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Phytochemical analysis, free radical scavenging capacity and antimicrobial properties of *Impatiens bicolor* plant

*[1]Anwer, N., [2]Waqar, M. A., [2]Iqbal, M., [2]Mushtaq, M. and [3]Sobia, A.

[1]*Department of Chemistry, Government College University, Lahore- 38000, Pakistan*
[2]*Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad-38040, Pakistan*
[3]*Department of Chemistry, Government College University, Faisalabad-38000, Pakistan*

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Abstract

The air dried powdered plant materials were extracted with different organic solvents, screened for phytochemicals and analyzed for their biological activities. The antioxidant potential was evaluated by scavenging of DPPH radical, TPC and TFC, while antimicrobial activity by disc diffusion assay against a set of bacterial and fungal strains. Ethylacetate extracts showed higher free radical scavenging capacity and phytochemical analysis revealed the presence of alkaloids, tannins, steroids, saponins and flavonoids. In biological assay, the extracts showed the antimicrobial activity comparable with standard antibiotics.

Introduction

Medicinal plants correspond to a wealthy source of antimicrobial agents. The plant materials used in traditional medicine are readily available in rural areas at relatively cheaper than modern medicine (Mann *et al*., 2008; Mahesh and Satish, 2008). Plants naturally produce a wide variety of secondary metabolites which have a good antibacterial, antifungal, anti-pests effect and hence are of pharmaceutical importance. These compounds are also responsible for antioxidant potential which offered a defensive mechanism against oxidant produced in living systems such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). The ROS and RNS are produced as by-products in aerobic organisms during oxidation which was considered to be vital phenomenon to many forms of life for their normal physiological function. But now it is believed that most of ROS and RNS, produced as by product of cellular metabolism are very harmful to body regarding cell and tissue damage. The ROS such as superoxide anions (O-2), hydroxyl radical (OH) and nitric oxide (NO) initiate degenerative processes in body by inactivating enzymes and destroying important cellular components and have been implicated in the pathology of a vast variety of diseases including cancer, atherosclerosis, diabetic mellitus, hypertension, AIDS and aging. Therefore, antioxidant potential of medicinal plant is important in view of the free radical theory of aging and associated diseases (Wallace, 1999; Lee *et al*., 2000; Biglari *et al*., 2008; Govindarajan *et al*., 2006). According to world health organization report on infectious diseases, overcoming antibiotic resistance is the major issue for the next millennium. Hence, the last decade witnessed an increase in the investigations on plants as a source of human disease management (Bisignano *et al*., 1996; Satish *et al*., 1999; Woldemichael *et al*., 2003).

Impatiens bicolor Linn (*Balsaminaceae*) is an annual 45-60 cm tall herb, distributed in northern areas (Murree, Nathia Gali and Miran Jani) of Pakistan. The genus *Impatiens* is rich in organic acids, anthraquinones and flavonoids. Charles and Hagen (1996) have reported the isolation of three monoglucosides of kaempferol, quercetin and pelargonidin from the stem of I. balsamina. Similarly salicylic acid, sinnapic acid, cafeic acid, scopletin, 2-hydroxy, 1,4-naphthoquinone and 2-methoxy 1,4-naphthoquinone had been extracted and purified from the stem of *I. balsamina* (Bhom and Towers, 1962). Panichayupakaranaut (1998) isolated a new biscoumarin, 4, 40-biisofraxidin, from the roots of *I. balsamina*. The extracts of *I. balsamina* also showed a long lasting skin moisturizing effect and prevent dryness, rough skin chap, dandruff and splitting hair ends, hence are used to prepare lotions, creams, hair tonics, cosmetics, bath preparations and detergents (Toki *et al*., 2000). The extracts of *I.*

*Corresponding author.
Email: *anwargcu@gmail.com*

biflora showed antibacterial and antifungal properties and three flavanone glycosides and six flavonoid glycosides have been reported from the leaves of *I. bicolor* (Hassan and Tahir, 2005). According to earlier reported studies the medicinal plants are the best sources to obtain a variety of newer herbal drugs (Hassawi and Kharma, 2006). So this study was designed to explore antimicrobial, antioxidant potential and phytochemical constituents of *I. bicolor* native to Pakistan.

Material and Methods

Plant material and reagents

The plant material *I. bicolor* was collected from Ayubia Park, Muree and was identified at the Department of Botany, GC University, Lahore. Air dried branches and leaves were powdered and extracted with different solvents (n-hexane, chloroform, ethyl acetate and methanol) using soxhlet aparatus. Crude extracts were filtered and concentrated at reduced temperature using rotary evaporator (EYELA, SB-651, Rikakikai Co. Ltd. Tokyo, Japan). The Linoleic acid, folin ciocalteu reagent, gallic acid, trichloro-acetic acid, sodium nitrite, ammonium thiocyanate, ferric chloride, ferous chloride, potassium fericyanate, butylatedhydroxytoluene (99.0%), dimethylsulfoxide, ferric chloride, iodine and 2, 2 diphenyl-1-picryl-1-hydrazyl were purchased from Sigma Aldrich (St, Louis, MO, USA) (USA), while Anhydrous sodium carbonate, chloroform, methanol, ethylacetate, n-butanol and n-hexane were of analytical grade (Merck, Germany) and microbial culture media and standard antibiotic discs were purchased from Oxoid (UK).

Sample preparation

The collected samples were dried under ambient conditions, grinded and were extracted with different solvents (n-hexane, chloroform, ethylacetate (Et-oAc) and methanol (MeOH)) using soxhlet apparatus for about 24h. The extracts were filtered and evaporated on rotary evaporator (N-N Series, Eyela, Rikakikai, Tokyo, Japan) to yield residue.

Phytochemical analysis

The samples for phytochemical tests were prepared as described by Sofowara (1993). For tannins determination, 0.5 mL of extract was dissolved in 1 mL of water and 1-2 drops of ferric chloride. Blue color was observed for gallic tannins and green black for catecholic tannins (Lyengar, 1995). For Saponin foam test was applied as; extract was shaken with small amount of water and the presence of foam until 10 min revealed the presence of saponin. Flavonoids were tested by alkaline Reagent test as; extracts were treated with few drops of sodium hydroxide solution and the formation of intense yellow color which became colorless on addition of dilute acid indicated the presence of flavonoids (Roopashree et al., 2008). For steroids, two mL of acetic anhydride was added to 0.5 mL extract along with 2 mL H_2SO_4. The color changed from violet to blue or green indicates the presence of steroids. Alkaloids were measured by Mayer's reagents. The alcoholic extract was evaporated to dryness and the residue was heated on a boiling water bath with 2% hydrochloric acid. After cooling, the mixture was filtered and treated with a few drops of Mayer's reagent. The samples were then observed for the presence of turbidity or yellow precipitation (Siddiqui and Ali, 1997).

DPPH radical scavenging assay

The free radical scavenging activity of different extracts of *I. bicolor* were measured by using stable radical, DPPH method (Lee *et al*., 1999). Briefly, a 1.0mL extracts at different concentrations (25, 50, 100, 200, 300 and 400µg/mL) in methanol was mixed with 2.0mL of methanolic solution of DPPH (10mg/L). The mixture was shaken vigorously and allowed to stand for at room temperature for 5 min. then absorbance was measured at 517 nm against methanol as the blank in a spectrophotometer. Lower absorbance of reaction mixture indicated higher free radical scavenging activity. The percent of DPPH discoloration of the sample was calculated according to the formula.

Antiradical activity= 100 × (1- A sample / AControl).
Acontrol = Absorbance of DPPH• solution (containing all the reagents except test sample.
Asample = Absorbance of DPPH• solution, 5 minutes after adding the extract.

Determination of total antioxidant activity

Total antioxidant activities of extracts were evaluated by phosophomolybdenum through complex formation method (Lee *et al*., 1999). For this purpose 0.1 ml solution of extracts and standards (Gallic acid and BHT) in methanol (0.5mg/ml) were combined with 1.9 ml of reagent solution (0.6 MH_2SO_4, 28 mM sodium phosphate and 4mM ammonium molybdate). The blank solution containing 2ml reagent was used as control. The vials were capped, incubated in a water bath at 95˚C and absorbance was measured at 695 nm at different time intervals after cooling the samples to room temperature. The total antioxidant activity of extracts of different parts of *I. bicolor* was determined in comparison with gallic acid (GA) and butylated hydroxy toluence (BHT).

Determination of total phenols

Total phenolic content of different solvent extracts were determined by using Folin-Ciocalteu reagent (Sultana *et al*., 2007) using Gallic acid (GA) as standard. Briefly, 0.1 ml of different solvent extracts was combined with, 2.8 ml of 10% Na_2CO_3 and 0.1 ml of Folin-Ciocalteu reagent. After 40 minutes absorbance was measured at 725 nm by using U.V. visible spectrophotometer. Total phenolic were determined as mg equivalent of GA per gm of dry extract by commuting with standard calibration curve obtained from different concentration of Gallic acid.

Antimicrobial Activity

The extracts of I bicolor were individually tested against a fungal and microbial strains including; *Proteus micabilus*, *Salmonella typhimorium*, *Bacillus subtillus*, *Bacillus lichaniform* and *Escherichia coli*.

Nutrient agar and potato dextrose were used for bacterial and fungal growth, respectively. The biological activity of extracts was determined using disc diffusion method CLSI (The Clinical and Laboratory Standards Institute (CLSI, 2007). Nutrient agar (Oxoid, UK) 28 g/L was suspended in distilled water, mixed well and distributed homogenously. The medium was sterilized by autoclaving at 121^0C for 15 min. Before the medium was transferred to Petri plates; inoculum (100 µL/100 mL) was added to the medium and poured in sterilized Petri plates. Now, small filter paper discs were laid flat on growth medium containing 100 µL of extracts. The Petri plates were then incubated at 37°C for bacteria and 30°C for fungus for 24 hours. The extracts having antibacterial activity, inhibited the bacterial growth and clear zones were formed after 24 h which were measured in mm using zone reader. Ciprofloxacin and fluconazole were used as control against bacteria and fungus, respectively.

Statistical analysis

One way ANOVA was performed using Statistica (version 8.1. Stat soft Inc, Tulsa Okahoma, USA) at 95% confidence interval of mean and all the experiments were seeded in triplicate and data thus obtained was reported as mean ± SD (Steel *et al*., 1996).

Results and Discussion

Phytochemical composition

The results of phytochemical analysis of *I. bicolor* are summarized in Table 1. The Flavonoids and Saponins and triterpenoids were present in all extracts where as sugars were present only in methanolic extract. All the extracts were found free of tannins while phenols were found in all extracts except n-hexane. It is revealed that the phytochemicals are well known due to their medicinal, antimicrobial and antioxidant activity (Wanga *et al*., 1996). Flavonoids have been reported to possess antibacterial, antioxidant, anti-inflammatory, antiallergic, antimutagenic, and vasodilatory activity. Saponins showed hypocholesterolemic and antidiabetic properties, while steroids are well known due to analgesic properties (Alan and Miller, 1996; Rupasinghe *et al*., 2003; Sultana *et al*., 2008). The presence of biologically important phytochemicals in *I. bicolor* extracts, as tested in our study, may contribute to their medicinal value and potential sources for useful drugs. Further phytochemical and pharmacological investigations as well as characterization of the active compounds from *I. bicolor* should be conducted for traditional uses and potential therapeutic applications.

Table 1. Phytochemical analysis of *I. bicolor*

Sr. no.	Chemical test	Compounds present	*n*-hexane	ethyl acetate	Chrofor m	mehtan ol
1	FeCl3	Phenols	--	++	++	++
2	Urea-HCl	Sugar	--	--	--	++
3	Lead Acetate	Flavonoids	++	++	++	++
4	Dragondroffs	Alkaloids	--	++	--	++
5	Cobalt Thiocyanate	Alkaloids	--	++	--	++
6	Butanol-HCl	Tannins	--	--	--	--
7	Cerric Sulphate	Saponins and triterpenoids	++	++	++	++
8	1%AlCl3	Flavonoids	++	++	+	++
9	Benedict test	flavonoids	++	++	++	++
10	Iodine/KI	Alkaloids	++	++	+	++

Key: + Present, - Absent

Antioxidant activity

A great number of aromatic, spicy, medicinal and other plants contain chemical compounds that exhibit antioxidant properties. Numerous studies were carried out on some of these plants, which resulted in a development of natural antioxidant formulations for food, cosmetic and other applications (Sayyah *et al*., 2004). However, scientific information on antioxidant properties of various plants, particularly those that are less widely used in culinary and medicine is still rather scarce. Therefore, the assessment of such properties remains an interesting and useful task, particularly for finding new sources for natural antioxidants, functional foods and nutraceuticals (Miliauskas and Venskutonis, 1996). For antioxidant activity, different assays were used including scavenging activity on DPPH radicals and phenolic content were also measured given in Table 3. The determination of antioxidant activity of *I. bicolor* plant MeOH, n-hexane, n-BuOH, EtOAc and $CHCl_3$ were used. Total phenolic contents were found to be ranged from 104-532 GAE mg/g. The ability of different solvents

Table 2. The DPPH radical scavenging activity of extracts of *I. bicolor*

Sr. no	Conc. (µg/ml)	n-hexane		Choroform		Methanol		ethyl acetate	
		Abs	%age	Abs	%age	Abs	%age	Abs	%age
1	400	0.169	33.9	0.141	45	0.055	78.5	0.025	92.2
2	300	0.183	29.1	0.153	40.46	0.076	69.14	0.040	84.2
3	200	0.195	24.12	0.175	31.9	0.099	61.47	0.075	70.62
4	100	0.21	18.28	0.191	25.1	0.123	52.14	0.096	62.6
5	500	0.235	8.56	0.2	9.2	0.148	42.4	0.133	48.24
6	25	0.249	3.1	0.24	6.61	0.175	31.9	0.159	38.13
7	Gallic acid	0.0201	92.5	0.020	92.5	0.020	92.5	0.020	92.5
8	B HT	0.057	77.8	0.057	77.8	0.057	77.8	0.057	77.8
9	blank	0.257	0	0.257	0	0.257	0	0.257	0

Values are mean ± SD of triplicate experiment.
Abs: Absorbance

Table 3. Antimicrobial activity of *I. bicolor*

Extract bicolor	Hexane	Chloroform	Ethyl acetate	Methanol
P. micabilus	11	2	6	8
S. typhimorium	6	6	11	7
B. subtillus	4	3	8	11
B. lichaniformis	-	-	-	-
E. coli	2	1	5	10

Inhibition zone (mm) diameter around test disc

to extract TPC was found as: methanol > EtOAc > $CHCl_3$ > n-hexane as shown in the figure. The effect of different solvent systems on the amount of TFC was significant ($P<0.05$). The DPPH radical is a stable free radical which is successfully used to estimate free radical-scavenging activity. Antioxidants neutralize DPPH by donating hydrogen or electron (Archana *et al*., 2005). The DPPH radical scavenging activity of different extracts of *I. bicolor* is shown in Table 2. The DPPH radical scavenging activity of extract (10 mg/mL) ranged from 3-92%. EtOAc extract exhibited more scavenging activity than all other fractions of I. bicolor. Among the solvents, MeOH, $CHCl_3$, EtOAc, n-hexane and EtOAc showed maximum (92.2, 84.2, 70.62, 62.6 and 48.24%) radical scavenging activity for different concentrations versus control (BHT).

Antimicrobial activity

The antimicrobial activities of *I. bicolor* plant extracts in different solvent are shown in Table 3. The antibacterial activity was determined in comparison with ciprofloxacin, while antifungal activity verses fluconazole. Against *E. coli*, the higher activity was shown by methanolic extract followed by EtOAc, n-hexane and $CHCl_3$. For *P. micabilus*, n-hexane extract showed better activity followed by MeOH, EtOAc and $CHCl_3$ extract. Against *S. typhimorium*, EtOAc showed highest activity which is also statistically comparable with fluconazole and mehtanol also showed good activity against *S. typhimorium* followed by n-hexane and $CHCl_3$. Similarly *I. bicolor* plant leaves also showed a considerable activity against *B. subtillus* strains. Collectively EtOAc extract showed the highest activity which is also comparable with standard followed by MeOH, n-hexane and chloroform whereas *B. lichaniformis* found resistant against all extracts of *I. bicolor*.

According to (Moghadam *et al*., 2010) the antimicrobial activity of medicinal plant material would probability due to the presence of alkaloids, flavonoides such as harmine/harmadine/harmadol in *P. hermala* and these compound or most probably soluble in organic polar solvent. So, the variation in the antimicrobial activity of *I. bicolor* plant leaves used in this study might be attributed to the different nature of solvent. From last few decades, there has been considerable interest in extracts from plants with antimicrobial activities for controlling pathogens and/or toxin producing microorganisms. Antibiotics provide the main basis for the therapy of bacterial infections. However, the high genetic variability of bacteria enables them to rapidly evade the action of antibiotics by developing antibiotic resistance. Thus there has been a continuing search for new and more potent antibiotics and our results have shown that different extracts *I. bicolor* might be a good candidate against set of bacterial and fungal strain.

Conclusion

From present study, it is concluded that the *I. bicolor* plant extracts have a high phytochemical contents quite good free radical-scavenging capacity as well as antibacterial and antifungal activities. The extracts of *I. bicolor* in different solvents can be used as an antibacterial and antifungal agent practically against the microorganism used during present study. The antioxidant activity of *I. bicolor* plant leaves extracts in different observed suggests that it can be used for stabilization of different oxidation processes after a preliminary experiment. Furthermore, the ethylacetate is efficient solvent for extraction purpose and there is further need to identify the potent compound responsible for antimicrobial and antioxidant activity and their possible practical applications.

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