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Phyto-chemical analysis, radical scavenging, cytotoxic and antibacterial activities of *Viola indica* from Kashmir, India

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ABSTRACT

Objectives: Phytochemical analysis and biological activities of *Viola indica*. **Methods:** The powdered plant material of *Viola indica* was successively extracted with petroleum ether, ethyl acetate and methanol. The radical scavenging activity of methanolic extract was tested using 1, 1-diphenyl-2-picrylhydrazyl radical. Ethyl acetate and methanol extract was tested against Leukemia (THP-1), Lung (A-549), Colon (HCT-15), Cervix (Hela) and Prostrate (PC-3) cell lines at 100 µg/ml, respectively. The antibacterial activity of methanolic extract was also tested, against *Pseudomonas aeruginosa*, *Proteus vulgaris* etc. **Results:** The phytochemical analysis of different extract revealed the presence of flavonoids, alkaloids, tannins, saponins and phenols. The methanolic extract tested showed 51±0.2% radical scavenging activity at 40 µg/ml and maximum zone of inhibitions against *S. epidermidis* (29 mm), *S. aureus* (23 mm) and *P. vulgaris* (22 mm). The results of cytotoxic activity showed that methanolic and ethyl acetate extracts were potent only against the THP-1, Hela, PC-3 and HCT-15 cell cultures. **Discussion:** *V. indica* showed radical scavenging, cytotoxic and antibacterial activity due to flavonoids, alkaloids, tannins, saponins and phenols. **Conclusion:** As a part of our ongoing research this will be a positive move towards the development of new pharmaceuticals agents used in the treatment of various radical oxide associated diseases.

KEYWORDS

Viola indica;
Phytochemical analysis;
Radical scavenging activity;
Cytotoxic activity;
Antibacterial activity.

INTRODUCTION

Reactive oxygen species (ROS) comprise various forms of activated oxygen including superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), nitric oxide ($^{\cdot}NO$), and peroxy-nitrite ($ONOO^-$), which often are generated as by-products of biological reactions or from exogenous factors[1]. It is commonly recognised that ROS are involved in a variety of physiological and pathological processes, including cellular signal transduction, cell proliferation, differentiation and apoptosis, as well as ischemia– reperfusion, inflammation, and many neurodegenerative disorders[2]. ROS production can induce DNA damage, protein carbonylation and lipid peroxidation, leading to a variety of chronic health problems, such as cancer, ageing, Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis[3]. In healthy individuals, ROS

production is continuously balanced by natural antioxidative defense system. Oxidative stress is a process where the physiological balance between prooxidants and antioxidants is disrupted in favour of the former, leading to potential damage to the organism[4].

Dietary antioxidant intake may be an important strategy for inhibiting or delaying the oxidation of susceptible cellular substrates, and is thus relevant to disease prevention in many paradigms. Phenolic compounds such as flavonoids; phenolic acids, triterpenes and tannins have much attention for their high antioxidant activity[5].

The antimicrobial compounds found in plants are of interest because of antibiotic resistance which is becoming a worldwide public health concern especially in terms of food borne illnesses and nosocomial infections. The antimicrobial agents isolated so far from different aromatic and medicinal plants have been of great importance in the field of pharmaceutical and therapeutic industries in formulation of various potent drugs, needed to combat some antibacterial and antifungal diseases including respiratory infections, asthma, sinusitis and chronic bronchitis.

Viola indica commonly known as Banafsha is an herb which belongs to the family Violaceae. Violaceae is a family of about 900 species, mainly found in temperate regions of the world, where they are usually small perennial plants. Most species of this genus are found in the temperate Northern Hemisphere. In India it is found in Kashmir and West Bengal. It is used as a cough expectorant locally. Other species of this genus are used as analgesic, diaphoretic, blood purifiers and diuretics. So in light of the above mentioned wide applications, the current study was directed towards the screening of different extracts including ethyl acetate and methanolic of *V. indica* for its radical scavenging, cytotoxic and antibacterial activities. However the detailed literature survey revealed that there is no such literature available documenting the radical scavenging, cytotoxic and antibacterial activities of the various extracts of *V. indica*. Thus this will be the first report on *V. indica* growing in the Kashmir, India.

EXPERIMENTAL

Plant material

The aerial parts of *V. indica* were collected from Aru Pahalgam, District Anantnag Kashmir, India in the month of June 2010. The localities were the plant material was collected are usually situated between 2400-4600 m higher than sea level. The plant material was properly identified by A. H. Malik, Centre for plant Taxonomy and Biodiversity, University of Kashmir, Srinagar. The Voucher specimen of *V. indica* bearing specimen no. 690 was deposited at KASH herbarium in Centre for plant Taxonomy, and Biodiversity, University of Kashmir, Srinagar.

Chemicals

DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical was purchased from Sigma–Aldrich, Madrid, Spain. Dimethyl sulphoxide (DMSO), anhydrous sodium sulphate, petroleum ether, ethyl acetate, methanol and all other reagents were of analytical grade (SISCO, Mumbai, India).

Extraction

Dried and powdered plant material (220 g) of *Viola indica* was extracted, using soxhlet extractor with 100% organic solvents in increasing order of their polarity (petroleum ether, ethyl acetate and Methanol). The extracts obtained were concentrated under vacuum using rotary vapor evaporator at 40^0 C.

Phytochemical analysis

The phytochemical screening of the plant extracts was carried out according to method previously reported[6].

Alkaloids

The plant extracts (30 ml) were evaporated to dryness in an evaporating dish on water bath. Five ml of 2 N HCl were added and stirred while heating on the water bath for 10 minutes, cooled, filtered, and the filtrated was treated with a few drops of Mayer reagent. The samples were than observed for the presence of turbidity or precipitation.

Flavonoids

The plant extracts (75 ml) were evaporated to dryness on a water bath, cooled and the residue was defatted by washing several times with petroleum ether. The defatted residue was dissolved in 30 ml 80% ethanol and filtered. The filtrated was treated with a few drops of concentrated HCl and magnesium turnings (0.5

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g). The presence of flavonoids was indicative if a pink or magenta red color developed within 3 min.

Tannins

The plant extracts (25 ml) were evaporated to dryness on a water bath. The residue was extracted several times with n-hexane and filtered, the insoluble residue was stirred with 10 ml of hot saline solution, the mixture was cooled, filtered and the volume of filtrate was adjusted to 10 ml with more saline solution. To 5 ml of this solution, few drops of ferric chloride test reagent were added. An intense green blue or black colour was taken as an evidence for the presence of tannins.

Saponins

1 gm of each plant extract was dissolved in 10 ml of distilled water in a test tube and shaked vigorously for 1-2 minutes. The presence of saponins was indicated by characteristic honey comb froath at least 1 cm in height, which persisted for 30 minutes.

Phenols

1 gm of plant extract was dissolved in 10 ml of distilled water in two separate test tubes. To these test tubes few drops of alcoholic ferric chloride were added. An intense green colour was taken as an evidence for the presence of phenols.

Radical scavenging activity

The hydrogen atom(s) donation abilities of the methanolic extract was measured from the bleaching of purple coloured methanol solution of DPPH. This spectrophotometric assay uses stable radical DPPH as a reagent[7]. For this assay various concentrations 5, 10, 20, 30 and 40 μg/ml of the extract in methanol was added to 5 ml of 0.004% methanolic solution of DPPH. After 30 minutes incubation period at room temperature, the absorbance was read against blank sample at 517 nm. Ascorbic acid was used as positive control. The tests were carried in triplicate. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the following formula:

Percentage inhibition = $[(A_o - A_t) / A_o \times 100]$.

Where A_o was the absorbance of the control (blank, without extract) and A_t was the absorbance in the presence of the extract. The reaction involved:

$$\underset{\text{(Purple coloured)}}{\text{DPPH}\cdot} + \text{Sample} \rightarrow \underset{\text{(Yellow coloured)}}{\text{DPPH}_2} + \text{Sample (Radicals)}$$

Cancer cell lines and culture

The cell lines Leukemia (THP-1), Lung (A-549), Colon (HCT-15), Cervix (Hela) and Prostrate (PC-3) were cultured in RPMI-1640 medium, supplemented with Paclitaxel and Mitomycin-C (Sigma Aldrich, Madrid Spain) as positive controls. The cell lines were cultured in Indian Institute of Integrative Medicines (IIIM-CSIR) Jammu, India in a humidified atmosphere at 37 ºC in 5% CO_2. The cells were incubated for 48 hours after adding a sample and then kept in cold ice for 1 hour at 4 ºC. The plates containing optimum density of seeded cell suspensions were washed with distilled water and 0.4% of SRB solution (Sulpha-rhodamine -B) was added to each air dried plate for staining at room temperature for 30 minutes. The unbound-SRB solution was removed by washing the plates with 1% (v/v) CH_3COOH (Acetic Acid). The bound SRB-dye was solubilised by adding 100 ml of 10 mM unbuffered Tris base (pH=10.5) to each well and shaking for 5 minutes on shaker plateform. The absorbance was measured at 570 nm. The experiment was repeated thrice.

Cytotoxic activity

SRB-assay was performed against five human cancer cell lines namely THP-1, A-549, HCT-15, Hela and PC-3, which revealed increase in growth of inhibition during 48 hours incubation at a concentration of 100 μg/ml of methanolic and ethyl acetate extract. Dimethyl sulphoxide (DMSO) was used as negative control, which was also used as a dissolving solvent for different extracts, homogeneously. The results depicted that the inhibition of different human cancer cell lines of varying tissue origin with 100 μg/ml of different extract imparted significant cellular cytotoxic effects against all the cell lines that were tested. However the most promising results were obtained for ethyl acetate and methanol extracts against THP-1, HCT-15, Hela and PC-3 cancer cell lines.

Test micro-organisms and growth conditions

Gram-positive and Gram-negative strains were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH) Chandigarh, India. The bacterial strains used were

Pseudomonas aeruginosa MTCC 1688, *Proteus vulgaris* MTCC 426, *Bacillus subtilis* MTCC 441, *Staphylococcus epidermidis* MTCC 435 and *Staphylococcus aureus* MTCC 96. Bacterial strains were grown on nutrient agar plates at 37 ^{0}C and maintained on nutrient agar slants.

Antibacterial activity

The antibacterial susceptibility test of methanolic extract was carried out using the agar well diffusion assay[8] with some modification. Briefly the overnight cultures of the indicator strains of bacteria were added to 20 ml of liquid nutrient agar. The contents of the tubes were transferred to petri plates. After the 10 minutes of solidification of the agar petri plates at room temperature, the punched wells on the plates were filled with 2 mg/ml of methanolic extract. The incubation was carried out for 24 h at 37 ^{0}C for bacteria. Antimicrobial activity was assessed by measuring the diameter of the growth-inhibition zone in millimeters (including disc diameter of 6 mm) for the test organisms comparing to the controls. Kennomycin (Merck, India) 10 µg per disc was used as positive controls for bacteria. The experiment was performed in triplicate.

RESULTS

Phytochemical analysis

The phytochemical analysis of different extracts of *Viola indica* revealed the presence of alkaloids, flavonoids, tannins, saponins and phenols. However, the results showed that the *Viola indica* contained flavonoids, tannins, alkaloids and saponins in good amount. Results of phytochemical analysis are depicted in the form of TABLE 1. Since flavonoids and tannins are responsible for various pharmacological activities, including anti-inflammatory, antioxidant, antibacterial, anti-allergic, asthma, and anti-histamine activity. Therefore the methanolic and ethyl acetate extract of the *Viola indica* was screened for its possible radical scavenging, cytotoxic and antibacterial activity using DPPH radical, SRB, Agar well diffusion assay, respectively.

TABLE 1 : Components of *Viola indica* extracts based on preliminary phytochemical screening.

Test	Aqueous	Ethyl acetate	Methanol	Petroleum ether
Tannin	-	-	+	NT
Flavonoids	+	-	++	NT
Alkaloids	-	+	+	NT
Saponins	NT	-	++	NT
Phenols	+	+	++	NT

++= appreciable amount, += moderate amount, (-) = Not present, NT= Not tested

Radical scavenging activity

Methanolic extract of *V. indica* showed a good result of radical scavenging activity at a concentration ranging from 5 to 40 µg/ml. The results of radical scavenging activity are arranged and depicted in the form of Figure 1 and TABLE 2. As shown in the results methanolic extract reacted directly with DPPH radicals and quenched them to different degrees with increased activities at higher concentration. At the concentration of 40 µg/ml, the scavenging activity of methanolic extract reached a plateau of 51±0.2%, whereas ascorbic acid reached 71±1.9% at the same concentration. Generally, antioxidants will react with DPPH, a nitrogen-centered radicals converted to 1, 1-diphenyl-2-picryl hydrazine, due to its hydrogen-donating ability, at a very rapid rate. The DPPH radical scavenging assay is commonly employed in evaluating the ability of antioxidants to scavenge free radicals. This method has been used extensively to predict the radical scavenging activity because of the relatively short time required for analysis. The change in absorbance at 517 nm is used as a measure of the scavenging effect of a particular sample for DPPH radicals. The more rapidly the absorbance decreases, the more potent the antioxidant activity of the sample in terms of its hydrogen atom-donating capacity[9,10].

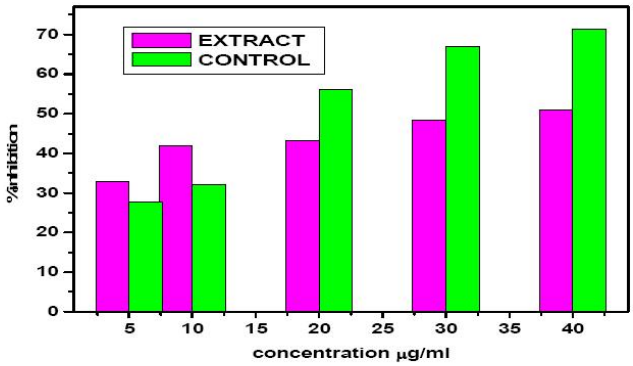

Figure 1 : Radical scavenging effect of methanolic extract in comparison to control ascorbic acid.

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TABLE 2 : *In-vitro* radical scavenging activity of methanolic extract of *Viola indica*.

MeoH.E (µg/ml)[a]	%RSA (MeoH.E)[b]	%RSA (Ascorbic acid)[c]
5	33±1.0	27.6±1.0
10	42±1.0	32.1±1.3
20	43.1±1.0	56.1±1.5
30	48.3±1.3	67.1±2.9
40	51±0.2	71.3±1.9

a = Concentration of MeoH.E i.e methnolic extract in µg/ml, b = Percentage of radical scavenging activity (RAS) of methanolic extract (MeoH.E), c = Percentage of radical scavenging activity (RAS) of positive control ascorbic acid.

Cytotoxic activity

In order to understand the effects of methnolic and ethyl acetate extract of *V. indica* on human cancer cell lines, experiments were carried using cultured Leukemia (THP-1), Lung (A-549), Colon (HCT-15), Cervix (Hela) and PC-3 cell lines by Sulph-rhodamine-B assay. All cell lines were submitted to maximum concentration of 100 µg/ml of ethyl acetate and methanolic extracts of *V. indica* for 48 hours. Both ethyl acetate and methnolic extract reduced the viability of these cell lines at above mentioned concentration. As shown in TABLE 3, these extracts were active against THP-1, HCT-15, Hela and PC-3 cancer cell lines tested. The percentage of dead cells in case of THP-1, HCT-15, Hela and PC-3 was found in the order of 81, 91, 75,61% and 70, 66, 65, 55%, for methanol and ethyl acetate extracts respectively, for the extract concentration of 100 µg/ml.

TABLE 3 : *In-vitro* cytotoxic activity of methanolic and ethyl acetat extract of *V. indica*.

Material	Conc. (µg/ml)	Leukemia (THP-1)[a]	Lung (A-549)[a]	Colon (HCT-15)[a]	Cervix (Hela)[a]	Prostrate (PC-3)[a]
EA.*E*	100	70±0.37	0±0.79	66±0.13	65±0.39	55±0.3
MEOH.*E*	100	81±0.10	14±0.93	91±0.53	75±0.32	61±0.96
Paclitaxel	$1x10^{-6}$	13±0.83	61±0.12	17±0.87	6±0.37	7±0.33
Mitomycin-C	$1x10^{-6}$	23±0.61	43±0.31	21±0.95	4±0.35	67±0.31

a= % growth inhibition against a particular cell line, EA.E= Ethyl acetat extract, MeoH.E= Methanolic extract.

Antibacterial activity

The methanolic extract from *V. indica* showed antibacterial activity at a concentration of 2 mg/ml against all the tested Gram-positive and Gram-negative bacteria i.e., *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Bacillus subtilis*, *Staphylococcus epidermidis* and *Staphylococcus aureus*. The methanolic extract of *V. indica* showed the maximum zone of inhibitions against *S. epidermidis* (29 mm), *S. aureus* (23 mm) and *P. vulgaris* (22 mm). However, the zone of inhibitions shown by extract against *P. aeruginosa* and *B. subtilis* was 18 and 20 mm, respectively. The data pertaining to the antimicrobial potential of the methanolic extract of *V. indica* are presented in the form of TABLE 4. Recently, there has been considerable interest in extracts from plants with antimicrobial activities for controlling pathogens and/or toxin producing microorganisms in foods[11-13].

TABLE 4 : *In-vitro* antibacterial activity of methnolic extract of *viola indica* and reference antibiotics determined with the agar diffusion method.

Test Microorganisms	MeoH. E[a]	Kennomycin	M.control[b]
P. aeruginosa	18±0.62	30±0.21	-
P. vulgaris	22±0.56	30±0.21	-
S. aureus	29±0.14	30±0.21	-
B. subtilis	20±0.65	30±0.21	-
S. epidermidis	23±0.84	30±0.21	-

MeoH.E[a] =Zones of inhibition of Methanolic extract in mm, M.control[b]=Methnol pure was used control, (-) = No inhibition

DISCUSSION

Investigation of extracts of *viola indica* indicated the presence of alkaloids, flavonoids, tannins, saponins and phenols, which may be responsible for its *in-vitro* radical scavenging, cytotoxic and anti-bacterial activities. Phenol and phenolic compounds such as flavonoids have been shown to posse's significant radical scavenging activities. Phenolic compounds can be simple with a single aromatic ring bearing at least one hydroxyl group. Polyphenols have at least two subunits such as flavonoids or three or more phenol subunits called tannins.

Li, and co-workers[14] reviewed the biological activities of tannins and observed that tannins, whether total or pure compound have remarkable activity in cancer prevention and anticancer activity. In addition to its antimicrobial and anticancer activities, tannins are potent antioxidants[15]. Flavonoids which are also among the constituents of *V. indica* extracts exhibit a wide range of biological activities which include antimicrobial, anti-

inflammatory, anti-analgesic, anti-allergic effects and anti-oxidant properties[16]. Flavonoids ability of scavenging hydroxyl radicals highlights many of their health-promoting functions in organisms, which are important for prevention of diseases associated with damage of membrane, proteins and DNA[17]. Flavonoids in human diet may reduce the risk of various cancers, as well as prevent menopausal symptoms[16]. Alkaloids, a nitrogen containing class of compounds reported to inhibit various pathogenic bacteria's growth[18]. Lastly, saponins which are responsible for numerous pharmacological properties[19] were also present in both ethyl acetate and methanolic extract of *viola indica*. The observations above support the use of *viola indica* ethyl acetate and methanolic extract in herbal cure remedies.

CONCLUSION

The current study indicates that *V. indica* could be used as a potential source of radical scavenging and cytotoxic agents. The results of antibacterial activity of *Viola indica* could also support to discover some new classes of antibiotic substances which could serve as selective agents for infectious disease.

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