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Original Article

Investigation of Phytochemical constituents from *Eulophia epidendraea*

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Abstract

The tuber and leaf of *Eulophia epidendraea* was phytochemically examined. Chromatographic procedures led to the isolation of four phytochemicals in *β*-sitosterol (**I**), *β*-sitosterolglucoside (**II**), *β* – amyrin (**III**) and lupeol (**IV**) from the tuber and also four flavonoids of apigenin, luteolin, kaempferol, and quercetin were identified from the leaves of *E. epidendraea*.

Keywords: Orchidaceae, *Eulophia epidendraea*, tuber, leaf, Phytochemicals

Introduction

Higher plants are major sources of natural products such as pharmaceuticals, agrochemicals ingredients of flavor and fragrance, food additives, and pesticides (Balandrin and Klocke,1988). The pharmaceutically well known phychemical of morphine was isolated from opium poppy, *Papaver somniferum* by Sertuner (Burger,1960) quinine from *Chinchona officinalis* (Cragg *et al*.,2002), reserpine from *Rauvolfia serentina*, ephedrine from *Ephedra vulgaris* and taxol from *Taxus brevifolia* (Wani *et al*.,1971). These phytochemicals constitute some of the most exiting chemotherapeutic agents currently available for use in a clinical medicine.

Orchid *Eulophia epidendraea* (Retz.) Fischer belongs to the family Orchidaceae, Which has been traditionally used by the local people of Yadav community for the treatment of tumour, abscess and healing of wound (Maridass *et al.,* 2008). Recently, pharmacological studies on the wound - healing activity of tuber extract of *E. epidendraea* were reported. The present study was, therefore, carried out to identify the chemical constituents of the tuber and leaf of *Eulophia epidendraea* (Retz.) Fischer.

Materials and Methods

Plant materials

The orchid *Eulophia epidendraea* (Retz.) Fischer was collected from Kambli Malaikovil Forest, (75°50'E and 9°20'N) near Tenkasi, Tirunelveli District, Tamil Nadu, India.

Solvent extraction and isolation

The tuber of *Eulophia epidendraea* (Retz.) Fischer was air - dried and powdered. About 1.0 kg of this powder was extracted with petroleum ether (30-60°C) benzene (80.3°C), chloroform (61°C), acetone (56°C) and methanol (65°C) in a Soxhlet apparatus. The extraction process was performed for 8h. The solvents were evaporated under reduced pressure. After determining the yields, sediment extracts were stored at 4°C for further study. The methanolic extract (8.0g) was then fractionated by column chromatography on silica gel and eluted with ethyl acetate (EtOAc) followed by EtOAc - MeOH, and gradient 25 ml fractions were collected: fractions1- 4 (EtOAc), fractions 5-17 (10% MeOH), fractions18-21 (20% MeOH), fractons 22-33 (30% MeOH), fractions 34-38 (40% MeOH), fractions 39-56 [EtOAc-MeOH-H2O (10:5:1v/v/v)]. Fractions 5-17 contained compound (**I**). Fractions 18- 21 contained (**II**); fractions 34-38 contained (**III**); fractions 39-56 contained (**IV**) respectively. Identifications were made by comparison with the data from previous IR, UV, NMR and mass spectra (Pandey *et al*., 1996; Mučaji *et al*., 2000).

Instrumentation

UV spectra were obtained on a Shimadzu UV-160 spectrophotometer, and IR spectra were determined in KBr discs on a Perkin-Elmer 781 spectrophotometer. ^{1}H NMR spectra were recorded with a Varian Gemini NMR spectrometer at 200, 400 MHz or with a Bruker Avance NMR spectrometer at 500 MHz in $CDCl_3$. ^{13}C NMR spectra were recorded with a Varian Gemini NMR spectrometer at 50, 100 MHz or with a Bruker Avance NMR spectrometer at 125 MHz in $CDCl_3$. EI - MS were obtained with a JEOL JMS - HX110

spectrometer and HREI - MS with a Finnigan MAT 95S spectrometer.

Isolation of leaf flavonoids

The powdered leaf of *Eulophia epidendraea* was studied using hydrolyzed extract following the method of Harborne (1973). About 100g of dried leaves were cut into small pieces and extracted in 20 ml of 2M HCl, then boiled in a water bath at 100 ^{o}C for 1hr. The hydrolyzed extract was allowed to cool and filtered through a filter paper to remove debris from the extract. The filtrate was treated twice with ethyl acetate; the upper layer containing flavones and flavonol was then separated from lower aqueous layer by a separating funnel. Amyl alcohol was added to the latter layer to extract anthocynidins. These extracts were allowed to evaporate to dryness overnight in a dark fume chamber. Then five drops each of ethanol (95%) and methanol (100%) were added to dissolve flavones and flavonol which were ready for spotting into the plates.

Thin layer chromatograpic (TLC) analysis

The hydrolyzed extracts were run single dimensionally in solvent forestal, at room temperature of 21 - 28 ^{o}C. The concentrated extracts were spotted on the lower left corner of the TLC plate using 5µl micropipette. Fifteen loads of the extracts were applied and allowed to dry using a hair dryer before each subsequent load. The diameter of the spot in each chromatogram was normally about 5mm. Authentic markers of flavones (luteolin and apigenin) and flavonols (myricetin, quercetin and kaempferol) obtained commercially were co-chromatographed. Identification of the hydrolyzed compounds of these extracts was made by examination of the spots under UV light and by changes in colour under day light after application of ammonia. Rf values of these spots in comparison with the Rf values of authentic markers, coupled with those values given for each known compound in Harborne (1973), were of great help in identification of these spots. Nine chromatographic spots were identified in this study. They indicated four known compounds and five unidentified compounds (Table -2).

Results and Discussion

The methanolic-tuber extract of *Eulophia epidendraea* was subjected to a series of chromatographic separations over silica-gel, resulting in the isolation of *β*-sitosterol (**I**), *β*-sitosterolglucoside (**II**), *β* – amyrin (**III**) and lupeol (**IV**). The structures of these compounds were determined by comparing their spectral data with those reported or analyzing their various ^{13}C and ^{1}H- NMR spectral data and determined in comparison with the literature data. Compound (**I**) was obtained as colourless needle. The molecular formula $C_{29}H_{50}O$ was assigned by HREI-MS spectrophotometer. On the basis of ^{1}H and ^{13}C-NMR spectral data from the previous literature compound (**I**) was established as *β* – sitosterol (Kovganko *et al*., 1999).

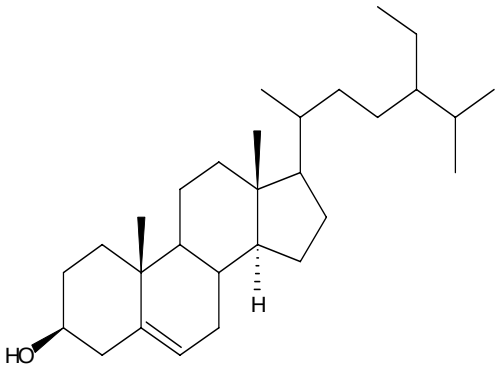

Fig.1. *β*- Sitosterol

In the present study, *β*-sitosterol was isolated from tuber of *Eulophia epidendraea*, and the same was reported in many species including *Tephrosia strigosa* and *Heliotropium indicum*, *Ajuga macrosperma* aerial part of *Brillantaisia palisatii, Elaphoglossum spathulatum, Parahancornia amapa, Conyza bonariensis, Lilium longiflorum* and *Tulipa gesneriana*, Zhongguo zhongyao, *Atractylodes chinenese* (Sreenivasulu and Sarma, 1996; Pandey *et al*., 1996; Dinda *et al*.,1997; Carvalhoa *et al*.,2001, Kong *et al*.,2001; Berrondo *et al*.,2003; Socolsky *et al*.,2003; Endoh *et al*.,1981). The occurrence of *β*-sitosterolglucoside was reported from plants such as *Lilium candidum, Olea europaea* and *Heliotropium indicum (*Pandey *et al*., 1996; Mučaji *et al*., 2000; Kadowaki *et al*., 2003). The intake of *β*-sitosterolglucoside capsule in marathon runner provide less inflammatory and reduced immunosuppressed activity excessive of physical stress (Bauic *et al*.,1999).

Compound (**II**) was obtained as colourless needle. The molecular formula $C_{35}H_{60}O_6$ was assigned by HREI-MS

spectrophotometer. On the basis of ^{1}H and ^{13}C-NMR spectral data from the previous literature compound (**II**) was established as *β* - sitosterolglucoside. (Swift, 1952). The ^{13}C-NMR spectrum of the *β* - sitosterol showed 35 carbon signal, including the signals corresponding to two olefinic carbon at δ121.9(C-6) and δ140.9 (C-5). Furthermore, the ^{1}H-NMR spectrum exhibited one olefinic proton signal at δ5.35 (H-6), two angular methyl groups at δ0.89(s,H-18), δ0.93(s,H-19) an isopropyl (δ0.86(H-26),0.89(H-27), 1.68 (H-25) and ethyl (δ0.66(H-29),1.26(H-28) group (Fig.1.)

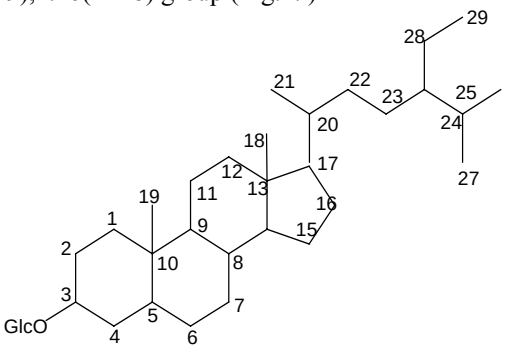

Fig.2. *β*- Sitosterolglucoside

Strong absorption due to many hydroxyl group (3400 cm^{-1}) in the IR spectrum and the signal in the ^{1}H-NMR (δ3.97 (H-5'),4.07(H-2'),4.30(H-3',4'), 4.43(H-6'α),4.58(H-6'β),5.06 (H-1') and ^{13}C-NMR (δ62.8 (C-6'), 71.7 (C-4'),75.4 (C-2'),78.6 (C-3'),102.6 (C-1') spectra suggested that the compound was a steroidal glycoside. This is the first report of steroidal glycoside from this orchid genus.

Beta-sitosterol (*β*-sitosterol) and its glucoside (*β*-sitosterolglucoside) are the most abundant sterols found in plants. In common with other phytosterols they are not endogenously synthesised in the human body and are derived exclusively from the diet (Ling *et al*.,1995). Although they differ from cholesterol by only an extra ethyl group in the side chain, they show profound biological effects in a number of experimental animal models. These include, inter alia, reduction of carcinogen-induced colon cancer, anti-inflammatory (Yamamoto *et al*., 1991), and anti-complement activity (Yamada *et al*., 1987). Bouic *et al*., (1996), reported that the *β*-sitosterol and *β* -sitosterol glucoside stimulate the proliferation of human peripheral blood lymphocytes and they can be used as an immunomodulatory agents. Donald *et al*., (1997) reported that the *β*-sitosterol and *β*-sitosterolglucoside were used in the treatment of pulmonary tuberculosis.

Compound (III)

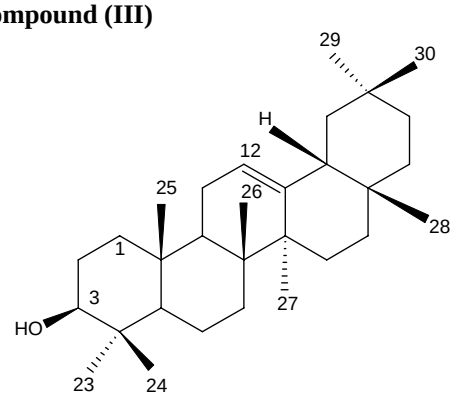

Fig.3. *β*- Amyrin

Compound *β*- Amyrin was obtained as colourless needle. The molecular formula was established as $C_{30}H_{50}O$ on the basis EI-MS: m/z 426 (M^{+}, 0.6%), 411(0.1%), 218 (100%), 272 (0.1%), 189 (30%), 135 (34%), 95(48%); UV spectrum showed the maxima at 240.2 nm (UV λ_{max}(MeOH); IR spectrum suggested the presence of IR γ_{max} (KBR) cm^{-1}: 3397, 2932, 1645, 1465, 1379, 1029, 900; ^{1}H (200MHZ, CDCl3): δ 5.12 (m, H-12), 3.23 (m, H-3), 1.13, 0.99, 0.97,0.94, 0.87(x2), 0.83, 0.79(CH_3). ^{13}C NMR (50.29 MHz,$CDCl_3$): δ 38.14 (C-1), 27.51(C-2), 79.12 (C-3), 38.88 (C-4), 55.27 (C-5), 18.44 (C-6), 33.03 (C-7), 38.82 (C-8), 47.81 (C-9), 37.00 (C-10), 23.48 (C-11),121.82 (C-12), 145.28 (C-13), 42.18 (C-14), 26.12 (C-15), 2737 (C-16), 32.04 (C-17), 47.22 (C-18), 46.93 (C-19), 31.34 (C-20), 34.83 (C-21), 37.26 (C-22), 28.22 (C-23), 15.47 (C-24), 15.72 (C-25), 16.97 (C-26), 25.26 (C-27),28.44 (C-28), 33.44 (C-29), 23.48 (C-30).

Compound (**IV**) was obtained as colourless needle. The molecular formula $C_{30}H_{60}O$ was assigned by HREI-MS spectrophotometer. On the basis of ^{1}H and ^{13}C-NMR spectral data from the previous literature compound (**IV**) was established as lupeol [Ref: Aratanechemuge *et al*., 2004].

In the present work, the compound *β*-amyrin was isolated from *Eulophia epidendraea* which was also found in several plants such as *Tephrosia strigosa* and *Heliotropium indicum*, *Brillantaisia palisatii*, *Lychnophora pinaster*,

Luxemburgia nobilis, *Chiococca braquiata, Parahancornia amapa, Atractylodes chinenese*, *Atractylodes chinenese* (Sreenivasulu and Sarma, 1996; Pandey *et al*., 1996, Carvalhoa *et al*.,2001; Ding *et al*.,2000; Berrondo *et al*.,2003, Silveira *et al*.,2005; Oliveira *et al*.,2002; Lopesa *et al*.,2004).

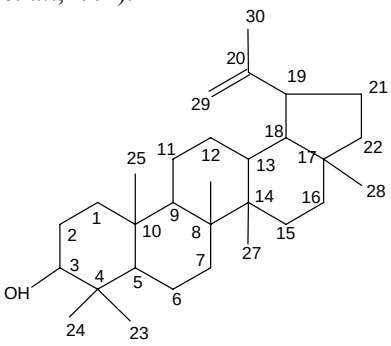

Fig.4. Lupeol

Similarly the compound Leupol isolated from *Eulophia epidendraea* was also reported from plants such as *Brillantaisia palisatii, Lychnophora pinaster, Parahancornia amapa,* (Ding *et al*.,2000; Carvalhoa *et al*.,2001; Berrondo *et al*.,2003, Silveira *et al*.,2005). Aratanechemuge *et al*.,(2004), reported that the suppression of growth of the HL-60 cells by lupeol results from the induction of apoptosis by this compound. Badami *et al*.,(2003) reported that the lupeol isolated from the bark of *Grewia tiliaefolia,* had weak cytotoxic properties.

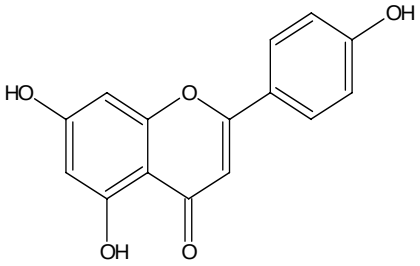

Fig.5. Apigenin

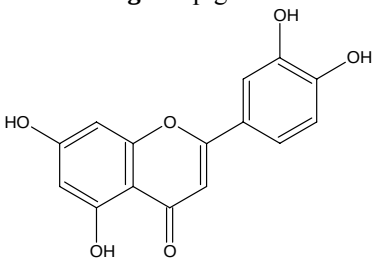

Fig.6. Luteolin

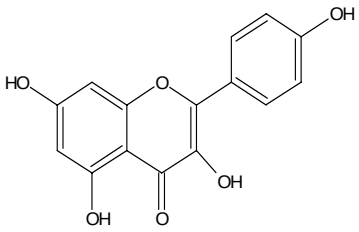

Fig.7. Kaempferol

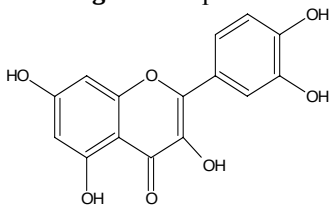

Fig. 8. Quercetin

Quercetin, a phytochemical belonging to the flavonoids, has antioxidant activities, inhibition of protein kinases (Davies *et al*., 2000) and DNA topoisomerases (Constantinou *et al*., 1995) regulate gene expression (Moon *et al*., 2003) and also modulate gene expression related to oxidative stress and in the antioxidant defence system (Moskaug *et al*.,2004). According to Van Wiel *et al*., (2001) and Tsanova- Savova and Ribarora (2002), the most common flavonoids in grape wine were flavonols (quercetin, kaempferol, and myricetin). Betes- Saura *et al*., (1996) detected quercetin, kaempferol in leaves and exocarps of grape *Vitis labruscana* cv. Kyoho and, *Vitis vinifera* L. fruits. *Lilium auratum, L. henryi, L.martagon, L. myrciphyllum* and *L. willmottiae. L. henryi, L. martagon, L. myrciphyllum, L. willmottiae, L. leichtlinii*.Apigenin and luteolin which were structurally elucidated from ^{13}C-NMR, where spectral data reported earlier (Loo *et al*., 1986; Wagner, 1976). These compounds were isolated from the roots of *Glossostemon bruguieri, Conyza bonariensis* (Meselhy, 2003; Kong *et al*., 2001). Luteolin was also isolated from the fruit of *Terminalia chebula (*Klika *et al*., 2004). So far anti-venom compounds isolated from plants include *β*-sitosterol, *β*-sitosterolglucoside, *β* - amyrin, kampferol and quercetin (Martz ,1992 ;Houghton *et al*.,1993; Abubakar *et al*.,2000; Reyes-Chilpa *et al*.,1994).t was concluded that the phytochemicals isolated from *Eulophia epidendraea* may appear to be a good resource of biologically active compounds.

Table- 1: ^{13}C- NMR ^{1}H- NMR data for lupeol (Aratanechemuge *et al*., 2004)

No.	^{13}NMR	^{1}H NMR
1.	38.7	1.65(1H,m),0.90(1H,m)
2.	27.4	1.59(1H,m),1.67(1H,m)
3.	79.0	3.20(1H,dd,J+5.03,11.5Hz)
4.	38.8	
5.	55.3	0.68(1H,m)
6.	18.3	1.40(1H,m),1.50(1H,m)
7.	34.3	1.32(1H,m),1.42(1H,m)
8.	40.8	
9.	50.4	1.29(1H,m)
10.	37.1	
11.	20.9	1.20(1H,m),1.40(1H,m)
12.	25.1	1.07(1H,m),1.68(1H,m)
13.	38.1	1.68(1H,m)
14.	42.8	
15.	27.4	1.00(1H,m)1.68(1H,m)
16.	35.6	1.37(1H,m) 1.48(1H,m)
17.	42.9	
18.	48.3	1.37(1H,m)
19.	47.9	2.38(1H,ddd,J=5.6,11.0,11.0Hz)
20.	150.9	
21.	29.8	1.37(1H,m),1.92(1H,m)
22.	39.9	1.37(1H,m),1.19(1H,m)
23.	27.9	0.97(3H,s)
24.	15.4	0.76(3H,s)
25.	16.1	0.83(3H,s)
26.	15.9	1.03(3H,s)
27.	14.5	0.94(3H,s)
28.	17.9	0.79(3H,s)
29.	109.3	4.54(1H,brs),4.67(1H,brs)
30.	19.3	1.68 (3H,s)

Table -2: Chromatoghapic identification of leaf phytochemicals

Spot No.	Mean Rf (x 100) in Forestal	Mean Rf (x 100) marker	Mean Rf (x 100) (Harborne ,1973)	Flavonoids	*Colour reactions*		
					Day light	UV-light	UV-ammonia
1.	-	79	83	Apigenin	Not visible	Ochre	Dull yellow
2.	22	29	-	Unknown1	Not visible	Light yellow	Bright yellow
3.	33	41	43	Unknown 2	Not visible	Light green	Olive green
4.	55	60	55	Kaempferol	Not visible	Yellow	Yellow
5.	66	60	66	Luteolin	Not visible	Ochre	Bright yellow
6.	38	44	41	Quercetin	Not visible	Yellow	Bright yellow
7.	72	78	-	Unknown3	Not visible	Yellow	Dark yellow
8.	68	-	-	Unknown 4	Not visible	Purple	Purple
9.	92	-	-	Unknown 5	Yellow	Bright yellow	Bright yellow

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