EXTRACTION, PHYTOCHEMICAL SCREENING AND ANTIOXIDANT POTENTIAL OF HYDROALCOHOLIC EXTRACT OF *VIOLA ODORATA LINN*

Abstract

Medicinal plants have bioactive compounds which are used for curing of various human diseases and also play an important role in healing. Secondary constituents contain alkaloids, flavonoids, phenol, saponin, steroids and tannins etc. Medicinal plants have anticancer, antimicrobial, antidiabetic, anti-diuretic and anti-inflammation activities etc. The increasing interest in powerful biological activity of secondary metabolites outlined the necessity of determining their contents in medicinal plants. *Viola odorata* (*V. odorata*, Violaceae) is a species of the genus *Viola* native to Europe and Asia and has been introduced to North America and Australasia. The herb is known as *Banafsa*, *Banafsha*, or *Banaksa* in India, where it is commonly used as a remedy to cure sore throat and tonsillitis. It was known to be used to relieve pain due to cancer. In the traditional system, it has been used in anxiety, insomnia and to lower blood pressure. It contains alkaloid, glycoside, saponins, methyl slicylate, mucilage and vitamin C. The plant has been reported to possess antioxidant, analgesic, antihypertensive and diuretic activities. The objectives of this study are to screen the phytochemicals, estimate the content of phenolic and flavonoid compounds and determine the antioxidant capacity of the *V. odorata* flowers. Qualitative analysis of various phytochemical constituents and quantitative analysis of total phenol and flavonoids were determined by the well-known test protocol available in the literature. The hydroalcoholic extract of flowers of *V. odorata* was studied for antioxidant activity on different *in vitro* models namely 1,1-diphenyl, 2-picryl hydrazyl (DPPH) assay and hydrogen peroxide radical scavenging method. Phytochemical analysis revealed the presence of phenols, flavonoids, tannins, saponins, alkaloids etc. The total phenolic and flavonoids content of *V. odorata* flower of hydroalcoholic extract was 0.521and 0.637mg/100mg respectively. The extract showed dose dependent free radical scavenging property in the tested models. *V. odorata* flower extract showed IC_{50} value 109.78µg/ml for DPPH method, which was comparable to that of ascorbic acid (IC_{50}=27.82µg/ml). For hydrogen peroxide method, IC_{50} value was found to be 85.85µg/ml, which compares favorably with ascorbic acid (IC_{50}=16.48µg/ml). The present study describes the phytochemical profile and antioxidant activity of *V. odorata* flower which will further used for medicinal applications.

Keywords: *Viola odorata*, Qualitative, Quantitative phytochemical, Antioxidant activity.

Introduction

Free radicals or highly reactive oxygen species are formed by exogenous chemicals or endogenous metabolic processes in the human body. These are capable of oxidizing bio-molecules viz nucleic acids, proteins, lipids and DNA and can initiate different degenerative diseases like neurological disorders, cancer, emphysema, cirrhosis, atherosclerosis, arthritis etc [1,2]. Antioxidants are the compounds which terminate the attack of free radicals and thus reduce the risk of these disorders [3]**.** Almost all organisms are protected up to some extent by free radical damage with the help of enzymes such as super-oxide dismutase, catalase and antioxidant compounds viz. ascorbic acid, tocopherol, phenolic acids, polyphenols, flavonoids and glutathione. Prior and Cao [4], reported that antioxidant supplements or dietary antioxidants protect against the damaging effects of free radicals. Presently, much attention has been focused on the use of natural antioxidants to protect the human body especially brain tissues from the oxidative damage caused by free radicals. In last two decades, several medicinal plants have shown such effectiveness through the traditional methods of psychoneuropharmacology [5]. Keeping this in view, the present study has been conducted to evaluate the antioxidant activity of *V. odorata* which are traditionally well known for their various activities. *V. odorata* (Violaceae) is popularly known as Banafshah. It is found in high altitudes of Himalayas, Europe, and throughout North America. It is a long trailing plant of a less than 6-in height. The plant has a thick and scaly underground stem, with rooting runners. It possesses heart-shaped leaves with scalloped or slightly serrated edges which are dark green, smooth, or sometimes downy underneath and grow in a rosette form at the base of the plant. Flowers are deep purple or blue to pinkish or even whitish-yellow in color [6,7]. The flowers of *V. odorata* contain the odorous principle, blue coloring matter and sugar, a glucoside. *Viola*-quercetin is found throughout the plant. Salicylic acid (natural aspirin) has also been obtained from this plant [8]. An alkaloid violine is found in the roots, leaves, flowers, and seeds of *V. odorata*. The essential oil of *V. odorata* contains ionine, saponins, glycoside, methyl salicylate, mucilage, vitamins A and C, and alkaloids [9]. Flowers of *V. odorata* contain 4.0% anthocyanins, 1.1% fl avonoids, 18.0% mucilage and 8.5% ash[10]. The whole aerial part of *V. odorata* including stem, flowers, and leaves are used in bronchitis, cancer, cough, fever, urinary infections, rheumatism, sneezing, and kidney and liver disorders [11,12]. Usual forms of the preparation is a syrup made from the petals of flowers of which 1 to 2 drachms may be given to infants for coughs and tightness of the chest. An infusion (2 drachms of the flower in a pint of warm water) is given as a cooling mixture in fever, in doses of 1–2 oz [6]. *V. odorata* was investigated for cytotoxicity and

antipyretic activity [13, 14]. The present study was focused to evaluate the phytochemical analysis and antioxidant activity of flowers of *V. odorata*.

Material and method

Plant material

Fresh flowers of *V. odorata* were collected from Minor Forest Produce Processing & Research Centre, Vindhya Herbals Bhopal. Plant material (flower part) selected for the study were washed thoroughly under running tap water and then were rinsed in distilled water; they were allowed to dry for some time at room temperature. Then the plant material was shade dried without any contamination for about 3 to 4 weeks. Dried plant material was grinded using electronic grinder. Powdered plant material was observed for their colour, odour, taste and texture. Dried plant material was packed in air tight container and stored for phytochemical and biological studies.

Chemical reagents

All the chemicals used in this study were obtained from Hi Media Laboratories Pvt. Ltd. (Mumbai, India), Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals and solvent used in this study were of analytical grade.

Defatting of plant material

53 gram shade dried powder of flowers of *V. odorata* was extraction with petroleum ether using maceration method. The extraction was continued till the defatting of the material had taken place.

Successive extraction with different solvents by maceration method

Plant material were extracted in four solvents of different polarity viz water, hydroalcoholic, ethyl acetate and chloroform. Powdered plant materials were extracted by maceration method. The resultant content was filtered with whatman filter paper no.1 and kept for evaporation of solvent to get the dry concentrated extract. The dried crude concentrated extract was weighed to calculate the extractive yield then transferred to glass vials (6 ×2 cm) and stored in a refrigerator (4°C), till used for analysis [15].

Phytochemical screening

Phytochemical screening to detect the presence of bioactive agents was performed by standard procedures [16, 17]. After the addition of specific reagents to the solution, the tests were detected by visual observation of color change or by precipitate formation.

Estimation of total phenolic content

The total phenolic content of the extract was determined by the modified Folin-Ciocalteu method [18]. 10 mg Gallic acid was dissolved in 10 ml methanol, various aliquots of 5-25µg/ml was prepared in methanol. 10mg of dried extracts of were dissolved in 10 ml methanol and filter. Two ml (1mg/ml) of this solution was used for the estimation of phenol. 2 ml of each extract or standard was mixed with 1 ml of Folin-Ciocalteu reagent (previously diluted with distilled water 1:10 v/v) and 1 ml (7.5g/l) of sodium carbonate. The mixture was vortexed for 15s and allowed to stand for 15 min for colour development. The absorbance was measured at 765 nm using a spectrophotometer.

Estimation of total flavonoids content

Determination of total flavonoids content was based on aluminium chloride method [19]. 10 mg quercetin was dissolved in 10 ml methanol, and various aliquots of 5- 25µg/ml were prepared in methanol. 10mg of dried extracts of were dissolved in 10 ml methanol and filter. Three ml (1mg/ml) of this solution was used for the estimation of flavonoid. 1 ml of 2% $AlCl_3$ methanolic solution was added to 3 ml of extract or standard and allowed to stand for 15 min at room temperature; absorbance was measured at 420 nm.

Antioxidant activity

DPPH radical scavenging assay

DPPH scavenging activity was measured by the spectrophotometer with slightly modification [20]. Stock solution (6 mg in 100ml methanol) was prepared such that 1.5 ml of it in 1.5 ml of methanol gave an initial absorbance. Decrease in the absorbance in presence of sample extract at different concentration (10- 100 µg/ml) was noted after 15 minutes. 1.5 ml of DPPH solution was taken and volume made till 3 ml with methanol, absorbance was taken immediately at 517 nm for control reading. 1.5 ml of DPPH and 1.5 ml of the test sample of different concentration were put in a series of volumetric flasks. Absorbance at zero time was taken for each concentration. Final decrease in absorbance was noted of DPPH with the sample at different concentration after 15 minutes at 517 nm. The percentage inhibition of free radical DPPH was calculated from the following equation: % inhibition = [(absorbance of control - absorbance of sample)/absorbance of control] × 100%.

Hydrogen peroxide

Scavenging activity of hydrogen peroxide (H_2O_2) by the plant extract was determined by the method of Ruch *et al.,* (1989) [21]. Ethanolic extract (4 ml) prepared in distilled water at various concentration was mixed with 0.6 ml of 4 mM H_2O_2 solution prepared in phosphate

buffer (0.1 M pH 7.4) and incubated for 10 min. The absorbance of the solution was taken at 230 nm. Ascorbic acid was used as a positive control compound. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples using following equation.

$$S\% = [(Acontrol - Asample)/Acontrol] \times 100$$

Where Acontrol = absorbance of the blank control (containing all reagents except the extract solution) Asample = absorbance of the test sample.

Results and Discussion

The crude extracts so obtained after each of the successive maceration extraction process were concentrated on water bath by evaporation the solvents completely to obtain the actual yield of extraction. The yield of extracts obtained from the flowers of the plants using chloroform, ethyl acetate, hydroalcoholic and water as solvents are depicted in the Table 1. The results of qualitative phytochemical analysis of the crude powder of flower of *V. odorata* were shown in Table 2. Hydroalcoholic and aqueous extracts of *V. odorata* showed the presence of alkaloids, diterpenes, glycosides, flavonoids, saponins, phenols, proteins and carbohydrate. The total phenolic content (TPC) was expressed as mg/100mg of gallic acid equivalent of dry extract sample using the equation obtained from the calibration curve: $y = 0.019x + 0.016$, $R^2 = 0.999$, where X is the gallic acid equivalent (GAE) and Y is the absorbance. Total flavonoids content was calculated as quercetin equivalent (mg/100mg) using the equation based on the calibration curve: $y = 0.032x + 0.002$, $R^2 = 0.999$, where X is the quercetin equivalent (QE) and Y is the absorbance. TPC of hydroalcoholic extract of *V. odorata* showed the content values of 0.521and fallowed by TFC were 0.637 Table 3 and fig. 1&2. DPPH radical scavenging assay measured hydrogen donating nature of extracts [22]. Under DPPH radical scavenging activity the inhibitory concentration 50% (IC_{50}) value of ***V. odorata*** hydroalcoholic flower extract was found to be 109.78µg/ml as compared to that of ascorbic acid (27.82µg/ml). A dose dependent activity with respect to concentration was observed Table 4 and fig. 3. Hydrogen peroxide is generated *in vivo* by several oxidase enzymes and by activated phagocytes and it is known to play an important role in the killing of several bacterial and fungal strains [23]. There is increasing evidence that, hydrogen peroxide, either directly or indirectly via its reduction product, OH^o, can act as a messenger molecule in the synthesis and activation of several inflammatory mediators [24]. When a scavenger is incubated with H_2O_2 using a peroxidase assay system, the loss of H_2O_2 can be measured. Table 5 & fig. 4 show the scavenging ability of *V. odorata* hydroalcoholic flower extract and ascorbic acid on hydrogen peroxide at different concentrations. Extracts was

capable of scavenging hydrogen peroxide in an amount dependent manner at all the tested concentrations. Hydrogen peroxide itself is a rather weak oxidant and most organic compounds (except for some sulfur containing molecules) are virtually inert to attack by it at ordinary environmental or cellular concentrations and temperatures. In the presence of reduced transition metal ions, however, hydrogen peroxide is converted to the much more reactive oxidant, hydroxyl radical in the cells by Fenton reaction. Besides this, studies have shown that other transition metals such as copper (I), cobalt (II) and nickel(II) also take part in the process [23]. Thus, the removing is very important for antioxidant defense in cell or food systems.

Table 1 Results of percentage yield of flowers extracts

Sr. No	Solvents	% Yield (W/W)
1	Petroleum ether	1.52
2	Chloroform	4.35
3	Ethyl acetate	3.78
4	Hydroalcoholic	9.33
5	Distilled water	5.44

Table 2 Result of phytochemical screening of extracts of *V. odorata*

S. No.	Constituents	Chloroform extract	Ethyl acetate extract	Hydroalcoholic extract	Aqueous Extract
1.	**Alkaloids** Hager's Test:	 -Ve	 -Ve	 +Ve	 -Ve
2.	**Glycosides** Legal's Test:	-Ve	-Ve	+Ve	-Ve
3.	**Flavonoids** Lead acetate Test: Alkaline test:	 -Ve -Ve	 +Ve -Ve	 +Ve +Ve	 -Ve +Ve
4.	**Diterpenes** Copper acetate Test:	-Ve	-Ve	+Ve	+Ve
5.	**Phenol** Ferric Chloride Test:	-Ve	-Ve	+Ve	+Ve
6.	**Proteins** Xanthoproteic Test:	+Ve	-Ve	+Ve	-Ve
7.	**Carbohydrate** Fehling's Test:	+Ve	-Ve	+Ve	+Ve
8.	**Saponins** Froth Test:	-Ve	-Ve	+Ve	+Ve
9.	**Tannins** Gelatin test:	 -Ve	 -Ve	 -Ve	 -Ve

Table 3 Results of total phenol and flavonoids content

S. No.	Extracts	Total phenol content	Total flavonoids content

		mg/100mg	
1	Ethyl acetate	0.253	0.365
2	Hydroalcoholic	0.521	0.637
3	Aqueous	0.425	0.569

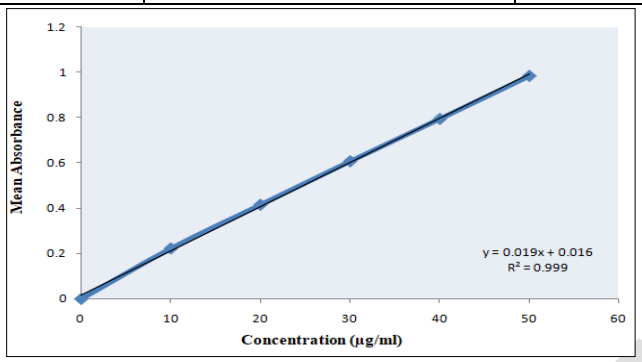

Figure 1 Graph of Calibration curve of Gallic acid

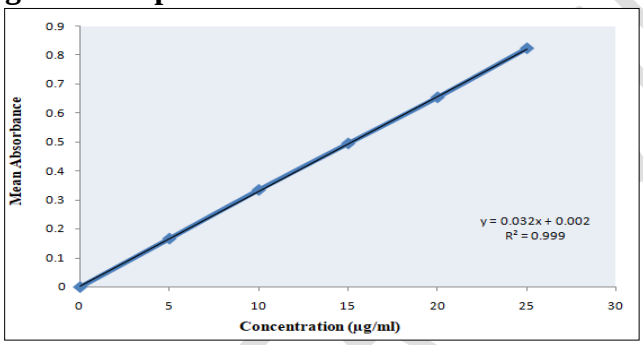

Figure 2 Graph of calibration curve of Quercetin

Table 4 % Inhibition of ascorbic acid and extract of *V. odorata* using DPPH method

S. No.	Concentration (µg/ml)	% Inhibition	
		Ascorbic acid	Hydroalcoholic extract
1	10	41.52	9.8
2	20	47.70	13.3
3	40	52.92	15.4
4	60	67.43	29.52
5	80	75.89	36.51
6	100	89.63	47.85
IC 50		**27.82**	**109.78**

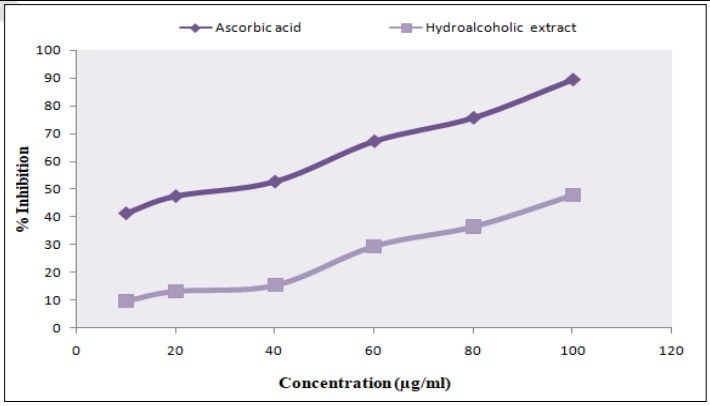

Figure 3 % Inhibition of ascorbic acid and extract of *V. odorata*

Table 5 % Inhibition of ascorbic acid and extract of *V. odorata* using H_2O_2 method

S. No.	Concentration (µg/ml)	% Inhibition	
		Ascorbic acid	Hydroalcoholic extract
1	20	46.58	15.85
2	40	67.52	23.52
3	60	76.52	30.54
4	80	83.52	48.21
5	100	91.85	59.52
IC 50		**16.48**	**85.85**

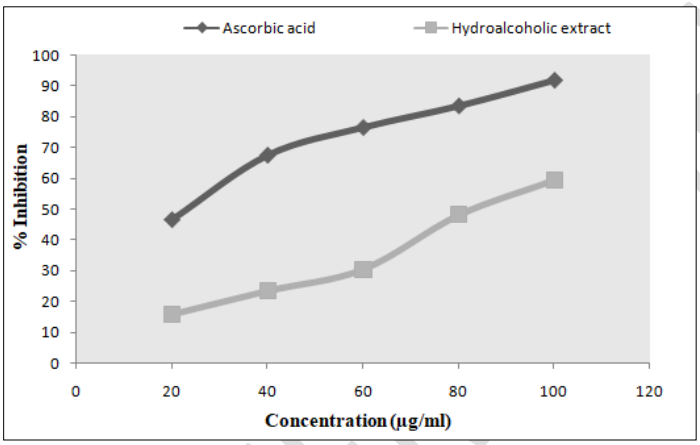

Figure 4 % Inhibition of ascorbic acid and extract of *V. odorata*

Conclusion

The results obtained in the present study clearly demonstrate that the extract, which can effectively scavenge various reactive oxygen species/free radicals under *in vitro* conditions. This may be due to the number of stable oxidized products that it can form after oxidation or radical scavenging. The broad range of activity of the extracts suggests that multiple mechanisms are responsible for the antioxidant activity. The multiple antioxidant activity of extract demonstrated in this study clearly indicates the potential application value of the plant. However, the *in vivo* safety of *V. odorata* needs to be thoroughly investigated in experimental rodent models prior to its possible application as an antioxidant ingredient, either in animal feeds or in human health foods. The above results showed that *V. odorata* hydroalcoholic flower extract could exhibit antioxidant properties. Further studies, on the use of above plants for their antioxidant role in various systems may provide potential natural antioxidants.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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