

## Eupatilin-7-O-2,6-Dirhamnosylgalactoside From The Leaves Of *Boerhaavia Diffusa* Linn.

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**ABSTRACT:** *Boerhaavia diffusa* (Nyctaginaceae), commonly known as Punarnava, 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 anti-oxidative 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 Eupatilin-7-O- $\alpha$ -rhamnosyl(1 $\rightarrow$ 2) $\alpha$ -rhamnosyl(1 $\rightarrow$ 6)- $\beta$ -D-galactopyranoside a non-catechol group flavone and two flavonol glycosides viz. Quercetin-3,7-di-O-glucoside and Quercetin-3-O- $\alpha$ -L-rhamnosyl(1 $\rightarrow$ 6)- $\beta$ -galactopyranoside from active catechol-group flavonoid fractions. The antioxidant activity of the isolates were determined against DPPH free radicals and their structures were elucidated by chromatographic, spectral (<sup>1</sup>H NMR, <sup>13</sup>C NMR, LC-MS) and hydrolytic methods (acid, H<sub>2</sub>O<sub>2</sub> and enzymatic).

**Keywords** - *Boerhaavia diffusa* linn. , eupatilin, antioxidant activity, flavonol glycoside, flavones

### I. Introduction

*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<sup>2</sup> 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)<sup>[1]</sup>. Only one species, i.e., *Punarnava* (*Boerhaavia diffusa*) is reported from Indian Himalayan region (Gaur, 1999<sup>[2]</sup>; Pangtey, 2005<sup>[3]</sup>). *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)<sup>[4]</sup>, asthma, jaundice, drosical swelling, worm infections, liver troubles, heart troubles, urinary tract infections, cough, cold and inflammation (Lad, 1999<sup>[5]</sup>; Guhabakshi *et al.*, 1999<sup>[6]</sup>). 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<sup>[7]</sup>); hypoglycemic (Chude *et al.*, 2001)<sup>[8]</sup>; antifibrinolytic (Jain and Khanna, 1989)<sup>[9]</sup>; anti-convulsant (Adesina, 1979)<sup>[10]</sup>; anti-nematodal (Vijaylakshmi *et al.*, 1979)<sup>[11]</sup>; anti-urethritis (Nadkarni, 1976)<sup>[12]</sup>; anti-bacterial (Olukoya *et al.*, 1993)<sup>[13]</sup>; anti-hepatotoxic (Misra, 1980<sup>[14]</sup>; Chandan *et al.*, 1991<sup>[15]</sup>); antiviral (Awasthi and Menzel, 1986)<sup>[16]</sup>; and antioxidant (Pari and Satheesh, 2004)<sup>[17]</sup>. 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, Kumaon 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<sub>2</sub>O layer (approx. 60 ml) remained. It was partitioned with 50 %

dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>). The CH<sub>2</sub>Cl<sub>2</sub> soluble fraction was separated and the remaining H<sub>2</sub>O 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<sub>2</sub>) 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<sub>2</sub> and FeCl<sub>3</sub>-K<sub>4</sub>Fe (CN)<sub>6</sub> 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<sub>2</sub>O 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<sub>2</sub>O, 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<sub>3</sub> 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<sub>3</sub> 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 334 (M)<sup>+</sup> and other prominent ion peaks at m/z 343, 329, 301, 167, 165, 139 and 111. <sup>1</sup>H NMR (in DMSO-d<sub>6</sub>, 400 MHz) showed signals at δ6.411 (1H, s), δ6.399 (1H,s), δ3.743 (3H,s), δ7.493 (1H, dd, J=1.733, J=0.587), δ3.765 (3H,s), δ6.940 (1H, dd, J=8.425, J=0.587), δ7.738 (1H, dd, J=8.425, J=1.733), δ3.950 (3H,s). Thus compound A has been deduced to be 2-(3, 4-dimethoxyphenyl)-5,7- dihydroxy-6-methoxychromen-4-one or Eupatilin. Further the CH- unsubstituted and – OCH<sub>3</sub> group substituted positions in aromatic systems were confirmed by HECTOR (<sup>13</sup>C NMR).

Two flavonol glycosides, E and F were isolated from most prominent antioxidant active catechol-group flavonoid positive fractions. The compound (E) appeared as dark purple fluorescent band on PC under UV light and gave positive colour reactions with Mg + HCl and α-naphthol. Complete acid hydrolysis of E with 2N HCl afforded Quercetin (CoPC) and Glucose (CoPC). H<sub>2</sub>O<sub>2</sub> oxidation of E gave Quercetin-7-O- β-D-glucopyranoside (CoPC) and glucose (CoPC). LC-MS (ESI-) of E gave a molecular ion peak at m/z 625 [M-H]<sup>-</sup> and other prominent ion peaks at m/z 463 (m/z 625- glucose) and m/z 301 (m/z 463- glucose), indicating the release of two glucose moieties from quercetin. <sup>1</sup>HNMR showed five aromatic signals for quercetin and two anomeric proton signals at δ 5.56 (1H,d, J=7.5 Hz) and δ 5.26 (1H, d, J=7.30 Hz) which have been attributed to two primary glucose moieties attached respectively to C-3 and C-7 positions of Quercetin. Thus compound (E) has been identified as Quercetin-3-O- β-D-glucopyranoside- 7-O- β-D-glucopyranoside. The compound (F) has been identified as Quercetin- 3-O-α-L-rhamnopyranosyl (1→6) - β-D-galactopyranoside. Its chromatographic behavior, hydrolytic products (acid, enzyme, H<sub>2</sub>O<sub>2</sub>) and spectral data (UV, <sup>1</sup>HNMR, <sup>13</sup>C NMR and MS) were found similar to those already reported in the literature to Quercetin- 3-O-α-L-rhamnopyranosyl (1→6) - β-D-galactopyranoside.

Compound (D) appeared as dark purple fluorescent band on PC under UV light which turned yellow-green with NH<sub>3</sub>, indicating the presence of 5-hydroxyl group in the flavone (Mabry et-al, 1970; Markham, 1982)<sup>[23]</sup>. The methanolic solution of D gave positive colour reactions with Mg + HCl and  $\alpha$ -naphthol, indicating a flavonoid glycosidic compound. Complete acid hydrolysis of D with 2-NHCl afforded eupatilin(CoPC), galactose (CoPC) and Rhamnose (CoPC). LC-FABMS (-) of D exhibited a molecular ion at m/z 797 [M-H]<sup>-</sup> and two prominent peaks at m/z 651 (m/z 797-rhamnose) and m/z 343 {m/z 797-(galactose + rhamnose)} supporting the ionization of two molecules of rhamnose and one molecule of glucose from eupatilin. Enzymatic hydrolysis of D with  $\alpha$ -rhamnosidase afforded a flavone 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<sub>3</sub>, indicating a flavone with free hydroxyl groups at 5- position.( Mabry et-al, 1970). Complete acid hydrolysis of D(a) with 2N HCl gave an aglycone, eupatilin (CoPC) and a sugar, galactose( CoPC). LC-MS (negative mode) of D(a) gave a molecular ion peak at m/z 505 and other prominent ion peaks at m/z 343 (m/z 505- galactose) indicating the release of galactose from the eupatilin galactoside. The compound D(a) was identified as eupatilin-7-O- $\beta$ -D-galactopyranoside on the basis of its <sup>1</sup>H NMR and <sup>13</sup>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.

<sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz) of D exhibited two doublets at  $\delta$ 1.274 (3H, d, J=6.900) and  $\delta$ 1.279 (3H, d, J=6.900) for the two methoxy substituents at C-3 and C-4 in the B-ring of the flavone and a singlet at  $\delta$ 3.950 for a methoxy group at C-6 of the A-ring. Three anomeric proton signals at  $\delta$ 5.299 (1H, d, J=10.260),  $\delta$ 4.509 (1H, d, J=2.920) and  $\delta$ 4.430 (1H, d, J=2.920) were attributed to galactose ( $\beta$ -configuration), rhamnose ( $\alpha$ -configuration) and rhamnose ( $\alpha$ -configuration), respectively. The terminal sugars, rhamnose moieties are attached to C-2'' and C-6'' of galactose (primary sugar) as evident from the <sup>1</sup>H NMR data. Thus the compound D has been identified as Eupatilin 7-O- $\alpha$ -L-rhamnopyranosyl (1 $\rightarrow$ 2)  $\alpha$ -L-rhamnopyranosyl (1 $\rightarrow$ 6)-  $\beta$ -D-galactopyranoside.

#### IV. Figures And Tables

<sup>13</sup>C NMR (DMSO-d<sub>6</sub>) data of Eupatilin (aglycone).(Table 1).

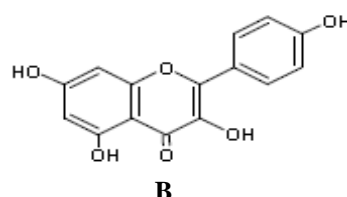
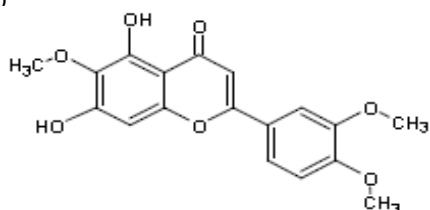
Shift	attribution	Shift	attribution
163.4	C-2	122.9	C-1'
103.3	C-3	109.3	C-2'
182.2	C-4	149.0	C-3'
131.3	C-6	152.0	C-4'
157.3	C-7	111.6	C-5'
94.4	C-8	120.0	C-6'
104.1	C-10	60, 55.8, 55.7	3 OCH <sub>3</sub> 's

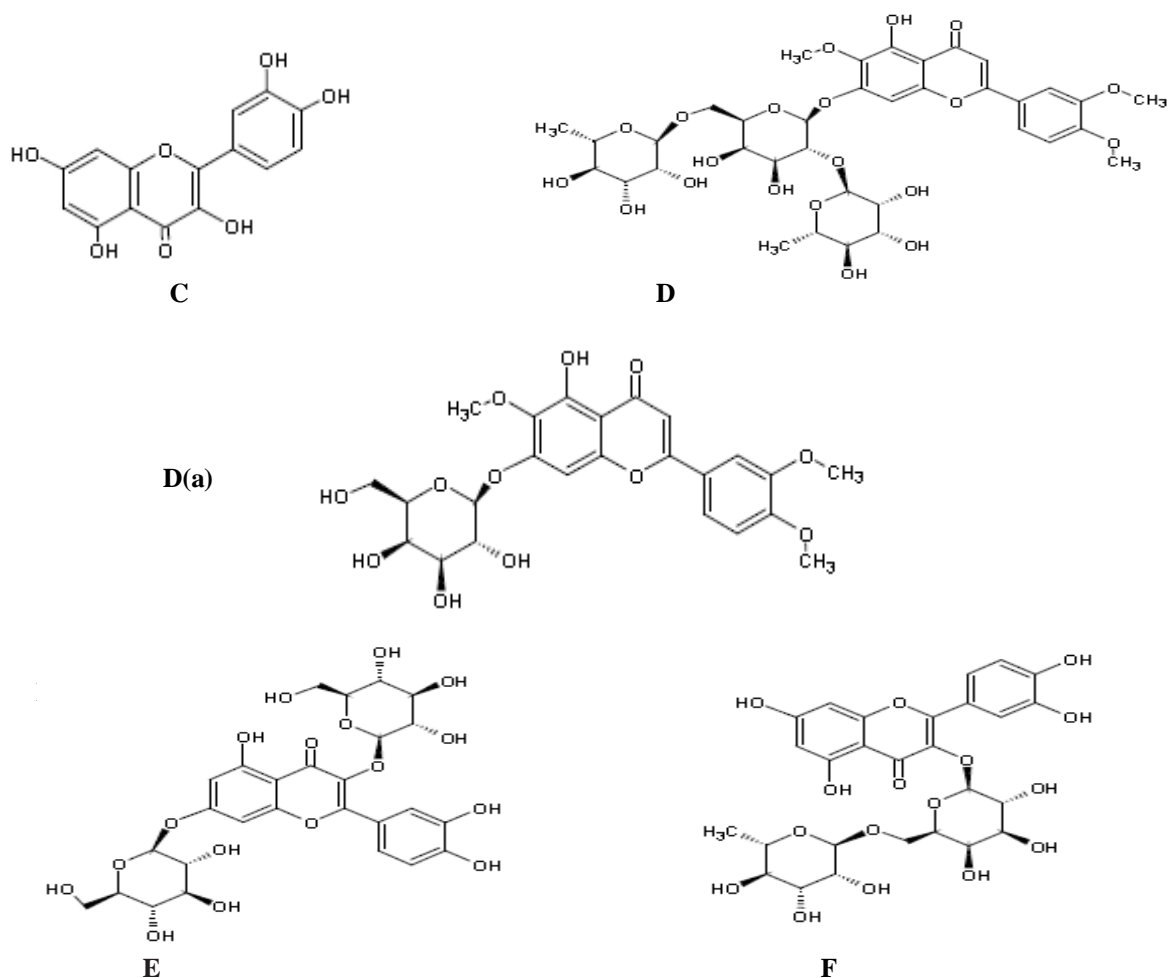
#### <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz) $\square$ ppm of D(a).

$\delta$ 3.950 (3H,s),  $\delta$ 3.764 (3H, s),  $\delta$ 3.742(3H, s),  $\delta$ 7.284 (1H, dd, J=8.395, J=1.654),  $\delta$ 6.984 (1H, dd, J=8.395, J=0.919),  $\delta$ 7.501 (1H, dd, J=1.654, J=0.919),  $\delta$ 6.419 (1H, s),  $\delta$ 6.751 (1H, s),  $\delta$ 3.997 (2H, d, J=5.119),  $\delta$ 3.440 (1H, td, J=5.119, J=2.680),  $\delta$ 3.443 (1H, dd, J=3.470, J=2.680),  $\delta$ 3.624 (1H, dd, J=10.170, J=3.470),  $\delta$ 3.256 (1H, dd, J=10.260, J=10.170),  $\delta$ 5.153 (1H, d, J=10.260)

#### <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz) $\delta$ ppm of D.

$\delta$ 3.950 (3H,s),  $\delta$ 3.765 (3H,s),  $\delta$ 3.741 (3H,s),  $\delta$ 1.274 (3H, d, J=6.900),  $\delta$ 1.279 (3H, d, J=6.900),  $\delta$ 7.658 (1H, dd, J=8.525, J=1.567),  $\delta$ 7.177 (1H, dd, J=8.525, J=5.424),  $\delta$ 7.559 (1H, dd, J=5.424, J=1.567),  $\delta$ 6.421 (1H,s),  $\delta$ 6.755 (1H),  $\delta$ 4.128 (2H, d, J=4.559),  $\delta$ 4.187 (1H, dq, J=10.260, J=6.900),  $\delta$ 4.129 (1H, dq, J=10.260, J=6.900),  $\delta$ 3.562 (1H, td, J=4.559, J=2.680),  $\delta$ 3.372 (1H, dd, J=10.260, J=10.170),  $\delta$ 3.360 (1H, dd, J=10.260, J=10.170),  $\delta$ 3.848 (1H, dd, J=3.470, J=2.680),  $\delta$ 3.904 (1H, dd, J=10.170, J=3.450),  $\delta$ 3.778 (1H, dd, J=10.170, J=3.450),  $\delta$ 3.512 (1H, dd, J=3.450, J=2.920),  $\delta$ 3.328 (1H, dd, J=3.450, J=2.920),  $\delta$ 3.750 (1H, dd, J=10.170, J=3.470),  $\delta$ 4.430 (1H, d, J=2.920),  $\delta$ 4.509 (1H, d, J=2.920),  $\delta$ 5.229 (1H, d, J=10.260),  $\delta$ 3.560 (1H, dd, J=10.260, J=10.170)





## 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*.<sup>[24]</sup> 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 Eupatilin-7-O-2,6-dirhamnosylgalactoside isolated from the above plant could be further investigated and used for preparing products for a specific purpose.

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