatory activity in murine hippocampal HT22 cells and BV2 microglia[7]. An assay of the antioxidant potential of the *in vitro* grown callus and the wild plant extract of *Viola patrinii* was determined by DPPH (α, α-diphenyl-β-picrylhydrazyl) method shown that the antioxidant activity of *in vitro* formed callus is higher than that of wild plant[8].Violae herba has been widely used in traditional Chinese medicine to treat carbuncles, boils, and other cutaneous and subcutaneous pyogenic infections. Sources of violae herba include *Viola patrinii*, *V. hederacea*, *V. arvensis*, and *V. odorata*. Previous phytochemical studies of *Viola* species have reported the isolation of cyclotides and several flavonoid glycosides[6,9-11]. The viola is rich in secondary metabolites including, flavonoids, alkaloid (violin, v*iola*-quercitin), essential oils including (ionones, alpha-ionone, betaionone and beta-dihydroionone, hydroquinone dimethyl ether, linolenic) and extensively used in diuretic, antiinflammatory, purogative properties, abdominal pain, skin disorders, upper respiratory complications (cough, sore throat and harash)[12-14]. In *Viola odorata* antioxidant activity is related to the amount of anthocyanins, one of the groups of flavonoids pigments. Anthocyanins occur in all tissues including leaves, stems, roots and flowers. Antioxidants like phenolic acids, polyphenols and flavonoids etc also show their effect by scavenging free radicals, preventing the generation of reactive oxygen species (ROS) or activating detoxifying proteins[15].The search for newer natural antioxidants and antimicrobials especially of plant origin has ever since increased. Antimicrobial potential of some plants of Uttarakhand were investigated[16-18]. In this study, the Traditional solvent extraction (TSE) methods were used for extraction of antioxidants[19]. The results can determine the natural antioxidants available in the plant parts having solubility in the specific solvent. Also, the extraction methods will emphasize on using the specific solvent (hexane, petroleum ether, chloroform, acetone, ethanol and water) for extracting antioxidants and polyphenolics. This study may provide insight for future extraction solvents and natural potent antioxidants which can be used as dietary supplements.

Materials and Methods

Plant Materials

The plant material was collected from Garhwal region of Uttarakhand, India. The plant material was identified from Botanical Survey of India, Dehradun. Voucher specimen of the plant was stored in the Dept.herbarium for future reference. The plant material was dried in the shade in an open air for 5-10 days to form the fine powder.

Preparation of Plant extracts

Plant parts were separated, washed with distilled water, dried under shade and pulverized. The plant extracts were prepared according to the method prescribed with little modifications[20]. Briefly 20 g portions of the powdered plant material was soaked separately in different solvents i.e. petroleum ether, hexane, chloroform, acetone, ethanol and distilled water on the basis of increasing polarity for 72 h. Each mixture was stirred every 24 h using a sterile glass rod. At the end of extraction, each solvent was passed through Whatmann filter paper No. 1 (Whatmann, England) The filtrates obtained were concentrated in vacuo using water bath at 30 ^{0}C .

Determination of *In vitro* Antioxidant activity

Determination of Total Phenolic Content (TPC)

The Total Phenolic Content of each extract obtained of each of the plant extract was determined[21] and the phenolic content was expressed as µg/g Gallic acid equivalents. In brief a 100 µl aliquot of the sample was added to 2 ml of 0.2% (w/v) Na_2CO_3 solution. After two minutes of incubation, 100 µl of 500ml/l Follin-Ciocalteu reagent added and the mixture was then allowed to stand for 30 minutes at 25^{0}C. The absorbance was measured at 750 nm using a UV-VIS Systronics spectrophotometer. The blank consist of all reagents and

solvents but no sample. The Total Phenolic Content (TPC) was determined using the standard Gallic acid calibration curve and was expressed as µg/g Gallic acid equivalents.

Determination of Antioxidant Activity by DPPH Radical Scavenging Method

The extract solution for the DPPH test[22] was prepared by re-dissolving 0.2 g of each of the dried extract in 10 ml of the specific solvent in which the extract was prepared. The concentration of DPPH solution was 0.025 g in 1000 ml of methanol. Two ml of the DPPH solution was mixed with 40 µl of each of the plant extract solution and was transferred to a cuvette. The reaction solution was monitored at 515 nm, after an incubation period of 30 minutes at room temperature, using a UV-Visible Systronics spectrophotometer. The inhibition percentage of the absorbance of DPPH solution was calculated using the following equation: Inhibition%= (Abst=0 min---Abst=30 min)/ Abst=0 min ×100 Where Abst=0 min was the absorbance of DPPH at zero time and Abst=30 min was the absorbance of DPPH after 30 minutes of incubation. Ascorbic acid (0.5 mM) dissolved in methanol was used as a standard to convert the inhibition capability of plant extract solution to the Ascorbic acid equivalent. IC_{50} is the concentration of the sample required to scavenge 50% of DPPH free radicals.

Superoxide Anion Radical Scavenging Activity

Superoxide Anion Radical scavenging Activity was measured according to the method[23] with some modifications. The different plant extracts were mixed with 3 ml of reaction buffer solution (pH, 7.4) containing 1.3 µM riboflavin, 0.02 M methionine and 5.1 µM NBT. The reaction solution was illuminated by exposure to 30W fluorescent lamps for 20 minutes and the absorbance was measured at 560 nm using Systronics UV-VIS double beam spectrophotometer. Ascorbic acid was used as positive control and the reaction mixture without any sample was used as negative control.

The Superoxide anion radical scavenging activity (%) was calculated as:

$$\frac{Ao - As}{Ao} \times 100$$

Phytochemical screening of the extracts

The portions of the dry extracts were subjected to the phytochemical screening using the method adopted[24,25]. Phytochemical screening was performed to test for alkaloids, saponin, tannins, flavanoids, steroids, sugars and cardiac glycosides.

Test for alkaloids

The 0.5 g of the plant extracts were dissolved in 5 ml of 1% HCl and was kept in water bath for about 2 minutes. 1ml of the filtrate was treated with Dragendroff's reagent Turbidity or precipitation was taken as indicator for the presence of alkaloids.

Test for Tannins

About 0.5 g of the sample were dissolved in 10 ml of boiling water and was filtered. Few ml of 6% $FeCl_3$ was added to the filtrate. Deep green colour appeared confirmed the presence of Tannins.

Test for Flavanoids

About 0.2 g of the extracts were dissolved in methanol and heated for some time. A chip of Mg metal was introduced followed by the addition of few drops of conc. HCl. Appearance of red or orange color was Indicator of the flavanoids.

Test for Saponin

About 0.5 g of the plant extracts were stirred with water in the test tube. Frothing persists on warming was taken as a evidence for the presence of saponin.

Test for Steroids

Salkowaski method was adopted for the detection of steroids. About 0.5 g of extracts were dissolved in 3 ml of chloroform and filtered. To the filtrate, conc. H_2SO_4 was added to form a lower layer. Reddish brown color was taken as positive for the presence of steroids ring.

Test for Cardiac glycoside

About 0.5 g of the extracts were dissolved in 2 ml of glacial acetic acid containing 1 drop of 1% $Fecl_3$. This was under laid with conc. H_2SO_4. A brown ring obtained at the interphase indicates the presence of deoxy sugar. A violet ring appeared below the ring while in the acetic acid layer a greenish ring appeared just above ring and gradually spread throughout this layer.

Test for reducing Sugars

1ml each of Fehling's solutions, I and II was added to 2 ml of the aqueous solution of the extracts. The mixtures were heated in a boiling water bath for about 2-5 minutes. The production of a brick red precipitate indicated the presence of reducing sugars.

Results

Antioxidant activity

In vitro antioxidant activity was determined by DPPH radical scavenging method and Superoxide anion radical scavenging assay. Amongst all the extracts, ethanol and water extract of whole plant of *Viola patrinii* exhibited potent antioxidant activity in comparison to that of acetone, chloroform, hexane and petroleum ether extracts. TPC in ethanol extract was found to be 425 µg/ml followed by water extracts having 350 µg/g gallic acid equivalents. IC_{50} value of ethanol extract was found to be 12.00 µg/ml followed by water extracts viz. 15.00 µg/ml in DPPH radical scavenging method. It was found that minimum is the value of IC50, maximum is the antioxidant activity. In Superoxide anion radical scavenging method ethanol extracts showed 85 % inhibition of superoxide followed by water extracts having 75 % inhibition. Ascorbic acid was used as the standard antioxidant having IC_{50} value, 78.17 µg/ml in DPPH radical scavenging method and causes 87.80 % inhibition of superoxide. The results are shown in **Table 1**, **2** and **3**; **Figure 1, 2** and **3.** The results of all the three procedures are totally correlated to each other and confirm the use of plant as natural antioxidant.

Table 1: Total Phenolic Content (TPC) (µg/g gallic acid equivalents) of solvent extracts of *Viola patrinii*

***Viola patrinii* (Solvent Extracts)**	**TPC (µg/g gallic acid equivalents)**
Ethanol extract (E)	425
Acetone extract (AC)	228
Water extract (W)	350
Hexane extract (H)	174
Chloroform extract (C)	86
Petroleum ether extract (PE)	80

Table 2: IC50 values of solvent extracts of *Viola patrinii* as determined by DPPH assay

***Viola patrinii* (Solvent Extracts)/ Positive Control**	**IC50 values**
Ethanol extract (E)	12.10
Acetone extract (AC)	18.60
Water extract (W)	15.00
Hexane extract (H)	20.00
Chloroform extract (C)	20.05
Petroleum ether extract (PE)	25.05
Positive Control, Ascorbic acid	78.17

Table 3: Percent inhibition of superoxide free radicals of solvent extracts of *Viola patrinii* as determined by Superoxide anion radical scavenging activity

***Viola patrinii* (Solvent Extracts)/ Positive Control**	**Percent inhibition of Superoxide free radicals**
Ethanol extract (E)	85.0
Acetone extract (AC)	62.0
Water extract (W)	75.0
Hexane extract (H)	56.0
Chloroform extract (C)	50.0
Petroleum ether extract (PE)	46.0
Positive Control, Ascorbic acid	87.80

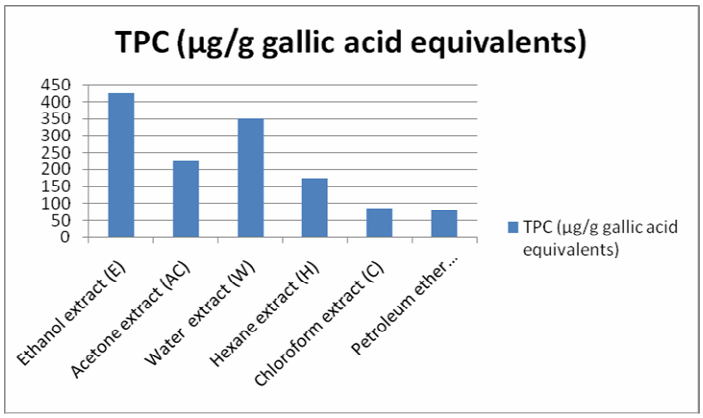

Figure 1: TPC (µg/g gallic acid equivalents) of solvent extracts of *Viola patrinii*

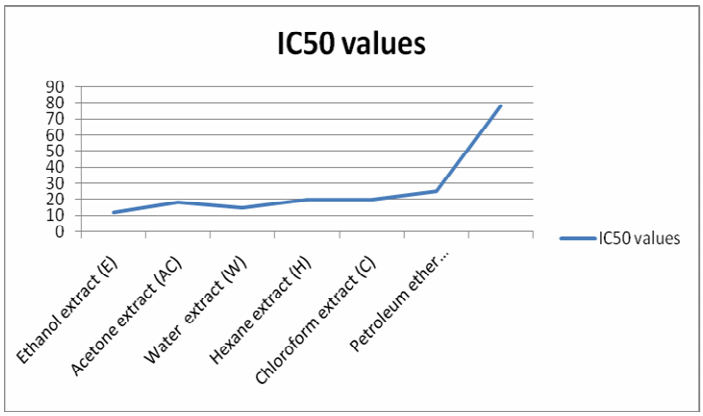

Figure 2: IC50 values of solvent extracts of *Viola patrinii* as determined by DPPH assay

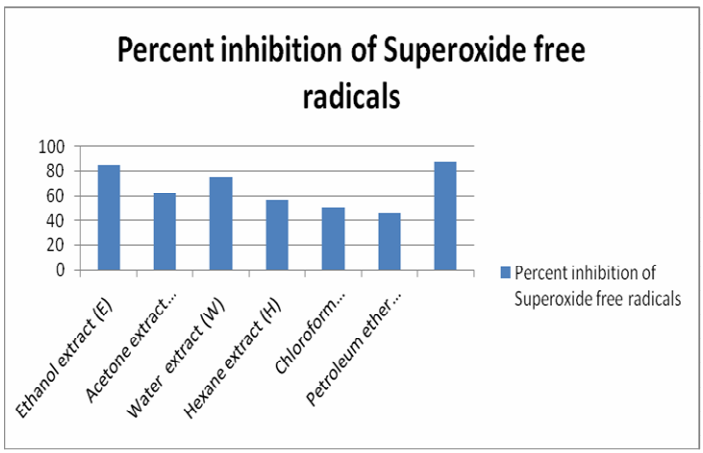

Figure 3: Percent inhibition of superoxide free radicals of solvent extracts of *Viola patrinii* as determined by Superoxide anion radical scavenging activity

Phytochemical Screening

Different conventional methods were followed to determine qualitatively the presence of phytochemical constituents present in the potent extracts. It was found that all the phytochemicals were present in all the extracts except steroids and saponin which were found only in hexane, chloroform and petroleum ether extracts. The results are indicated in **Table 4.** The study thus highlighted the importance of pharmacological importance and scientific investigation of plants from North West Himalaya Garhwal region through forward bio-prospection to uncover bioactive phytochemicals of interest and thus validates traditional medicine.

Table 4: Phytochemical screening of solvent extracts of *Viola patrinii*

Viola patrinii **(Solvent Extracts)**	**Phytochemicals**						
	Alkaloids	**Tannins**	**Flavanoids**	**Saponin**	**Steroids**	**Cardiac glycosides**	**Reducing Sugars**
Ethanol extract (E)	+	+	+	_	_	+	+
Acetone extract (AC)	+	+	+	_	_	+	+
Water extract (W)	+	+	+	_	_	+	+
Hexane extract (H)	+	+	+	+	+	+	+
Chloroform extract (C)	+	+	+	+	+	+	+
Petroleum ether extract (PE)	+	+	+	+	+	+	+

*+, presence; -, absence

Discussion

The present study illustrates that plant, Viola patrinii is the good source of antioxidant. A sufficient ingestion of natural antioxidants in food is therefore of great consequence for the defense of macromolecules against oxidative damage. The cells most frequently damaged by oxidative stress are unsaturated fatty acids in lipids, cholesterol, different functional polypeptides and proteins, and nucleic acids. Mechanisms of antioxidants consist of free radical quenching, transition metal chelating, reducing peroxide, and simulation of in vivo antioxidative enzyme activities[26] thus our study can be utilized further to isolate novel molecules responsible for antioxidant activity.

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