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Antioxidant, Antibacterial and Antifungal Activity of *Impatiens sulcata* Wallich in Roxb. Extracts

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ABSTRACT- *Impatiens sulcata* is an annual plant used in traditional Chinese medicine for treatment of several ailments, seeds are edible, plant paste is applied to prevent utricaria, itching, eczema, pimples and mucilage is used as an abortifacient. Methanolic extract of *Impatiens sulcata* and its various fractions were screened for phytochemical analysis, antioxidant potential by total phenolic content, total flavonoid content, total antioxidant activity, DPPH scavenging activity, reducing power and ABTS scavenging activity, while antimicrobial activity by disc diffusion assay against a set of bacterial and fungal strains. Petroleum ether and methanolic extract showed higher free radical scavenging activity and phytochemical analysis revealed the presence of saponins, flavonoids and triterpenoids. In biological assay, the extracts showed the moderate antimicrobial activity.

Key-words- *Impatiens sulcata*; Phytochemical analysis; Antioxidant potential; Anti-bacterial activity; Anti-fungal activity;

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INTRODUCTION

Plants offer an enormous source of medicinal compounds due to structural complexities and diversities of their secondary metabolites. There is a growing interest in the investigation of plants as a source of novel drug molecules owing to the presence of panoply chemical structures that are yet to be explored for biological activity.

Impatiens sulcata Wallich in Roxb. (Balsaminaceae) is a medicinal plant used in folk medicine for treatment of several ailments; seeds are edible; plant paste is applied to prevent utricaria, itching, eczema, pimples; mucilage is used as an abortifacient [1]. *Impatiens sulcata* syn. *Impatiens gigantea* Edgew is an annual or biennial herb 50 to 250 cm high, found in North-West Himalayas. The secondary metabolites isolated from *Impatiens* species include phenolics, flavonols, anthocyanin pigments, quinones and saponins [2,3].

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Impatiens species are reported to be useful as antifungal [4], antibacterial [5], anti-anaphylactic [6], anti-inflammatory [7], anti-pruritic/anti-dermatitis [8], anti-tumour [9], antibacterial [10], anti-helminthic [11] and anti-histaminic [12].

The present paper for first time reports the antioxidant potential and antimicrobial activity of *Impatiens sulcata* from the high altitude regions of North West (Garhwal) Himalayas, Uttarakhand, India. The aim of this research work was to determine the total phenolic contents, total flavonoid contents and evaluate the antioxidant potential, antibacterial activity and antifungal activity of *I. sulcata* wall extracts.

MATERIALS AND METHODS

Plant material: The aerial part of *Impatiens sulcata* wall were collected in May (2013) from North-West (Garhwal) Himalayas, Uttarakhand, India and identified (GUH 6528) H. N. B Garhwal University (A Central University), Srinagar Garhwal, Uttarakhand, India.

Preparation of Plant extract

The plant material was dried at room temperature (25ºC) and chopped into small pieces. The air dried powdered plant material was extracted with petroleum ether, ethyl acetate and methanol in soxhlet extractor. The extracts were concentrated by rotary vacuum evaporator (40ºC) and then air dried.

Phytochemical composition

The extracts of *Impatiens sulcata* were analyzed for the presence of saponins, triterpenoids and flavonoids. About 2gm of the powdered extract was boiled in 20mL of distilled water in a water bath and filtered. Filtrate (10mL) was mixed with distilled water (5ml) and shaken vigorously for a stable persistent froth. The frothing was mixed with few drops of olive oil, shaken vigorously and observed for the formation of emulsion to determine the presence of saponins. Extract (5mL) was mixed in chloroform (2mL) and concentrated H_2SO_4 (3mL) was carefully added to form a layer. The presence of reddish brown coloration at the interface indicated presence of triterpenoids [13]. Few drops of sodium hydroxide solution were added to extract (5mL), the formation/absence of yellow color which becomes colorless on addition of dil. acid indicated presence/absence of flavonoids [14].

Determination of total phenolic content

The total phenolic content (TPC) was determined according to the method described by [15]. The reaction mixture consisted of extract (0.5mL), Folin-Ciocalteu's reagent (2.5mL, 10% v/v) and saturated sodium carbonate solution (2.0mL). The resulting mixture was vortexed (15sec) and incubated (40°C, 30min) for color development. The absorbance of total phenolics was measured at 765nm. Standard gallic acid solutions were used for calibration curve and results were expressed as gallic acid equivalent per gram of extract (mg GAE/g).

Determination of total flavonoid content

The extract (500µL) was diluted appropriately and mixed with $NaNO_2$ (1mL, 5%). After standing for 6min, 10% $AlCl_3$ (1mL) and NaOH (10mL, 1M) were added to the mixture. The mixture was adjusted to 25mL with 70% ethanol and allowed to rest for 15min. The absorbance was measured at 510nm, with 70% ethanol as a blank control [16]. Rutin was used as a reference standard and the total flavonoid content was expressed as rutin equivalents per gram of extract (mgRE/g).

Total antioxidant capacity

Extract (0.3mL) was mixed with 3.0mL reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). Reaction mixture was incubated (95°C, 90min) and absorbance was measured at 695nm [17]. Total antioxidant capacity was expressed as ascorbic acid equivalent per gram extract (mg AAE/g).

Reducing power

Different concentration of extracts (50-500 µg/mL) in 1mL of alcohol was mixed with 2.5mL phosphate buffer (0.2M, pH 6.6) and 2.5mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20min and 2.5mL of 10% trichloroacetic acid was added. The reaction mixture was then centrifuged for 10min. Further, 2.5mL of the supernatant solution was mixed with 2.5mL of distilled water and 0.5mL of 0.1% $FeCl_3$. The absorbance was measured at 700nm [18].

DPPH free radical scavenging activity

A 2ml aliquot of solution was added to 2ml of $2x10^{-4}$ mol/L ethanolic DPPH solution. The mixture was shaken vigorously and the absorbance was measured at 517nm immediately. The decrease in absorbance was determined at 15 and 30min until the absorbance reached a steady state (after nearly 30 min). The mixture with the addition of standard antioxidants served as a positive control [19]. All the tests were performed in triplicate, and the inhibition rate was calculated according to the formula,

$$\% \text{ Inhibition of DPPH free radical} = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$$

ABTS free radical scavenging activity

ABTS free radical was produced by reacting 7mM ABTS aqueous solution with 2.4mM potassium per sulfate in the dark for 16h at room temperature [20]. Prior to assay, the solution was diluted in ethanol and equilibrated at 30°C to give an absorbance of 0.700±0.02 at 734nm. The stock solution of the sample extracts were diluted such that after introduction of 10µl aliquots into the assay, they produced between 20% and 80% inhibition of the blank absorbance. After the addition of 1ml of diluted ABTS solution to 10µl of sample or Trolox standards in ethanol, absorbance was measured exactly 30min after the initial mixing. Appropriate solvent blanks were also run in each assay and the percentage inhibition of the blank absorbance was calculated at 734nm. Triplicate determinations were made at each dilution of the standard and percentage inhibition calculated and plotted as a function of Trolox concentration. The antioxidant activity is expressed as trolox equivalent antioxidant capacity (TEAC).

Anti-bacterial activity

Antibacterial activity was evaluated by the disc diffusion method [21] with slight modification against gram-positive and gram-negative bacteria. Human bacterial pathogens *Escherichia coli* MTCC-443, *Salmonela typhirium* MTCC-1255, *Klebsiella pneumoniae* MTCC-432, and *Staphylococcus aureus* MTCC-737 were procured from the Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India.

Disc diffusion assay

Nutrient agar medium (20 ml) was poured into the plates to a uniform depth and allowed to solidify. The standard inoculum suspension (10^6 c.f.u./mL) was streaked over the surface of the media using a sterile cotton swab to ensure the confluent growth of the organism. Plant extract (10µL) was diluted with two volumes of 5% dimethyl sulfoxide, impregnated on filter paper discs, and used for the assays. On the surface of the plates, discs were placed with sterile forceps, pressed gently to ensure contact with the inoculated agar surface. Streptomycin (10µg $disc^{-1}$) was

used as a positive control and hexane as a negative control. The plates were incubated in the dark at 37°C (24 h) and the inhibition zones calculated. All experiments were carried out in triplicate.

Anti-fungal activity

The antifungal activity was tested by disc diffusion method. Three fungal strains select for check antifungal activity (*Trichorderma viride* MTCC 167, *Aspergillus niger* MTCC 2208 *and Aspergillus fumigatus* MTCC 4163) were procured from the Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. The Sabouraud dextrose agar plates were each similarly seeded with each fungal strain The 24 hrs. both culture of each bacterium and 7 days inoculated fungus culture were used to seed sterile sabouraud dextrose agar at 45°C respectively, and fungal plates were incubated at 25-28°C for 7 days after which diameter of zones of inhibition were measure. Each disc filled with extract [22-23].

RESULTS AND DISCUSSION

Phytochemical analysis

Flavonoids and triterpenoids were present in all extracts whereas saponins were present only in ethyl acetate and methanolic extracts of *I. sulcata* (Table 1). Flavonoids have been reported to possess antibacterial, antioxidant, anti-inflammatory, anti-allergic, anti-mutagenic and vasodilatory activity [24]. Terpenoids reported to antibacterial activity [25]. Saponins have been reported as hypocholesterolemic and as antidiabetics [26]. The presence of these secondary metabolites in the extracts of *I. sulcata* may be an indication of its medicinal potential.

Table 1: Phytochemical analysis of *I. sulcata*

Extract	Saponins	Flavonoids	Triterpenoids
PEIS	-	+	+
EAIS	+	+	+
MEIS	+	+	+

Note: PEIS, EAIS and MEIS are petroleum ether, ethyl acetate and methanolic extracts of *Impatiens sulcata*;
(-) = Absence and (+) = Presence

Total phenolic contents

Phenolic compounds such as flavonoid, tannins and phenolic diterpenes possess antioxidant activity. Folin-Ciocalteu method of determination of total phenolic content is based on the principle that oxidation of phenol by molybdotungs to phosphoric reagent yield a colored product that is estimated by measuring absorbance at 765nm. Gallic acid was used as reference standard and the phenolic contents of the extracts were expressed in mg Gallic acid equivalents per gram of extract (Table 2). The highest amount of phenolic content was found in the ethyl acetate extract (137±0.5773 mg GAE/g) followed by methanolic (125.3±2.90 mg GAE/g) and the petroleum ether extract (111.1±64.52 mg GAE/g) of *I. sulcata.*

Total flavonoid contents

Flavonoids possess a wide range of bioactivities including antioxidant activity. The presence of hydroxyl groups in the chemical structure of flavonoids is responsible for their antioxidant activity [27]. The determination of total flavonoid content using aluminum chloride is based on the formation of stable complex between aluminum chloride and keto and hydroxyl groups of flavonoids. The total flavonoid content of the extracts of *I. sulcata* is expressed as rutin equivalents in mg/g extract (Table 2). The petroleum ether extract (236.24 ± 1.7638 mg RE/g) showed the presence of higher flavonoid contents. The high amount of flavonoids in the petroleum ether, ethyl acetate (145.48±8.66 mg RE/g) and methanolic extracts (102.48±10.392 mg RE/g) suggested the possible antioxidant potential of the Impatiens sulcata extracts.

Total antioxidant capacity

Total antioxidant capacity determination by phosphomolybdenum method involves formation of a green phosphate/Mo^{5+} complex at acidic pH and is measured by absorbance at 695nm. The total antioxidant capacity of the extracts of *I. sulcata* is expressed as ascorbic acid equivalent (mg/g extract). The calibration curve of standard ascorbic acid standard solutions was used to determine the total antioxidant capacity of the extracts. The antioxidant capacity of petroleum ether, ethyl acetate and methanolic extracts are 76.08 mg AAE/g, 93.06 mg AAE/g and 92.97 mg AAE/g respectively (Table 2).

Reducing power

Reducing power of the extract is determined on the ability to reduce a yellow color Fe^{3+}/ferric cyanide complex to form Fe^{2+} ferrous complex. The amount of Fe^{2+} was monitored by measuring the formation of blue color at 700nm. A higher value of absorbance implies higher concentration of Fe^{+2} complexes and indicates higher reducing power. The methanolic extract demonstrated highest reducing power followed by ethyl acetate and petroleum ether extract of *I. sulcata* (Fig 1). BHA, a synthetic antioxidant demonstrated significant reducing power far better than the extracts and rutin. The results indicate that the methanolic extract of *I. sulcata* has a fair ability to act as electron donor and convert free radicals to stable products [28].

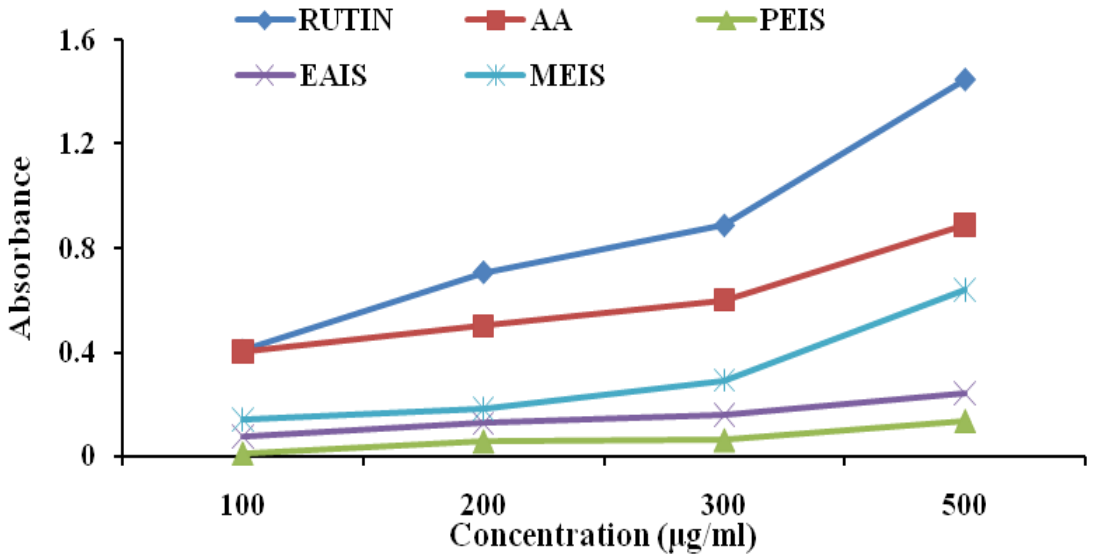

Fig. 1: Reducing power of *Impatiens sulcata* extracts

Note: PEIS, EAIS, MEIS and AA are petroleum ether, ethyl acetate, methanolic extracts and ascorbic acid of *Impatiens sulcata* respectively

DPPH free radical scavenging ability

The antioxidants scavenge DPPH free radical by their ability to act as hydrogen donor. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The antioxidant potential is determined by measuring decrease in absorbance of DPPH solution on addition of an antioxidant. The DPPH radical method is widely used to assess the antioxidant activity of the extracts. Antioxidant activity was assessed by determining the IC_{50} value of the extracts. Lower the IC_{50} value; higher is the antioxidant activity. The IC_{50} value of the petroleum ether, ethyl acetate and methanolic extract of *I. sulcata* is (95.37 µg/ml), (138.3737 µg/ml) and (95.3337 µg/ml) respectively (Table 2). The methanolic extract demonstrated highest free radical scavenging power compare to petroleum ether and ethyl acetate extract of *I. sulcata* (Fig 2).

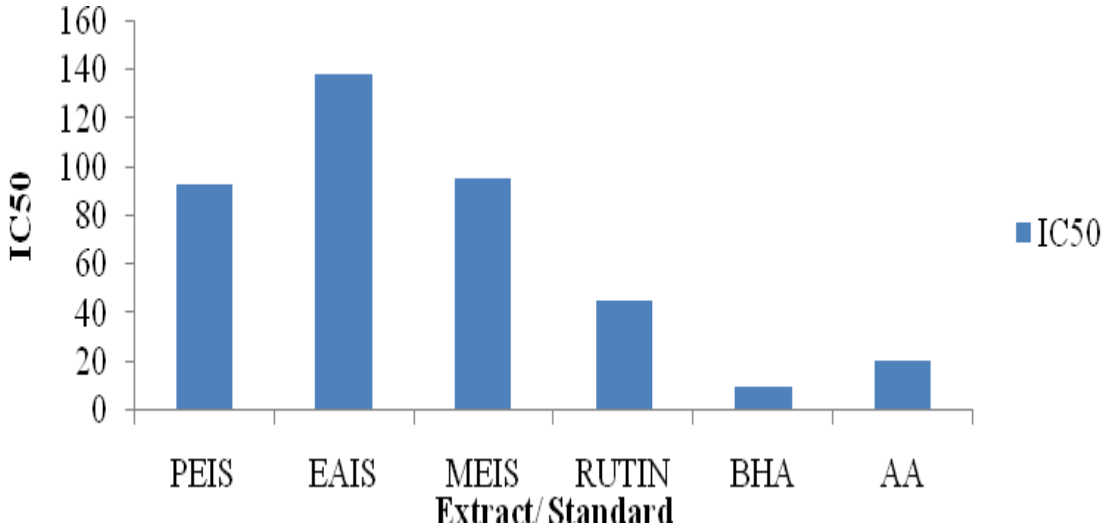

Fig. 2: DPPH free radical scavenging activity (IC_{50}) of *Impatiens sulcata* extracts

Note: PEIS, EAIS, MEIS, BHA and AA are petroleum ether, ethyl acetate, methanolic extracts, butylated hydroxy anisole and ascorbic acid of Impatiens sulcata respectively

ABTS radical cation scavenging assay

An antioxidant is added to preformed ABTS radical cation and after a fixed time period the remaining ABTS is quantified. The activity of the tested sample extracts is expressed as Trolox equivalent antioxidant capacity (TEAC) defined as micromolar Trolox solution having an antioxidant capacity equivalent to 1gm extract. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) a water soluble analog of vitamin E is used as standard to represent the antioxidant strength of sample. The extracts exhibited good ABTS radical scavenging ability as all of them were capable of decolorizing the ABTS radical color. The methanolic extract (10142.23) demonstrated highest Trolox equivalent activity whereas the petroleum ether extract (4690.78) and ethyl acetate (3424.56) exhibited (Table 2).

Table 2: Antioxidant potential of *Impatiens sulcata* extracts

Extract/ Standard	TPC[a] (mg GAE/g)	TFC[b] (mg RE/g)	TAOC[c] (mg AAE/g)	DPPH IC_{50}(µg/ml)	ABTS[d] (TEAC)
PEIS	111.1 (64.52)	236.24 (1.76)	76.08	95.37	4690.78
EAIS	137 (0.57)	145.48 (8.66)	93.06	138.37	3424.56
MEIS	125.3 (2.90)	102.48 (10.39)	92.97	95.33	10142.23
Rutin	-	-	-	45	-
BHA	-	-	-	10	-
Ascorbic acid	-	-	-	21	-

Note: PEIS, EAIS and MEIS are petroleum ether, ethyl acetate and methanolic extracts of *Impatiens sulcata* respectively.
BHA is butylated hydroxy anisole;
[a]Total phenolic contents (TPC) are expressed as gallic acid equivalent;
[b]Total flavonoid contents (TFC) are expressed as rutin equivalent;
[C]Total antioxidant activity (TAOC) is expressed as ascorbic acid equivalent;
[d]TEAC is trolox equivalent antioxidant capacity defined as micromolar trolox solution having antioxidant activity equal to 1g extract; values in parenthesis indicate SD (n=3)

Anti-bacterial activity

The antibacterial activity of the extract was evaluated against both gram positive and gram negative bacterial strains. The zone of inhibition for the extracts ranged from 10-21mm against the tested bacterial strains (Table 3). The activity was higher against gram positive *S. aureus* compared to gram negative strains *E. coli, S. typhirium* and *K. pneumoniae* bacterial strains. The antibacterial activity is related positively to the presence of total phenolic contents of the extract as ethyl acetate extract demonstrated higher activity than methanolic and petroleum ether extract. The activity of the extracts were however lower than the standard drug oxacillin (10µg/disc). However, the results are encouraging as they are from the natural extracts and suggest a need for further phytochemical work on the ethyl acetate and methanolic extract of *I. sulcata* Wall.

Table 3: Zone of inhibition (mm) antibacterial activity of *I. sulcata* extracts

Extract/ Standard	*E. coli* (MTCC-443)	*S. typhirium* (MTCC-1255)	*K. pneumoniae* (MTCC-432)	*S. aureus* (MTCC-737)
PEIS	-	-	11	13
EAIS	10	-	15	21
MEIS	14	-	12	-
Oxacillin	23	24	30	25

Note: PEIS, EAIS and MEIS are petroleum ether, ethyl acetate and methanolic extracts of *Impatiens sulcata*

Anti-fungal activity

In-vitro antifungal activity of the *Impatiens sulcata* extracts against the three fungal strains. The zone of inhibition for the extracts ranged from 12-23mm against the tested fungal strains. Ethyl acetate extract of *Impatiens sulcata* showed highest activity against the all three fungal strains (*Trichorderma viride* 22mm, *Aspergillus niger* 15mm and *Aspergillus fumigates* 18mm). Petroleum ether extract also showed similar activity against two fungal strains (*Trichorderma viride* 20mm and *Aspergillus fumigates* 23mm) and methanolic extract active against (*Trichorderma viride* 12mm and *Aspergillus fumigates* 14mm). The ethyl acetate extract showed excellent antifungal activity against all tested fungal strains. The antifungal activity is related positively to the presence of total phenolic contents of the extract as ethyl acetate extract demonstrated higher activity than methanolic and petroleum ether extract. The activity of the extracts were however lower than the standard drug ketoconazol.

Table 4: Antifungal activity in the different fractions of *Impatiens sulcata* of against three fungal strains

Fungal Name	MTCC (Code)	Ketoconazole	Pet. ether Extract	Ethyl acetate Extract	Methanol extract
Trichorderma viride	167	15Mm	20Mm	22Mm	12Mm
Aspergillus niger	2208	20Mm	-	15Mm	14Mm
Aspergillus fumigatus	4163	22Mm	23Mm	18Mm	-

Note: Mm means (millimetres) and (-) indicate (NIZ) No inhibitory zone

CONCLUSIONS

Petroleum ether, ethyl acetate and methanolic extracts of *Impatiens sulcata* show potent activity against *K. pneumonia*e. The activity was higher against gram positive *S. aureus* compared to gram negative strains *E. coli*, *S. typhirium* and *K. pneumoniae* bacterial strains. The antibacterial activity was related positively to the presence of total phenolic contents of the extract as ethyl acetate extract demonstrated higher activity than methanolic and petroleum ether extract. The ethyl acetate extract of *Impatiens sulcata* showed excellent antifungal activity against all tested fungal strains (*Trichorderma viride* 22mm, *Aspergillus niger* 15mm and *Aspergillus fumigates* 18mm). Further, the antifungal activity is related positively to the presence of total phenolic contents of the extract as ethyl acetate extract demonstrated higher activity than methanolic and petroleum ether extract.

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