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Antioxidant potential and chemical constituents of *impatiens bicolor* Royle

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Abstract – Alcoholic extract of *Impatiens bicolor* Royle and its various fractions were screened for antioxidant potential by DPPH radical scavenging activity and its chemical constituents were isolated and their chemical structures were determined by mass spectrometry and different NMR spectroscopy techniques. *Impatiens bicolor* fractions revealed significant antioxidant activity. Dichloromethane fraction of alcoholic extract yielded four compounds whose structures were elucidated as methyl-4-hydroxyl cinnamate (1), stigmasterol (2), stigmasterol 3-*O*-*β*-glucoside (3) and *β*-Sitosterol (4). These results indicated the potential of *Impatiens bicolor* to be further explored as natural source for new lead compounds.

Keywords – *Impatiens bicolor*, Balsaminaceae, Diphenylpicrylhydrazyl radical scavenging, steroids

1. Introduction

Plants are manufacturing units of important chemicals like essential oils, fatty oils, resins, mucilage, sterols, triterpenes, tannins, gums and others [1]. Most of these are potent bioactive compounds and can be used for therapeutic purpose or which are precursors for the synthesis of useful drugs [2]. These bioactive molecules differ from plant to plant due to their biodiversity and they produce a definite physiological action on the human body. These substances have been the cause of use of plants in traditional ethnomedicinal lore. There is growing interest in isolation, structure elucidation and bioactivity determination of these bioactive molecules.

Many cardinal bioactive compounds found in plants have received much attention due to their capacity to scavenge free radicals produced in human body. The oxidation induced in human body can result in cell membrane disintegration, membrane protein damage and DNA mutation, which can further initiate or propagate the development of many diseases, such as cancer, liver injury and cardiovascular disease [3]. The incessant exposure to chemicals and contaminants may enhance the quantity of free radicals in the body beyond its capacity to control them, and cause irreversible oxidative damage [4]. Therefore, antioxidants with free radical scavenging activities may have great significance in the prevention and therapeutics of diseases in which oxidants or free radicals are implicated [5].

Impatiens bicolor is an important medicinal plant distributed in northern areas in Murree, Nathia Gali, Swat and Miran Jani of Pakistan [6]. A literature survey revealed only one report on isolation of few flavonoids form this plant [7]. There is dearth of information on isolation of further molecules as well as its antioxidant potential. In this context as part of our research work on exploring the hidden potential of indigenous flora of Pakistan [8-10] we have screened the extract of *I. bicolor* for antioxidant potential and have isolated some known compounds from this plant. The present investigation will provide a broad base for the possibility of further detailed studies on *I. bicolor* for rationalizing its use in traditional medicinal knowledge.

2. Material and Methods

2.1. Plant Material

Whole plant of *Impatiens bicolor* Royle was collected from Khwazakhela, Swat, N.W.F.P. Pakistan, during September 2008. Taxonomist, Dr. Hassan Sher, Jahan Zeb Post Graduate College Saidu Sharif, Swat (Pakistan), identified the plant species. A voucher specimen numbered as 18-NH-4-008 was deposited in the National Herbarium Islamabad.

2.2. Extraction and Isolation

Shade-dried *I. bicolor* (10 kg) was grounded and extracted with MeOH and water at room temperature. The combined methanolic extract was filtered and evaporated under vacuum to obtain a thick greenish black gummy mass. It was fractionated into n-hexane; dichloromethane, ethyl acetate, n-butanol, aqueous fractions. All these fractions as well as crude extract were evaluated for antioxidant activity. Dichloromethane fraction (10 g) was subjected to silica gel column chromatography with EtOAc/ hexanes as eluting solvent, yielded compounds **1** (8mg), **2** (10 mg), **3** (100 mg) and **4** (14 mg).

2.3. Antioxidant Activity by DPPH Radical Scavenging Activity

Radical scavenging activity was determined as described previously by Shaheen et al [11]. Briefly reaction mixture containing 5 µL of test sample (1 mM in DMSO) and 95 µL of DPPH (Sigma, 300µM) in ethanol was taken in a 96-well microtiter plate and incubated in Elisa (multiple reader spectra Max-3400) at 37°C for 30 min. The absorbance was measured at 515 nm. Percent radical scavenging activity was determined by comparison with a DMSO containing control (Figure 1). IC_{50} values represent concentration of compounds to scavenge 50% of DPPH radicals. BHA (3-*t*-butyl-4-hydroxyanisole) was used as a positive control. All the chemicals used were of analytical grade (Sigma, USA). % Radical scavenging activity was calculated by using the following formula:

$$\text{Radical scavenging activity}\,\% = 100 - [(\frac{\text{ODsample}}{\text{ODcontrol}}) \times 100]$$

2.4. Isolation and Identification of Compounds

UV spectra were recorded on Schimadzu UV-240 spectrometer in MeOH. IR spectra were obtained as KBr discs on JASCO A-302 spectrophotometer. Optical rotation was recorded on *Glan-Taylor Prism*, *P*-2000. ^{1}H and ^{13}C NMR (400 MHz and 100 MHz) were recorded in CD_3OD on Bruker Av 600 NMR instrument. Column chromatography was conducted on silica-gel (Kiesegel 60; 70-230 mesh) and TLC was obtained on pre-coated silica-gel, Merck-F_{254} aluminum sheets (0.25 mm thickness).

Methyl-4-hydroxyl cinnamate (1):

UV (MeOH) λ_{max}: 314, 226 and 210 nm.

IR ($CHCl_3$) ν_{max}: 3382 and 1682 cm^{-1}.

EI MS *m/z*, (rel. intest.): 178 [M^+] (90), 147 [M-OMe] $^+$ (100), 119 [M-$C_2H_3O_2$] $^+$ (60) and 91 [M-$C_2H_3O_2$-CO] $^+$ (30).

^{1}H-NMR ($CDCl_3$, 400 MHz, δ): δ 7.62 (1H, d, *J* = 15.9 Hz, H-7), 7.41 (2H, d, *J* = 8.4 Hz, H-3 and H-5), 6.82 (2H, d, *J* = 8.6 Hz, H-2 and H-6), 6.28 (1H, d, *J* = 15.9 Hz, H-8), 3.77 (3H, s, OMe).

^{13}C-NMR ($CDCl_3$, 100 MHz,δ): 127.2 (C-1), 130.5 (C-2 and C-6), 116.5 (C-3 and C-5), 158.5 (C-4), 168.4 (C-9), 144.5 (C-7), 115.4 (C-8) and 51.2 (OMe).

Stigmasterol (2):

Colorless crystalline solid,

$[\alpha]_D^{26}$ = + 51.0 (MeOH, *c* = 0.30),

IR ($CHCl_3$) ν_{max}, cm^{-1}: 3432 (OH), 1648 (C=C).

EIMS *m/z* (rel. int. %): [M]$^+$ 412 (10), 396 (16), 379 (25), 369 (33), 351 (73), 327 (66), 300 (68), 270 (21).

HREIMS *m/z*: 412.3921 (calcd. for $C_{29}H_{48}O$, 412.3926).

^{1}H-NMR (CDCl3, 400 MHz, δ): 5.32 (1H, m, H-6), 5.16 (1H,dd, *J* = 15.3, 8.0 Hz, H-22), 5.02 (1H, dd, *J* = 15.3, 8.0 Hz, H-23), 3.26 (1H, m, H-3), 0.89 (3H, d, *J* = 6.5 Hz, H_3-29), 0.81 (3H, d, *J* = 6.6 Hz, H_3- 26), 0.83 (3H, t, *J* = 7.0 Hz, H_3- 29), 0.80 (3H, d, *J* = 6.5 Hz, H_3-27), 0.80 (3H, s, H_3-19), 0.67 (3H, s, H_3-18).

^{13}C-NMR ($CDCl_3$, 100 MHz, δ): 140.7 (C-5), 138.6 (C-22), 129.5 (C-23), 121.6 (C-6), 71.3 (C-3), 57.5 (C-14), 56.2 (C-17), 51.8 (C-24), 49.7 (C-9), 42.9 (C-13), 42.1 (C-4), 40.3 (C-20), 39.1 (C-12), 37.7 (C-1), 36.2 (C-10), 32.5 (C-8), 32.1 (C-25), 31.6 (C-7), 31.4 (C-2), 28.2 (C-16), 25.3 (C-28), 24.6 (C-15), 21.4 (C-27), 21.0 (C-21), 20.9 (C-11), 19.5 (C-19), 19.3 (C-26), 12.6 (C-18), 12.1 (C-29).

Stigmasterol 3-*O*-*β*-glucoside (3):

Colorless needles

$[\alpha]_D^{25}$ = - 51.0 (MeOH, *c* = 0.21).

IR (KBr) $\nu_{max,}$ cm^{-1}: 3458 (OH), 1646 (C=C).

EIMS *m/z* (rel. int. %): [M-Glu] $^+$ 412 (76), 394 (25), 379 (24), 369 (38), 351 (72), 300 (66), 273 (26).

HR-FAB-MS *m/z*: 575.4231 [M+H] $^+$ (calcd. for $C_{35}H_{58}O_6$ + H = 575.4233).

^{1}H-NMR ($CDCl_3$, 400 MHz, δ): 5.23 (1H, br. d *J* = 5.4 Hz,H-6), 5.14 (1H, dd, *J* = 15.2,8.0 Hz, H-22), 5.02 (1H, dd, *J* = 15.3, 8.0 Hz, H-23), 4.77 (1H, d, *J* = 7.5 Hz, H-1′), 3.86 (1H, m, H-3), 3.85 - 4.41 (m, Glc-H′), 1.01 (3H, s, H3-19), 0.90 (3H, d, *J* = 6.2 Hz, H_3-21), 0.83 (3H, d, *J* = 6.6 Hz, H_3-26), 0.82 (3H,t, *J* = 7.0 Hz, H_3- 29), 0.80 (3H, d, *J* = 6.5 Hz, H_3-27), 0.66 (3H, s, H_3-18).

^{13}C-NMR (CD3OD ,400 MHz, δ): 141.3 (C-5), 138.4 (C-22), 129.3 (C-23), 121.5 (C-6), 102.3 (C-1′), 79.2 (C-3′), 76.3 (C-5′), 74.1 (C- 2′), 70.7 (C-4′), 62.2 (C-6′), 57.3 (C-14), 56.2 (C-17), 52.1 (C-24), 50.8 (C-9), 43.7 (C-4), 43.1 (C-13), 40.3 (C-20), 39.7 (C-12), 37.3 (C-1), 36.6 (C-10), 32.8 (C-25), 32.5 (C-2), 31.8 (C-7), 31.7 (C-8), 28.9 (C-16), 25.5 (C-28), 24.5 (C-15),21.9 (C-21), (C-24), 21.7 (C- 27), 21.5 (C-11), 19.5 (C-19), 19.1 (C-26), 12.6 (C-18), 12.1 (C-29).

***β*-Sitosterol (4):**

Colorless crystalline solid,

$[\alpha]_D^{26}$ = -35.0 (MeOH, *c* = 0.15).

IR ($CHCl_3$) ν_{max}, cm^{-1}: 3450 (OH), 3050 (C-H), 1650 (C=C).

EIMS *m/z* (rel. int. %): [M] $^+$ 414 (10), 399 [M-Me] (16), 396 [M-H_2O] (18), 381 [M-Me-H_2O] (70).

^{1}H-NMR (CDCl3, 400 MHz, δ): 5.22 (1H, m, H-6), 3.31 (1H, m, H-3), 1.02 (3H, s, H_3-19), 0.91 (3H, d, *J* = 6.4 Hz, H_3- 21), 0.83 (3H, t, *J* = 7.0 Hz, H_3- 29), 0.80 (3H, d, *J* = 6.5 Hz, H_3-26), 0.79 (3H, d, *J* = 6.5 Hz, H_3-27), 0.78 (3H, s, H_3-19), 0.69 (3H, s, H_3-18).

^{13}C-NMR ($CDCl_3$, 100 MHz, δ): 140.8 (C-5), 121.9 (C-6), 71.5 (C-3), 57.4 (C-14), 56.2 (C-17), 51.6 (C-24), 49.9 (C-9), 42.8 (C-13), 42.2 (C-4), 40.3 (C-20), 39.1 (C-12), 37.7 (C-1), 36.2 (C-10), 32.5 (C-8), 32.1 (C-25), 31.6 (C-7), 31.4 (C-2), 28.2 (C-16), 25.3 (C-28), 24.6 (C-15), 21.4 (C-27), 21.0 (C-21), 20.9 (C-11), 19.4 (C-19), 19.3 (C-26), 12.6 (C-18), 12.1 (C-29).

3. Results and Discussion

The results indicated that dichloromethane, ethyl acetate and n-butanol fraction caused 82%, 50% and 34 % inhibition while crude extract showed minimum inhibition i.e.1.75 % only. Excellent free radical scavenging property present in these fractions of *I. bicolor* may be the reason for its effectiveness in its ethnopharmacological uses against different ailments. Our results are in partial

comparison with those described recently [12], however this difference may be attributed to source of collection, solvent used and method followed to determine free radical scavenging activity as all these factors affect the antioxidant capacity of plant or part of plant being investigated. Further studies for evaluation of antioxidant capacity using other methods (e.g. various biochemical assays both *in vivo* and *in vitro*) are essential to characterize the antioxidant profile completely.

Figure **1** was obtained as needle shaped crystals, from the dichloromethane fraction, by using EtOAc/hexanes (1:15) as eluting solvent system.

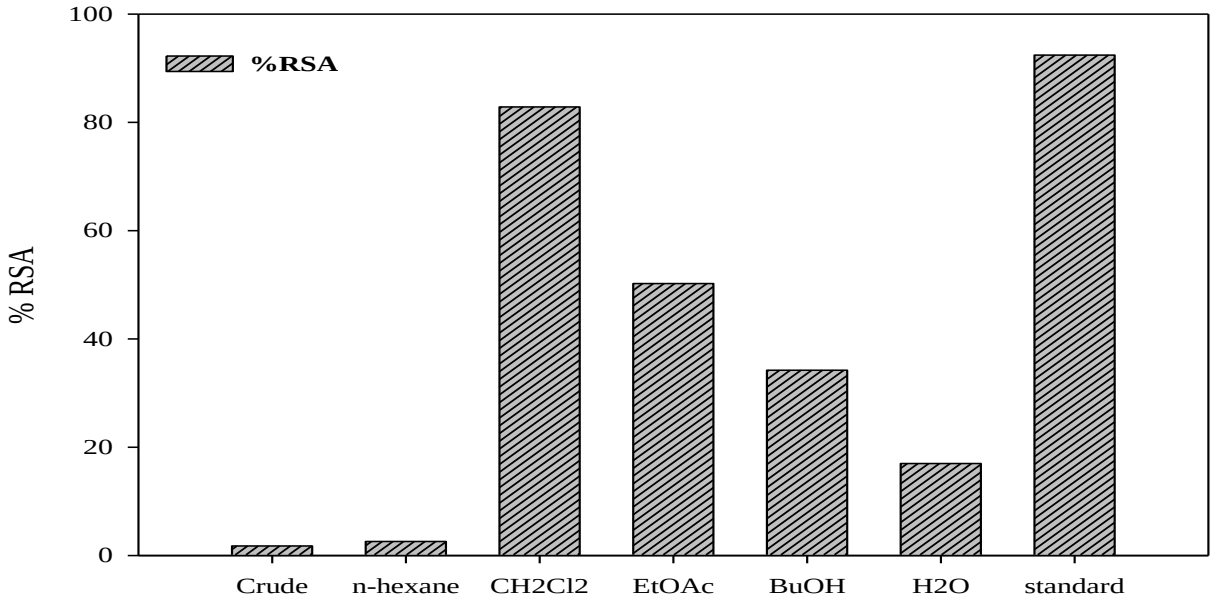


Figure 1. DPPH radical scavenging activity of crude extract and fraction of *Impatiens bicolor* Royle

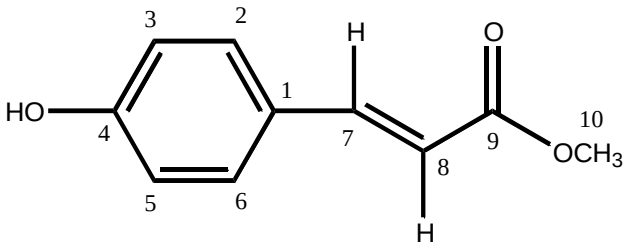
EI MS exhibited [M^+] peak at *m/z* 178. Molecular formula of **1**, $C_{10}H_{10}O_3$, was obtained from EI MS and ^{13}C-NMR spectroscopy. The UV spectrum displayed absorption bands at 314, 226 and 210 nm, while IR spectrum exhibited the absorptions for hydroxyl (3382 Cm-1) and α, β-unsaturated ester (1682 cm^{-1}). The EI MS spectrum of 1 displayed fragments ion peaks at m/z 147 [M-OMe] $^+$, 119 [M-C2H3O2] $^+$ and 91 [M-C2H3O2-CO] $^+$, which indicated the presence of methyl cinnamate as a basic skeleton. The ^{1}H-NMR spectrum exhibited a pair of doublet at δ 7.62 and 6.28 (1H, each, J = 15.9 Hz), another pair of ortho coupled signals at δ 7.41 and 6.82 (2H, each, J = 8.4 Hz). A 3H singlet at δ 3.77 of methoxy group. ^{13}C-NMR spectrum (BB, DEPT) of **1** displayed resonances for 10 carbon signals including one methyl, six methine and three quaternary carbons. All the physical and spectral properties unambiguously matched with the reported values [13].

Figure **2** obtained as colorless crystals. The molecular formula was established as $C_{29}H_{48}O$ by high resolution mass spectrometry which indicated M^+ peak at *m/z* 412.3920 (calcd. for $C_{29}H_{48}O$, 412.3926). The IR spectrum showed absorption bands for hydroxyl group (3432 cm^{-1}). The ^{1}H -NMR showed resonances for two tertiary methyl groups at δ 0.80 (3H, s, H_3-19) and 0.67 (3H, s, H_3-18). ^{1}H-NMR also displayed resonances for three olefinic protons at δ 5.32 (1H, m, H-6), 5.16 (1H, dd, J = 15.3, 8.0 Hz, H-22), 5.02 (1H, dd, J = 15.3, 8.0 Hz, H-23), while an oxygenated methine signal at δ 3.26 (1H, m, H-3). The ^{13}C -NMR (Broad band and DEPT) displayed 29 carbon signals including six methyl, nine methylene, eleven methine and three quaternary carbons, corresponding to stigmasterol [14].

Figure **3** was obtained as amorphous solid. Its molecular formula was determined as $C_{35}H_{58}O_6$ by HR FAB MS which displayed pseudo molecular ion peak at *m/z* 575.4231 [M+H] $^+$ (calcd.for $C_{35}H_{58}O_6$ + H = 575.4233). EI MS spectrum of **3** showed characteristic fragmentation pattern similar to compound **2**. The ^{1}H NMR of **3** was found to be similar to **2,** with only the different in additional resonances at δ 4.77 (1H, d, J = 7.5 Hz) and signals at δ 3.84 – 4.44, which were due to the glucose moiety. The correlation between carbon **3** of aglycone and the H-1′ of glucose revealed the point of attachment of glucose. The ^{13}C-NMR displayed 35 carbon signals, out of them, 29 carbons for the stigmasterol moiety and the remaining 6 for the sugar molecule. The orientation of the glucose was confirmed to be a β linkage due to the coupling constant of the anomeric proton (J = 7.5 Hz).The structure of **3** was then confirmed as stigmasterol 3-O-β-D-glucoside [14].

Figure **4** was found to be similar with compound **2,** with only the difference of absent of two olefinic proton signals of H-22 and H-23. In the light of EI MS, ^{1}H- and ^{13}C NMR, structure of compound **4** was determined as β-sitosterol. All the physical and spectral data

unambiguously matched with the reported data in literature.



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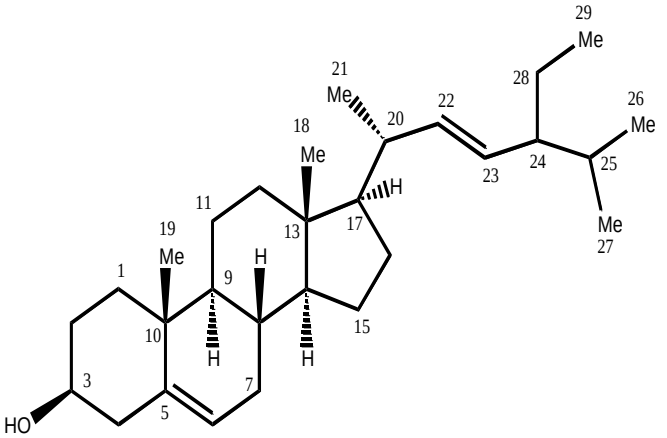

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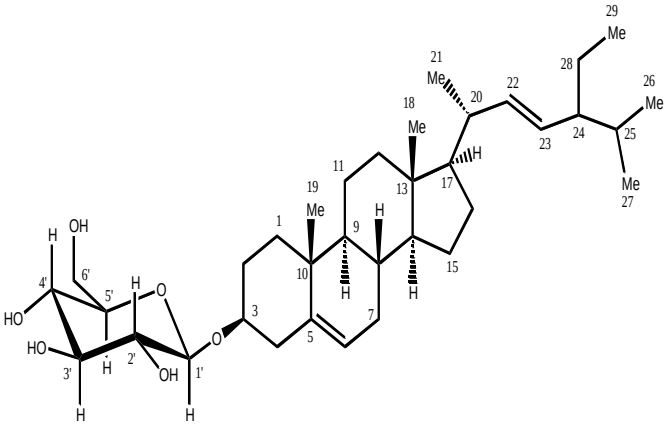

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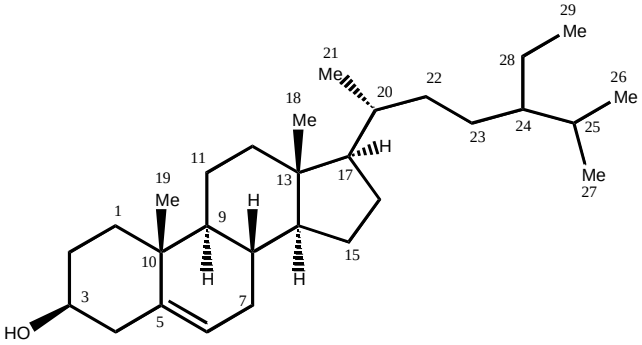

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4. Conclusion

The current phytochemical studies of *I.bicolor* revealed the isolation of four new source known compounds namely Stigmasterol, Methyl 4-hydroxycinnamate, *β*-Sitosterol, Stigmasterol-3- *O*- *β*-glucoside and were fully characterized by spectroscopic studies. Furthermore, the dichloromethane fraction shows excellent DPPH antioxidant radical scavenging activity.

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