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Full Length Research Paper

Antioxidant activity and phenolic compounds from *Colchicum luteum* Baker (Liliaceae)

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The ethanolic extract from corms of the *Colchicum luteum* Baker (Liliaceae) was investigated phytochemically. During phytochemical studies, compounds 1 - 4 were isolated from the *n*-butanol fraction. These compounds were identified as colchicines 1, β - Lumicolchicine 2, chlorogenic acid 3 and 3', 4', 5.7-Tetrahydroxyflavone 4, on the basis of different modern spectroscopic techniques. Chlorogenic acid 3', 4', 5.7-Tetrahydroxyflavone were isolated for the first time from this species. On the basis of scavenging activity of the stable 1, 1-diphenyl-2-picrylhidrazyl (DPPH) free radical, the crude ethanolic extract and subsequent fractions of *C. luteum* Baker offered promising antioxidant activity. The highest activity was displayed by chloroform fraction (91%), while the overall range was found (56 - 91%), expressed some correlation with the isolated compounds.

Key words: *Colchicum luteum*, Liliaceae, chlorogenic acid, luteolin, antioxidant.

INTRODUCTION

The plant *Colchicum luteum* Baker belongs to family Liliaceae (Huxley, 1992). The Liliaceae are mostly perennial herbs from starchy rhizomes, corms or bulbs comprising about 289 genera and 4,000 species. The leaves are alternate or less often opposite or whorled. The flowers are nearly always bisexual and actionmorphic. The genus *C.* includes 42 species, most of which are endemic to Middle East and South Africa to Western Europe and Asia (Brickell 1984; Mabberley 1997). The corms of the *C. luteum* Baker are extensively used for the treatment of gout, rheumatism and diseases of the liver and spleen (Chopra et al., 1986). The corms are also used as blood purifier (Shinwari et al., 2003). The poison alkaloid colchicine is extracted from *C. luteum* Baker, used for the treatment of Behcet's syndrome (Miyachi et al., 1981; Wechsler 2002) and a topical remedy for penile condylomata acuminate (Von 1978; Von 1981). Excellent enzyme inhibition and antimicrobial activities was shown by the crude methanolic extract and subsequent fractions of the *C. luteum* Baker against lipoxygenase (Bashir et al., 2006; Bashir et al., 2006).

Phytochemically, the mainstay of the genus, *Colchicum*, is alkaloids including *C. luteum* (Miyachi et al., 1981). From the colchicum, thirty one different alkaloids have been isolated (Capraro and Brossi, 1984). Colchicine is the main alkaloid isolated from all species of the genera, *Colchicum* (Ondra et al., 1995). The major phenolic compounds obtained from the genus, *Colchicum*, are 4-hydroxy-3-methoxybenzaldehyde (vanillin), 4-hydroxybenzoic acid (vanillic acid), 3-(3-hydroxyphenyl)-2-propanoic acid (coumaric acid), 3-(3, 4-dihydroxyphenyl)-2-propanoic acid (caffecic acid), and 3, 4, 5, 7 tetrahydroxyflavone (luteolin). In the light of the present advancement in the field of natural products, the global interest in the herbal remedy is the standardization of these products to ensure their purity (Mansoor, 2004). Development of a quality assurance or standardization system for medicinal plants according to the specification of WHO (2000) is a great challenge to provide high quality and safe product to the consumer. Like most third world countries, the rural people of Pakistan still rely on the ingenious system of treatment to greater extent (Khattak et al., 1985). For this purpose, *C. luteum* Baker was screened for the isolation of biologically active molecules.

Abbreviations: RSA, Free radical scavenging activity; **^{1}H-NMR,** proton nuclear magnetic resonance spectroscopy; **DPPH,** stable 1, 1-diphenyl-2-picrylhidrazyl; **HR-EIM,** human resource-enterprise incentive management; **HMBC,** heteronuclear multiple bond correlation.

MATERIAL AND METHODS

Plant material

C. luteum Baker, as a whole plant was collected from Sherengel, upper Dir, KPK (Pakistan) during the month of February – March, 2004. The plant material was identified by Prof. Dr. Jahandar Shah, Plant Taxonomist and Vice Chancellor of Malakand University and verified by Prof. Dr. Abd-ur-Rashid, Department of Botany, University of Peshawar.

Extraction

The corms of the *C. luteum* Baker was washed with water and dried in shade. The shade dried corms of the plant was pulverized into fine powder in the PCSIR laboratories, Peshawar. The powdered plant material (8 kg), was soaked in ethanol, at room temperature for 15 days, occasional shaking. Then the ethanol soluble materials were filtered. The filtrate was concentrated under vacuum at 40 - 50°C using rotary evaporator. As a result, a yellow crude extract (296 g) was obtained.

Fractionation

The crude ethanolic extract (246 g) was suspended in distilled water (500 ml) and partitioned with *n*-hexane (3 x 500 ml), chloroform (3 x 500 ml), ethyl acetate (3 x 500 ml) and n-butanol (3 x 500 ml) to yield the *n*-hexane (26 g), chloroform (59 g), ethyl acetate (32 g), n-butanol (35 g) and aqueous (68 g) fractions, respectively. For other biological activities about 50 g of the crude ethanolic extract was reserved.

General experimental conditions

The melting point of the compounds was recorded in glass capillary tubes using Buchi-535 melting point apparatus. Optical rotations were measured using JASCO DIP-360 digital polarimeter. The Ultraviolet (UV-visible) spectra were recorded in methanol using Hitachi UV-3200 spectrometer. The Infrared (IR) spectra were measured using JASCO IRA-1 (Japan Spectroscopic and Shimadzu IR-460 Infrared spectrometers). Proton Nuclear Magnetic Resonance (^{1}H NMR) spectra were taken in $CDCl_3$, CD_3OD and $(CD_3)_2CO$ using TMS as an internal standard at 300, 400 or 500 MHz on Bruker AC-300, AM-300, AM-400 or AMX-500 Nuclear Magnetic Resonance (NMR) spectrometer with Aspect-3000 data systems at a digital resolution of 32 K. The ^{13}C-NMR spectra were recorded in CD_3OD, $CDCl_3$ and $(CD_3)_2CO$ at 75, 100 or 125 MHz on the same instruments.

Spraying reagents

Ceric sulphate [$Ce(SO_4)_2$] reagent was used for the visualization of the compound. Ceric sulphate (0.1 g) and trichloroacetic acid (0.1 g) were dissolved in 4 ml of distilled water. The solution was boiled and concentrated sulphuric acid was added drop wise until the disappearance of turbidity.

Column chromatography

Column chromatography was carried out on silica gel 60 (E. Merck), mesh size 230 - 270. Recoated silica gel GF-254 preparative plates (20 x 20, 0.5 mm thick) (E. Merck) were used for preparative chromatography.

Isolation

The n-butanol soluble fraction was subjected to column chromatography (CC) over silica gel eluting with $CHCl_3$ / MeOH (95: 5, 90: 10, 80: 20, 50: 50) in the increasing order of polarity. The fraction eluted with (80: 20) was resubjected to repeated column chromatography eluting with $CHCl_3$ / MeOH (87: 13) to offered compound **1** (21 mg) and compound **2** (14 g). The fraction that was obtained from $CHCl_3$ / MeOH (50: 50) was then subjected to repeated column chromatography over silica gel eluting with $CHCl_3$ / MeOH; (40: 60) to offered compound **3** (13 mg) and compound **4** (17 mg).

Characterization of colchicine (1)

Colorless needles M.P; [148 - 150°C], UV λ max/nm: 350.5, 243 nm,
IR v max/ (KBr) cm^{-1}: 1660, 1610, 1555 and 1495.
^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.62 (1 H, d, J = 6 Hz), 7.70 (1 H, s), 7.41 (1 H, d, J = 11 Hz), 6.91 (1 H, d, J = 11 Hz), 6.54 (1 H, s), 4.68 (1 H, dt, J_1 = 12 Hz, J_2 = 6 Hz), 4.04 (3 H, s), 3.95 (3 H, s), 3.90 (3 H, s), 3.70 (3 H, s), 2.50 (2 H, m), 2.36 (1 H, m), 2.02 (1 H, m), 1.96 (3 H, s).
EIMS m/z (rel. int. %): 399 (7), 337 (20), 275 (65), 260 (90), 210 (15), 185 (55), 145 (10).
HREI-MS m/z: 399.7624 (Calcd for $C_{22}H_{25}O_6$, 399.7619).

Characterization of β-Lumicolchicine (2)

Colorless needles M.P; [190°C]: UV λ max/nm: 380.5, 373nm, IR v max/ (KBr) cm^{-1}: 1860, 1710, 1655 and 1595.
^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.66 (1 H, d, J = 3 Hz), 6.50 (1 H, s), 6.20 (1 H, d, J = 7 Hz), 4.82 (1 H, m), 4.09 (1 H, dd, J_1 = J_2 = 3 Hz), 3.96 (3 H, s), 3.90 (3 H, s), 3.85 (3 H, s), 3.70 (3 H, s), 3.60 (1 H, dd, J_1 = 3 Hz, J_2 = 2 Hz), 2.77 (2 H, dd, J_1 = 15 Hz, J_2 = 9 Hz), 2.60 (1 H, dd, J_1 = 15, J_2 = 9 Hz) , 2.04 (3 H, s), 2.00 (1 H, m).
FAB-MS m/z: 354 (M+1) +, 193, 165, 136.
HREI-MS m/z (rel. int. %): 354.1530 (Calcd for $C_{16}H_{18}O_9$, 354.1524).

Characterization of chlorogenic acid (3)

Pale yellow amorphous powder, M.P; [208 - 211°C]: UV λ max (MeOH) nm: 328, 297 sh, 245 sh, 218.
IR v max (KBr) cm$^{-1:}$ 3400, 3200 - 2500, 1705, 1640, 1610, 1520, 1180, 1035.
^{1}H NMR (CD_3OD, 500 MHz): δ 2.00 (H-1, dd, J = 13.8, 9.0 Hz, H-2ax), 2.02 (1H, dd, J = 13.8, 4.0 Hz, H-6eq), 2.09 (1H, dd, J = 3.68), 3.4 Hz, H-6ax) 2.13 (1H, dd, J = 13.8, 4.5 Hz, H-2eq), 3.68 (1H, dd, J = 8.7, 3.3, H-4), 4.12 (1H, m, H-5), 5.28 (1H, ddd, J = 9.0, 8.7, 4.5 Hz, H-3), 6.12 and 7.51 (each 1H, d, J = 15.9 Hz, H-α, β), 6.74 (1H, d, J = 8.0 Hz, H-5'), 6.90 (1H, dd, J = 8.0, 2.1 Hz, H-6'), 7.01 (1H, d, J = 2.1 Hz, H-2').
^{13}C NMR: Table 1.
FAB-MS m/z: 354 $(M+1)^+$, 193, 165, 136.
HREI-MS m/z (rel.int. %): 354.1530 (Calcd for $C_{16}H_{18}O_9$, 354.1524).

Characterization of luteolin (4)

M.P; [320°C], [α] D ^{25}: + 43.5 (C = 0.04, MeOH):UV λ max/ (60 % MeOH) nm: 256, 267 sh, 348
^{1}H NMR (CD_3OD, 500 MHz) δ 7.39 (1H, dd, J) 1.4 and 8.3 Hz, H-6'), 7.37 (1H, d, J) 1.4 Hz, H-2'), 6.86 (1H, d, J) 8.3 Hz, H-5'), 6.42 (1H, d, J) 1.2 Hz, H-8), 6.66 (1H, s, H-3), 6.18 (1H, d, J) 1.2 Hz, H-6.

Table 1. ^{13}C-NMR (CD_3OD, 125 MHz) of cholinergic acid.

Position	Chemical shift δ	Position	Chemical shift δ
C-1	76.3	C-1’	127.8
C-2	38.2	C-2’	115.2
C-3	72.0	C-3’	146.6
C-4	73.6	C-4’	149.4
C-5	71.4	C-5’	116.5
C-6	38.9	C-6’	123.0
C-7	177.5	C-9’	168.9
C-α	115.2	C-β	147.1

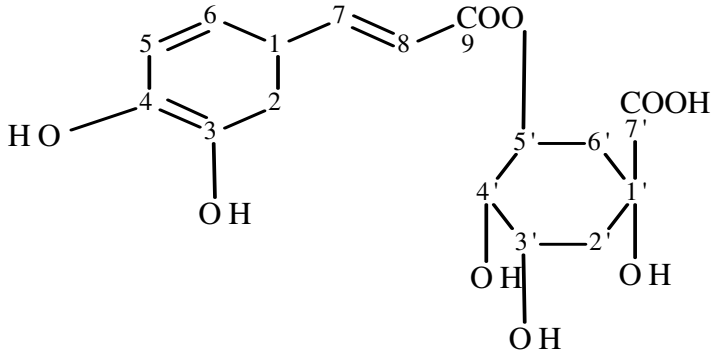

Figure 1. Compound 35’-O-caffeoyl quinic acid (chlorogenic acid).

^{13}C NMR (75 MHz, $CDCl_3$): δ 164.4 (C-2), 103.3 (C-3), 182.2 (C-4), 161.9 (C-5), 99.3 (C-6), 164.5 (C-7), 94.3 (C-8), 157.7 (C-9), 104.2 (C-10), 122.0 (C-1’), 113.8 (C-2’), 146.2 (C-3’), 150.1 (C-4’), 116.5 (C-5’), 119.5 (C-6’):EI-MS m/z (% rel.int.): 286 $[M]^+$ (100), 217 (10), 199 (20). 175 (20), 151 (85), 133 (50), 107 (10).
HREI-MS m/z: 286.3341 (Calcd for $C_{15}H_{10}O_6$, 286.4317).

Free radical scavenging assay

The crude ethanolic extract and subsequent fractions were tested for potential antioxidant activity on the basis of scavenging activity of the stable 1, 1-diphenyl-2 picrylhidrazyl (DPPH) (Waka Ltd. Japan) free radical (Cotelle et al., 1996). A solution of DPPH was prepared by dissolving DPPH (5 ml) in the methanol (2 ml) and the solution was kept in the dark at room temperature. Different concentrations of the test samples were prepared in the micro titer plates (Molecular Devices) at λ 517 nm. Percentage inhibition of the samples was determined by comparison with the methanol treated control group. Quercetin (Sigma, USA) and ascorbic acid (Sigma, USA) were used as positive control. All the analysis were performed in triplicate and averaged.

RESULTS AND DISCUSSION

The compounds 1 and 2 were identified as colchicine and β-lumicolchicine, by comparison of their spectral data with the available in the literature (Danieli et al., 1980; Duandeun et al., 1988; Grewe and Wulf, 1951; Chapman et al., 1963).

Compound 3 was isolated as a pale-yellow amorphous powder. The HR-EIM spectrum exhibited the molecular ion peak at m/z 354.1524 corresponding to the molecular formula $C_{16}H_{18}O_9$ (calculated for $C_{16}H_{18}O_9$, 354.1524). The UV spectrum showed maximum absorption UV max (MeOH) nm as 328, 297 sh, 245 sh, 218, while IR max (KBr) cm^{-1} as 3400, 3200 - 2500, 1705, 1640, 1610, 1520, 1180, 1080, 1035 cm^{-1}(Figure 1). These reading of UV and IR of the compound (3) showed the phenolic character of that compound. The ^{1}H-NMR spectrum indicated aromatic protons as an ABX system at H 6.77 - 7.04 and two trans olefinic protons as an AB system at 6.26 and 7.55 (d, J = 15.8 Hz), indicating the presence of an (E)-caffeic acid moiety. On the other hand, two methylene protons H 2.04 (2H, d, J = 8.2 Hz) with 2.11 (1 H, d, J = 11.3 Hz) and 2.23 (1 H, d, J = 11.5 Hz) with three methine protons H 3.72 (dd, J = 8.2/3.0 Hz), 4.17 (d, J = 3.0 Hz) and 5.33 m together with the corresponding carbon resonance at C 38.20 t, 73.48 d, 71.50 d and 71.90 d, respectively, showed the presence of a quinic acid moiety (Ihsan and Ogihara, 2002). The heteronuclear multiple bond correlation (HMBC) experiment was performed in order to confirm the location of the (E)-caffeoyl moiety and the quinic acid. This experiment showed the correlation peak between H-5’ (H 5.33 m) of the quinic acid and the carbonyl carbon resonance (C 168.66, C-9) of caffeic acid. These assignments showed that the structure of compound (3) is 5’-O-

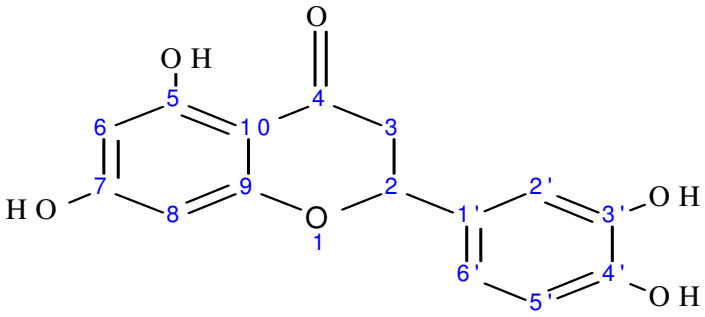

Figure 2. Compound 4 [3' 4' 5, 7-Tetrahydroxyflavone (luteolin)].

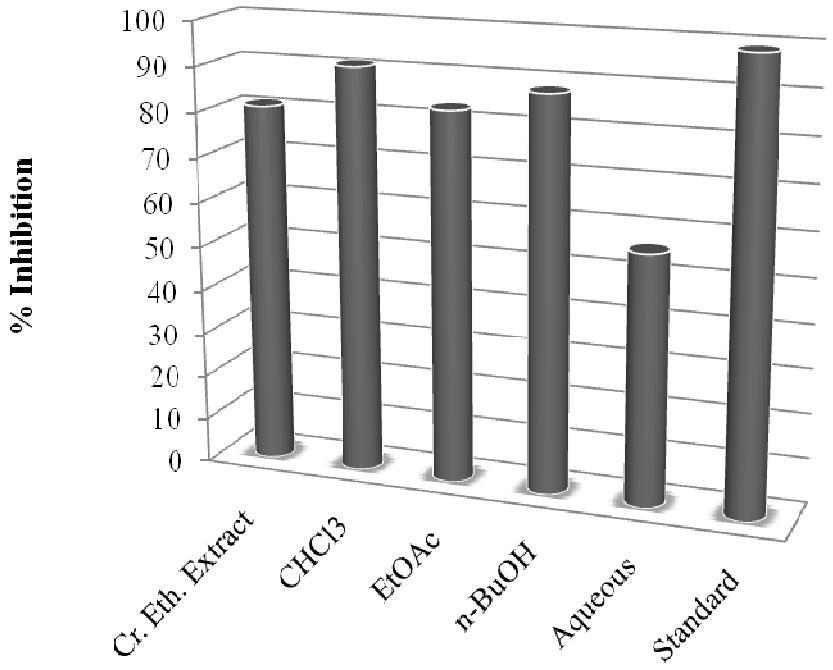

Figure 3. DPPH free radical scavenging activity of the crude extract and fraction of Colchicum luteum Baker 200 µg/ml.

caffeoyl quinic acid (chlorogenic acid) as mentioned in literature (Cheminat et al., 1988).

The high resolution mass spectrum of compound 4 (Figure 2) showed $[M]^+$ peak at m/z 286.3341 (calculated for $C_{15}H_{10}O_6$, 286.4317). The UV spectrum showed λ / (60% MeOH) at 256, 267 sh, 348 nm suggesting it to be a flavonoid (Voirin, 1983). The IR spectral analysis showed the presence of a hydroxyl group (3327 cm^{-1}), the presence of α, β unsaturated carboxyl group (2928 cm^{-1}) and the presence of a methoxyl group (1730 cm^{-1}).

^1H NMR spectra indicated a significantly deshielded signal in the region of 12 - 13 ppm. This resonance may be attributed to the hydroxyl proton OH (5) of flavonoids that participates in a strong six membered ring intra-molecular hydrogen bond with CO (4) and therefore, is strongly deshielded (Jaffrey, 1997). As a rule, the ^1H NMR resonances of the OH groups appear at room temperature as broad signals especially in protic solvents, owing to the mobility of the hydrogen and its fast exchange, on the NMR time scale, with the protons of the solvent. ^1H NMR spectroscopy of compound (4) showed that the OH (5) signals appeared as sharp singlet. Since the region of 12 - 13 ppm in the ^1H NMR spectrum is not as crowed as the aromatic one, the identification of the flavonoids can be based only on 1D proton NMR spectroscopy, without the need for 2D proton NMR. On the basis of above evidences of IR, ^1H NMR analysis and comparison of ^{13}C NMR values with the values in the literature (Ludwing et al., 2004) and EI-MS (Glasl et al., 2002), the structure of compound (4) was established as 3' 4' 5, 7-tetrahydroxyflavone (luteolin).

The results of free radical scavenging activity (RSA) are presented in Figure 3. The crude ethanolic extract and its subsequent fraction presented an outstanding free

radical scavenging activity on the stable DPPH free radical. The crude ethanolic extract afforded an excellent (81%) RSA. Interestingly, fractionation offered mix change in antioxidant activity. The highest antioxidant activity was shown by chloroform fraction (91%) among the tested fractions, followed by n-butanol fraction that displayed (88%) RSA. While the ethyl acetate fraction displayed (83%) RSA. On the other hand, aqueous fraction offered the least (56%) RSA in the assay.

Literature revealed that chlorogenic acid and luteolin have strong antioxidant properties on stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical (Mee et al., 2001; Hsia et al., 2005; Sinisa, et al., 2000), but no such evidences on colchicine type compounds. Therefore, this plant species need further phytochemical analysis to explore mechanism of this activity and other uses of the plant in traditional medicine.

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