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Eupalitin-3-O-α-rhamnosyl (1→2) α-rhamnosyl (1→6)-β-D-galactopyranoside From The Aerial Parts Of Punarnava

Navabha Joshi and Dr. D. L. Verma

Research Scholar (Department of Chemistry, S. S. J. campus, Kumaon University, Almora-263601, Uttrakhand, India)
Mobile no. (9027088024) Email: joshinavabha@gmail.com
Professor, Department of Chemistry, S. S. J. campus, Kumaon University, Almora-263601, Uttrakhand, India)

Abstract

Punarnava, a herb from Kumaon region of Uttrakhand India, has been used to cure various ailments since vedic, unani and ayurvedic periods. Keeping in view the medicinal importance of the punarnava extracts among various ethnic groups of the Himalayas, the reducing potential of aqueous methanolic extract derived from the aerial parts of the plant was evaluated. The n-butanol soluble fraction from water-ethanolic extract, which was found to be a potent antioxidative and highly enriched with quercetin glycosides, was fractionated on whatmann N3 PC using BAW (Butanol-Acetic acid-Water in the ratio 4:1:5, v/v, upper layer) as a developing solvent. Antioxidant activity-guided fractionation of chromatographically isolated flavonol positive fraction led to the isolation and characterization of Eupalitin-3-O-α-rhamnosyl (1→2) α-rhamnosyl (1→6)-β-D-galactopyranoside, a non-catechol group flavone and two flavonol glycosides including Quercetin-3-O-α-L-rhamnosyl (1→6)-β-galactopyranoside from active catechol-group flavonoid fraction. The antioxidant activity of the isolates were determined against DPPH free radicals and their structures were elucidated by chromatographic, spectral (^{1}H NMR, 13C NMR, LC-MS) and hydrolytic methods (acid, H_2O_2 and enzymatic) .

Keywords : punarnava, eupalitin, antioxidant activity

I. Introduction

Punarnava [Boerhaavia diffusa (Nyctaginaceae)], is a flowering plant distributed in

tropical, subtropical and temperate regions of the world at an elevation of 700 ft to 7000 ft. It is a profusely branched, perennial, creeping and pubescent, ascending herb with a height of about 70 cm. The leaves of the plant are simple, thick, fleshy, short petiole, ovate-oblong and up to 5.5x3.3 cm^2 in area. Its flowers are minute, yellow and white colored present four to ten together forming axillary and terminal panicles. Flowering in the plant is observed in the months of October and November. Punarnava is distributed over the hot Himalayan valley from 500 ft to 6000 ft. Forty species of Boerhaavia have been reported, six of which viz., B.diffusa, B.chinensis, B.erecta, B.repens, B.rependa, and B.rubicunda are found in India (Chopra, 1969; CSIR, 1988) [1]. Only one species, i.e., Punarnava (Boerhaavia diffusa) is reported from Indian Himalayan region (Gaur, 1999 [2]; Pangtey, 2005[3]) . Boerhaavia diffusa is a traditional medicinal plant of Kumaon Himalayan region. In Ayurvedic, Siddha, Unani and Homeopathic systems of medicine, the plant has been used to treat nephritic syndrome (Singh and Udupa, 1972) [4], asthama, jaundice, drosical swelling, worm infections, liver troubles, heart troubles, urinary tract infections, cough, cold and inflammation (Lad, 1999 [5]; Guhabakshi et al., 1999 [6]) . In Kumaon hills Boerhaavia diffusa leaves have also been used as a vegetable for better health (Pangtey, 2005) . The extracts derived from different parts of the plant exhibit a wide range of biological activities, viz. anti-inflammatory (Bhalla et al., 1971 [7]) ; hypoglycemic (Chude et al., 2001) [8]; antifibrinolytic (Jain and Khanna, 1989) [9]; anti-convulsant (Adesina, 1979) [10] ; anti-nematodal (Vijaylakshmi et al., 1979) [11]; anti-urethritis (Nadkarni, 1976) [12] ; anti-bacterial (Olukoya et al., 1993) [13] ; anti-hepatotoxic (Misra, 1980 [14] ; Chandan et al., 1991[15]) ; antiviral (Awasthi and Menzel, 1986) [16]; and antioxidant (Pari and Satheesh, 2004) [17] . The present investigation reveals the isolation and identification of flavonol-glycosides from antioxidant activity-guided fractionation of ethyl acetate (EtOAc) and n-butanol (n-BuOH) soluble fractions of aqueous methanolic extract of Boerhaavia diffusa.

II. Materials And Methods

1. Plant material: The aerial parts of Boerhaavia diffusa were collected from Kathgodam, a part of terai bhabar and foothill forming region of Kumaon. The flower bearing twig of the plant was authenticated by Prof. P.C. Pandey, Department of Botany, Kumaun University, S.S.J. campus, Almora-263601, Uttarakhand, and deposited in the same department with herb specimen No. FL-P-003.
2. Extraction and isolation of flavonoid positive fractions: Three kg dried and powdered aerial parts of Boerhaavia diffusa were extracted sequentially with 80% methanol (MeOH) and 50% aqueous MeOH by cold percolation method for six days. The two extracts were filtered and combined. The combined extract was concentrated in vacuo until only H_2O layer (approx. 60 ml) remained. It was partitioned with 50 % dichloromethane (CH_2Cl_2) . The CH_2Cl_2 soluble fraction was separated and the remaining H_2O layer was further partitioned with diethyl ether, ethyl acetate and n-butanol successively. Each partition was examined for antioxidant activity against DPPH (free

radical) by the standard thin layer autobiography (SiO_2) method (Cuendet et al., 1997) [18]. Diethyl ether, EtOAc and n-BuOH soluble fractions produced three, four and three positive spots, respectively against DPPH reagent.

The diethyl ether soluble antioxidative fraction produced three spots on TLC and was evaluated for total flavonoid positive compound by 2 DPC using BAW (4:1:5, upper layer) and 30% HOAc as a developing solvent. 2 DPC examination revealed the presence of five fluorescent spots under UV (360 nm) light, three of which were identified as flavonoids on the basis of color reactions with NA (Naturstoff reagent-A) , ammoniacal silver nitrate, $ZrOCl_2$ and $FeCl_3$-K_4Fe $(CN)_6$ reagents. Among three flavonoid positive compounds, only one was identified to catechol-grouping flavonoid (Mabry and Markham, 1975) [19]. The three flavonoid positive constituents were isolated by RPPC using 30% acetic acid (HOAc) as a developing solvent. Each component was finally purified by sephadex LH-20 CC using 50% aqueous methanol as an eluent. The three components from diethyl ether fractions were referred to as A, B and C in order of their decreasing mobilities on PC with 30% HOAc as a developing solvent.

The n-BuOH soluble anti-oxidative fraction was adsorbed on cellulose CC and eluted initially with H_2O and then with 20%HOAc, 30% HOAc, 40% HOAc and 50% HOAc successively. On eluting CC with 30 % HOAc, two dark purple fluorescent bands were observed and each was eluted and collected separately by monitoring under UV light. The eluates derived from faster and slower moving band were referred to as Frac.I and Frac. II respectively and the former was found to have more antioxidant potential amongst the two. RPPC of Frac. I using BAW (4:1:5, v/v, upper layer) as a developing solvent afforded two broad dark purple fluorescent bands which were eluted and collected separately in order of their decreasing mobilities and named as Frac. I (a) and Frac. I (b) respectively. Frac. I (a) , negative to catechol grouping flavonoid and less anti-oxidative compared to Frac. I (b) , was chromatographed on sephadex LH-20 CC using 40% aqueous methanol as an eluent. A broad dark purple fluorescent band was observed on CC. It was eluted and referred to as compound D. Frac.I (b) , positive to catechol-grouping flavonoids and highly anti-oxidative was adsorbed on sephadex LH-20 CC and eluted successively with H_2O, 10%MeOH, 20% MeOH, 30% HOAc, 40% HOAc and 60% HOAc. A total of 50 fractions, 100 ml each, were collected. Each fraction was examined on cellulose (Merck) TLC using 15% HOAc as developing solvent and catechol grouping flavonoids were characterized by spraying with ammonical $AgNO_3$ and NA (reagents) . Chromatographically similar catechol grouping flavonoids bearing fractions were combined. Two major fractions derived from fractions 30-40 (50% MeOH) and fractions 42-49 (60% MeOH) afforded two compounds, (E) and (F) respectively.

III. Results And Discussion

The Compounds B and C were identified as Kaempferol and Quercetin respectively on the basis of comparison with their respective standards by PC. . The spectral data of the two compounds were also found in agreement with the literature values of

Kaempferol and Quercetin . (Agrawal, 1989 [20]; Markham and Geiger, 1994) [21].

The compound A appeared as dull yellow fluorescent streak on PC under UV light both in the absence and presence of NH_3 vapors indicating a flavone with free hydroxyl group at 5-position (Mabry et-al, 1970) [22]. EIMS of the compound A exhibited a molecular ion peak at m/z 330 $(M)^+$ and two prominent ion peaks at m/z 184 (A^+) and 121 (B^+) indicating the presence of one hydroxyl and two methoxyl groups in the A-ring and one hydroxyl group in the B-ring respectively. ^{1}H NMR (in DMSO-d_6, 400 MHz) showed three signals in the aromatic region at δ6.40 (1H, s) , δ6.94 (2H, d, J=8.5 Hz) , δ8.06 (2H, d, J=8.5 Hz) which were assignable to H-8, H-3′/5′ and H-2′/6′, respectively. Two signals in the aliphatic region at δ3.76 (3H, s) and δ3.87 (3H, s) were identified to $–OCH_3$ groups at C-6 and C-7 positions present in the A ring. Thus compound A has been deduced to be 4′, 5-dihydroxyl-6, 7-dimethoxyflavonol or Eupalitin. Further the CH-unsubstituted and $–OCH_3$ group substituted positions in aromatic systems were confirmed by HECTOR (^{13}C NMR) .

Compound (D) appeared as dark purple fluorescent band on PC under UV light which turned yellow-green with NH_3, indicating the presence of 5-and 4′-hydroxyl groups in the flavonoid. (Mabry et-al, 1970; Markham, 1982) [23]. The methanolic solution of D gave positive colour reactions with Mg + HCl and α-naphthol, indicating a flavonoid glycosidic compound. Complete acid hydrolysis of D with 2-NHCl afforded eupalitin (CoPC) , galactose (CoPC) and Rhamnose (CoPC) . LC-FABMS (-) of D exhibited a molecular ion at m/z 783 $[M-H]^-$ and other prominent peaks at m/z 637 (m/z 783-rhamnose)-and m/z 329 {m/z 783-(galactose + rhamnose) $\}^-$ supporting the ionization of two molecules of rhamnose and one molecule of glucose from eupalitin. Enzymatic hydrolysis of D with α-rhamnosidase afforded a flavonol glycoside, represented as D (a) . The compound D (a) appeared as dark purple fluorescent spot on PC under UV light which turned yellow-green with NH_3, indicating a flavonoid with free hydroxyl groups at 4′ and 5-positions. (Mabry et-al, 1970) . Complete acid hydrolysis of D (a) with 2N HCl gave an aglycone, eupalitin (CoPC) and a sugar, galactose (CoPC) . LC-MS (negative mode) of D (a) gave a molecular ion peak at m/z 491 and other prominent ion peaks at m/z 329 (m/z 491-galactose) indicating the release of galactose from the eupalitin galactoside. The compound D (a) was identified as eupalitin-3-O-β-D-galactopyranoside on the basis of its ^{1}HNMR and ^{13}C NMR (aglycone) . The rhamnose sugar was identified from the mixture obtained by the complete acid hydrolysis of the compound (D) . Thus it has been established that both the rhamnose moieties substitute the hydroxyl groups of primary sugar, galactose.

^{1}HNMR (DMSO-d_6, 400 MHz) of D exhibited three anomeric proton signals at δ5.73 (1H, d, J=7.3Hz) , δ 5.10 (1H, d, J=1.5Hz) and δ4.45 (1H, d, J=1.5Hz) which were attributed to galactose (β-configuration) , t-rhamnose (α-configuration) and t-rhamnose (α-configuration) , respectively. The terminal sugars, rhamnose moieties are attached to C-2″ and C-6″ of galactose (primary sugar) as evident from the ^{1}H NMR data. Thus the compound D has been identified as Eupalitin-3-O-α-L-rhamnopyranosyl (1→2)-α-L-rhamnopyranosyl (1→6)-β-D-galactopyranoside.

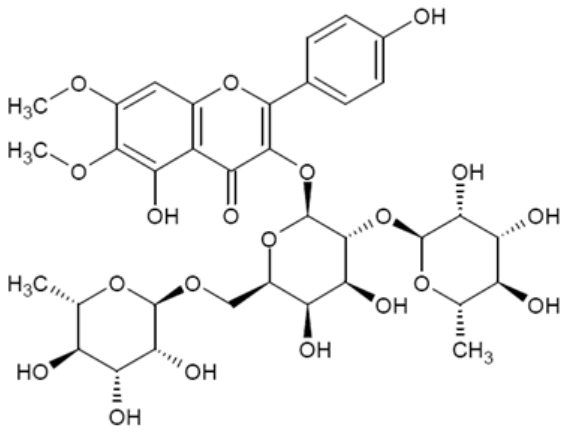
Figures And Tables

^{13}C NMR (DMSO-d_6) data of Eupalitin (aglycone) . (Table 1) .

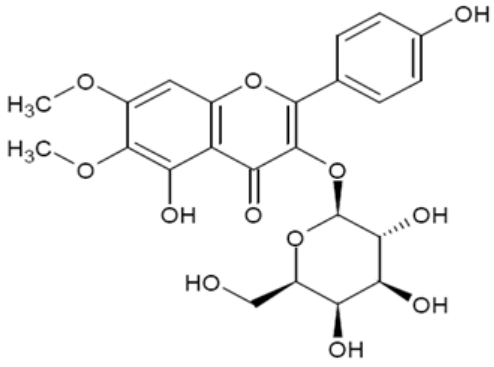
Shift	attribution	Shift	attribution
91.3 116.0 130.0	C-8 C-3′/C-5′ C-2′/C-6′	56.30 60.0	-OCH_3 (C-7) -OCH_3 (C-6)

^1H NMR (DMSO-d_6, 400 MHz) δppm of D (a) .
3.81 (3H, s) , 3.71 (3H, s) , 6.94 (2H, d, J=8.5 Hz) , 8.08(2H, d, J=8.5 Hz) , 6.40 (1H, s) , 5.40 (1H, d, J=7.5 Hz) , 12.56 (1H, br s)

^1H NMR (DMSO-d_6, 400 MHz) δppm of D.
6.38 (1H, s) , 6.86 (2H, d, J=8.5 Hz) , 8.04 (2H, d, J=8.5 Hz) , 12.58 (1H, br s) , 5.73 (1H, d, J=7.3 Hz) , 4.45 (1H, d, J=1.5 Hz) , 5.10 (1H, d, J=1.5 Hz) , 1.20 (3H, d, J=6.5 Hz) , 1.28 (3H, d, J=6.5 Hz) , 3.71 (3H, s) , 3.81 (3H, s) , 3.06- 4.02 (m).



D



D(a)

V. CONCLUSION

Flavonoids exhibit a wide range of activities so their source plants could be used to prepare various medicines and cosmetics to meet the increasing global demand. In higher plants, Flavonoids are involved in UV filtration. Some flavonoids have inhibitory activity against organisms that cause plant disease e.g. Fusarium oxysporum.[24] Flavonoids might induce mechanisms that affect cancer cells and inhibit tumor invasion. Flavonoids are most commonly known for their antioxidant activity. The activities of the flavonoids especially Eupalitin-3-O-α-rhamnosyl (1→2)

α-rhamnosyl (1→6)-β-D-galactopyranoside isolated from the above plant could be further investigated and used for preparing products for a specific purpose.

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