0971-4693/94 Euro 10.00
Allelopathy Journal 34 (1): 155-178 (2014) *International Allelopathy Foundation* 2014
Tables: 6, Figs : 19

Chemical characterization of antifungal constituents of *Emblica officinalis*

C. ARORA CHUGH[1*] and D. BHARTI

Department of Chemistry, Banasthali University, Rajasthan-304022, India.
E. Mail: charuarora77@gmail.com

(Received in revised form: July 11, 2014)

ABSTRACT

Hexane and methanol extracts of *Emblica officinalis* were screened for their antifungal activity against fungal pathogens [*Fusarium oxysporum* and *Rhizoctonia solani*] causing stem canker and potato wilt, respectively. Fractions collected from the methanol extract proved more effective against both test pathogens. Six compounds [(E) -oct-4-ene-1, 2, 3, 4, 5, 6, 7, 8-octanol; 3,4,5-trihydroxybenzoic acid; propyl 3, 4, 5-trihydroxybenzoate; 3, 5, 7, 3', 4'-penta-hydroxy flavone; 2,3,7,8-tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione; and 1,2-dihydroxyethyl]-3,4-dihydroxyfuran-2(5H)-one] were isolated and characterized from different bioactive fractions of methanol extract. Fraction 112 and 106 possessing 1,2-dihydroxyethyl-3,4-dihydroxyfuran-2(5H)-one and 2,3,7,8-tetrahydroxy- chromeno [5,4,3-cde] chromene - 5,10-dione proved most inhibitory (2 mg per disc) to *F. oxysporum* and *R. solani,* respectively. We observed that whole fraction exhibited more inhibition than pure constituent(s) isolated from the respective fraction(s). Perhaps this observation was synergistic effect of isolated bioactive constituent with other chemical constituents present in the fraction.

Key words: Antifungal potential; 1,2-dihydroxyethyl-3,4-dihydroxyfuran-2(5H)-one; *Emblica officinalis*; *Fusarium oxysporum; Rhizoctonia solani;* 2,3,7,8-tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione

INTRODUCTION

E. officinalis (euphorbiaceae family) is a deciduous tree used in Chinese herbal medicine, Tibetan medicine and Ayurvedic medicine (11, 12, 31). *E. officinalis* contains numerous constituents [alkaloids, benzenoid derivatives, diterpenes, furano-lactones, flavonoids and sterols] in various concentrations. It possesses medicinal properties [analgesic, antipyretic (13), anticancer (19), antioxidant (32), antivenom (23), antitussive (26), antimicrobial (28), antitumour (15), antiulcerogenic (29), hypoprotective (14), cytoprotective (17) and antidiarrheal (30)]. It helps in memory enhancing (36), curing ophthalmic disorders (7) and lowering cholesterol level (24). It is often used in the form of *Triphala,* which is an herbal formulation containing fruit of *E. officinalis*, *Terminalia chibula* and *Terminalia belerica* in equal proportion (32). However, its potential role for pests and pathogens control in crops is not explored. Few studies have been carried out to evaluate the bio efficacy of E. officinalis to control the bacterial and fungal pathogens of crop like potato (2-3).

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[*] Correspondence author.[1*] Department of Chemistry, Guru Ghasidas Central Univrsity, Koni, Bilaspur- 495009, Chattis Garh, India.

Therefore, this study aimed to standardize the procedures for isolation of active principles from potential plants collected from various locations in India and to develop microanalytical methods for estimation, identification of active principles and determine their bioefficacy against *Rhizoctonia solani* and *Fusarium oxysporum* causing black scurf and wilt of potato.

MATERIALS AND METHODS

I. Collection of Plant Material: The leaves samples of *E. officinalis* were collected in February 2009 from Herbal Garden, Banasthali University, Banasthali, Rajasthan. The sample (voucher number BVH-786313) was deposited in herbarium, Botany Division, Department of Bioscience and Biotechnology, Banasthali University. Plant material was dried in shade at 35ºC for 10 days. The shade dried leaves of *E. officinalis* were grinded in mixer, and stored in airtight containers.

II. Extraction: Three kg leaf power was extracted with organic solvents [hexane, chloroform and methanol] in increasing order of polarity using soxhlet apparatus. The extracts were concentrated separately in rotavapour and evaporated to dryness.

III. Collection of fungal strains: Two test fungal pathogens, *Rhizoctonia solani* and *Fusarium oxysporum* (causing scurf and wilt diseases of potato) were collected from Plant Pathology Laboratory, Central Potato Research Institute, Modipuram, Meerut. Both the fungal pathogens were grown on potato dextrose agar (PDA) medium and incubated at 28 ±1° C for 1 to 3 days depending on the growth of pathogens.

IV. Media preparation: To prepare Potato Dextrose Agar (PDA) 39 g potato dextrose agar of Hi-media make was dissolved in 1.0 L distilled water, the medium thus prepared is known as potato dextrose agar. It was sterilized in autoclave. PDA was used for growing fungal culture. To avoid bacterial contamination, streptomycin was added at 30 mg/l before pouring the medium to petridish.

V. Evaluation of Antifungal Activity: Paper disc method (4, 16) was used for initial screening of antifungal activity of plant extracts. This method is based on diffusion capacity of test chemical(s) through agar medium. Fungal plug were placed at the center of assay plate containing sterilized PDA and allowed to grow. After circular growth of about 2-3 cm diameter four sterilized paper disc (two loaded with 20µl extract/ fraction and two with same amount of solvent) were placed at equal distance from center in order to see the effect of plant extract on the growth of fungal pathogen. Inhibition zones were measured after 36 hrs. of incubation. Dumb bell shaped growth of fungus was observed in case of extracts containing growth inhibitory component(s).

Food poisoning technique (17) was used to find percent inhibition. For this purpose 50µl plant extract fraction was spread to each Petridish after pouring the sterilized medium, while in control treatment equal amount of pure solvent was added. The fungal plug was placed at the centre of Petridish. Growth of fungus was recorded after one to three days depending upon the growth of pathogen. The percent inhibition was calculated using the formula of Vincent (37).

$$\text{Percent Inhibition} = (C-T)/C \times 100$$

Where C is the growth in control in mm and T is growth in treatment in mm.

All the experiments were carried out in triplicate and average value was used for interpretation of results.

VI. Activity guided fractionation of hexane and methanol extracts of *E. officinalis* : Hexane and methanol extracts of *E. officinalis* were found to possess antifungal potential against the test pathogens. So these two extracts were subjected to column chromatography as under:

(i). The glass column (5 cm×100 cm) was packed with 350 g silica gel using hexane as solvent. Twenty g hexane extract mixed with 30 g silica gel to form the slurry was loaded on top of the column. Hundred fractions (75 ml each) were collected using solvent system in eluotropic series. Biocidal activities of collected fractions were studied and further activity guided fractionations were carried out. Active fractions were further purified by column chromatography and preparatory TLC using suitable solvent systems.

(ii). Methanol extract of *E. officinalis* was fractionated using column chromatography. The glass column (5cm × 100 cm) was packed with 350 g activated silica gel. Twenty six g methanol extract pre-absorbed in 30 g silica gel was quantitatively transferred to silica gel column. The column was eluted with 500 ml chloroform followed by 250 ml chloroform: ethyl acetate in the ratio of 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, ethyl acetate 100%; ethyl acetate: acetone, 80:20, 60:40, 40:60, 20:80, acetone 100% and acetone: methanol, 80:20, 60:40, 40:60. Finally the column was washed with 500 ml methanol. Bioactive fractions were further purified by column chromatography and preparatory TLC using suitable solvent systems. Biocidal activities of collected fractions were evaluated against *F. oxysporum* and *R. solani*. Further, activity guided fractionation was done with column chromatography and preparatory TLC using suitable solvent systems.

(iii). U.V.-VIS spectra were obtained on Shimadzu double beam spectrophotometer (UV-150-02). I.R. Spectra were recorded on Shimadzu and Perkin Elmer FTIR Spectrophotometer. Mass spectra were acquired in the positive ion mode on Waters (Q-Micromass) spectrophotometer. Electronic Balance of Shimadzu was used for weighing. Brukar Avance III (400 MHz ZH079807) instrument was used to record NMR spectra. ^{1}H-NMR, ^{13}C-NMR, HMBC, HMQC, IR, U.V.-VIS and mass spectral studies were carried out at Sophisticated Analytical Instrumentation Facility, Panjab University, Chandigarh and (CIL) Central Instrumentation Laboratories, Mohali, Chandigarh.

VII. STATISTICAL ANALYSIS

Correlation coefficient (r)

The correlation coefficient (r) was calculated using the following equation:

$$r = \frac{n\sum xy - \sum x\sum y}{\sqrt{\{[n\sum x^2 - (\sum x)^2][n\sum y^2 - (\sum y)^2]\}}}$$

Here n is the number of data points.

The limits of r are from+1 to-1 as follows:

(i) r = +1, perfect positive correlation, increase in one variable is accompanied by the increase in the other.

(ii) r = -1, perfect negative correlation, decrease in one variable is accompanied by the decrease in the other.

Coefficient of Determination (‘r^2’)

Although correlation coefficient is a good measure of the strength of the association, but it has got no literal interpretation. The squared values of r, r^2 called coefficient of determination, however have a very clear meaning. It gives the measure of the proportion of variation in one variable associated with variations in the other. For example, if the value of r = 0.8, then $r^2 = 0.64$. It means that 64% variations in the value of inhibition zones are associated with variation in plant extracts, the remaining 36% can be attributed to some other unknown factors. The value of r^2 ranges from 0 to 1.

Significance test

The significance test (t test) was calculated by the formula:

$$t = r\,\frac{\sqrt{(n-2)}}{\sqrt{1-r^2}}$$

Here, n is number of observations.

The observed value of ‘t’ is then compared with the critical value of t obtained for n-2 degrees of freedom at 5% significance level from the t distribution table.

RESULTS AND DISCUSSION

Antifungal Potential of Fractions of Hexane Extract

Bioactive constituents from the fractions of hexane extract were tested for antifungal activity (Table 1a). Fractions 8-9, 44 -48, 61-65, 86-89 and 97-100 possessed components having antifungal activity against both test fungal pathogens [*Rhizoctonia solani* and *Fusarium oxysporum*]. These fractions may contain broad spectrum fungicidal constituent(s) or more than one active constituent possessing antifungal activity against different test pathogens. Fractions 18-19, 30-34, 54-57 and 82-85 possessed constituent(s) having activity only against *R. solani*. Fraction 13-15, 20-21 and 69-72 exhibited activity only against *F. oxysporum*. These fractions contained specific fungicidal constituent(s).

Table 1a. Antifungal activity of fractions collected from hexane extract of *E. officinalis*

Fraction number	Solvent system used for elution	Fungal Pathogens			
		F. oxysporum		*R. solani*	
		Inhibition (%)	Inhibition zone (mm)	Inhibition (%)	Inhibition zone (mm)
8	Hexane: Chloroform 75:25	40	2	40	2
9		40	2	40	2
10-12		0	0	0	0
13		20	1	0	0
14		20	1	0	0
15	Hexane: Chloroform 50:50	20	1	0	0
16-17		0	0	0	0
18		0	0	36	3
19		0	0	38	3
20		40	2	0	0
21		40	3	0	0
22-29	Hexane: Chloroform 25:75	0	0	0	0
30		0	0	40	3
31	Chloroform100%	0	0	42	3
32		0	0	40	3
33		0	0	35	2
34		0	0	45	4
35-38		0	0	0	0
39-43	Chloroform: Ethyl acetate75:25	0	0	0	0
44		66	6	56	5
45		50	5	56	5
46		72	7	57	6
47	Chloroform: Ethyl acetate 50:50	55	5	55	5
48		59	6	57	5
49-53		0	0	0	0
54		0	0	58	5
55	Chloroform: Ethyl acetate 25:75	0	0	60	5
56		0	0	61	6
57		0	0	63	6
58-60		0	0	0	0
61		58	6	68	7
62		56	6	68	7
63	Ethyle acetate100%	62	7	67	7
64		59	6	60	6
65		58	6	64	6
66-68		0	0	0	0
69		67	6	0	0
70	Ethyl acetate: Acetone 75:25	68	6	0	0
71		56	5	0	0
72		69	7	0	0
73-78		0	0	0	0

79-81	Ethyl acetate: Acetone 50:50	0	0	0	0
82		0	0	63	6
83		0	0	65	6
84		0	0	61	6
85		0	0	67	6
86	Ethyl acetate: Acetone 25:75	55	5	59	5
87		57	5	55	5
88		58	6	68	7
89		58	6	65	6
90-94		0	0	0	0
95-96	Methanol 100%	0	0	0	0
97		65	6	61	6
98		65	6	60	6
99		66	6	65	6
100		68	7	69	7

Table 1b. Mean Statistical data for effect of fractions of methanol extract of *E. officinalis* on the fungal growth

Fungal pathogen	Correlation coefficient (‘r’)	Coefficient of determination (‘r^2’)	‘t’ (at 5% significance)
F. oxysporum	0.986	0.972	58.57
R. solani	0.989	0.978	66.41

Tabulated value of t =1.66 for degree of freedom 98 at 5% significance level.

The results were statistically analyzed and values of r^2 and ‘t’ in (Table-1b) indicated that 97.2 and 97.8% inhibition in *F. oxysporum* and *R. solani*, respectively, was caused by fractions of hexane extract of *E. officinalis* used, while rest 2.8%, and 2.2% was due to other unknown and uncontrolled factors. The calculations related to significance test (‘t’ test) revealed that ‘t’ values calculated for all fungal pathogens were much higher than critical value (1.66) noted from ‘t’ distribution table for 98 degree of freedom at 5% significance level. This suggested that there were less than 5% chances of error in drawing the conclusions. Hence the null hypothesis may be safely rejected at 5% significance level and the results were significant. Bioactive constituents present in hexane extract having potential antifungal activity against test pathogens could not be purified and characterized due to their insufficient quantity.

Antifungal Potential of Fractions of Methanol Extract

Methanol extract of *E. officinalis* exhibited better antifungal potential than hexane extract (Table-2a). Fraction numbers 16-31 eluted with chloroform: ethyl acetate showed 20 - 69% growth inhibition with inhibition zone of 1-7 mm against both test pathogens. Fraction 30 containing (E) -Oct-4-ene-1, 2, 3, 4, 5, 6, 7, 8-octanol as major constituent exhibited 45 and 57 % fungicidal potential against *F. oxysporum* and *R. solani,* respectively. Fraction numbers 32-42 showed significant antifungal potential (40-68%) against the test pathogens. Fraction numbers 43-46 did not possess bioactive constituents,

Table 2a. Antifungal activity of methanol extract of *E .officinalis*

Fraction no.	Solvent system used for elution	*F. oxysporum*		*R. solani*	
		Inhibition (%)	Inhibition zone (mm)	Inhibition (%)	Inhibition zone (mm)
1-6	Chloroform 100%	0	0	0	0
7-15	Chloroform: Ethyl acetate 9:1	0	0	0	0
16	Chloroform: Ethyl acetate 8:2	20	1	21	1
17		21	1	20	1
18		22	1	23	2
19		21	1	22	2
20		30	1	30	2
21		32	2	30	2
22	Chloroform: Ethyl acetate 7:3	33	2	32	2
23		30	2	40	3
24		34	2	39	3
25		37	2	41	3
26		37	3	40	3
27		36	3	43	4
28	Chloroform: Ethyl acetate 6:4	40	3	43	4
29		39	3	43	5
30		45	3	57	6
31		40	3	69	7
32		0	0	45	4
33		0	0	48	4
34		0	0	51	5
35	Chloroform: Ethyl acetate 5:5	43	3	54	5
36		40	3	45	4
37		45	4	49	5
38		49	4	49	5
39		50	4	50	5
40		52	5	52	6
41	Chloroform: Ethyl acetate 4:6	54	5	54	6
42		55	5	68	7
43-46		0	0	0	0
47	Chloroform: Ethyl acetate 3:7	65	6	69	8
48		59	5	69	8
49		69	7	0	0
50		55	5	0	0
51		69	6	0	0
52		0	0	0	0
53-55	Chloroform: Ethyl acetate 2:8	0	0	0	0
56		61	6	61	6
57		69	6	61	6
58		69	6	63	6
59		67	6	65	7
60	Ethyl acetate 100%	64	6	66	7
61		69	7	67	8
62		68	4	70	8
63		65	6	68	7
64		71	7	69	7
65		61	6	71	7

66	Ethyl acetate: Acetone 8:2	75	8	70	8
67		65	6	71	8
68		73	8	69	7
69		68	7	68	7
70		74	8	71	8
71		0	0	59	5
72	Ethyl acetate: Acetone 6:4	0	0	62	6
73		0	0	70	8
74		61	5	66	6
75		68	6	71	8
76		55	4	75	8
77		67	6	68	7
78	Ethyl acetate: Acetone 4:6	0	0	70	8
79		65	6	0	0
80		66	6	69	8
81		61	6	0	0
82		0	0	0	0
83		55	5	0	0
84	Ethyl acetate: Acetone 2:8	66	6	0	0
85		69	6	0	0
86		65	6	68	8
87		67	7	58	6
88		71	8	0	0
89		66	6	0	0
90		69	7	58	6
91	Acetone 100%	67	7	67	7
92		66	7	69	7
93		0	0	69	7
94		0	0	58	6
95		70	7	69	7
96	Acetone: Methanol 8:2	59	5	55	6
97		68	6	70	8
98		69	6	70	8
99		74	8	72	9
100		71	7	0	0
101		73	8	0	0
102		69	7	0	0
103		0	0	65	6
104	Acetone: Methanol 6:4	0	0	74	8
105		58	5	73	7
106		74	8	77	8
107		71	7	75	9
108		72	8	65	6
109	Acetone: Methanol 4:6	70	6	61	6
110		0	0	69	7
111		0	0	50	5
112	Methanol 100%	75	8	69	6
113		67	7	61	6
114		69	7	61	6
115		72	8	65	6

Table 2b. Mean Statistical data for the effects of fractions of methanol extract of *E. officinalis* on fungal growth

Fungal pathogen	Correlation coefficient ('r')	Coefficient of determination ('r^2')	't' (at 5% significance)
F. oxysporum	0.983	0.966	316.11
R. solani	0.949	0.900	31.97

Tabulated value of t = 1.645 for degree of freedom 113 at 5% significance level

as these fractions did not exhibit any activity against test pathogens. Fractions 47-51 possessed significant inhibitory potential (59-69%) against *F. oxysporum*. Fraction number 47 and 48 eluted with $CHCl_3$ possessed highly potent constituent(s) against *R. solani*. Fraction 56-70 exhibited significant antifungal potential against both test pathogens. These fractions may either possess broad spectrum antifungal constituent(s) or they may possess more than one active constituent having specific activity against the test pathogens. The results were subjected to statistical analysis and values of r^2 and 't' (Table-2b) indicated 96.6%, 90.0% inhibition of *F. oxysporum* and *R. solani,* respectively, was caused by fractions of methanol extract of *E. officinalis*, while rest 3.4%, and 10.0% was due to other unknown and uncontrolled factors. The calculations for significance test ('t' test) revealed that the calculated 't' for both fungal pathogens were much higher than critical value (1.645) noted from 't' distribution table for 113 degree of freedom at 5% significance level. This suggested that there are < 5% chances of error in drawing the conclusions. Hence the null hypothesis may be safely rejected at 5% significance level and the results were significant. Fraction number 60 eluted with 100% ethyl acetate possessed 3,4,5-trihydroxybenzoic acid as major constituent showing 64 and 66% growth inhibition against *F. oxysporum,* while, *R. solani* respectively. Fraction numbers 71-72 did not show growth inhibition in *F. oxysporum* but caused 66-71% growth inhibition in *R. solani*. Fraction numbers 74-77 and 79-81 showed significant inhibition against *F. oxysporum*. Fraction number 80, possessing 3,4,5-trihydroxybenzoic acid and propyl 3, 4, 5-trihydroxybenzoate as major constituents, showed 66-69 % growth inhibition against *F. oxysporum* and *R. solani*. The 3, 5, 7, 3', 4'-penta-hydroxy flavone was isolated as major constituent from fraction number 87. This fraction exhibited significant activity against both of test pathogens. The 2,3,7,8-tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione and 1,2-dihydroxyethyl]-3,4-dihydroxyfuran-2(5H)-one (eluted from acetone: methanol 6:4 and methanol 100% respectively) isolated from fraction no 106 and 112, respectively, have exhibited significant antifungal potential (69-77 %).

Identification of compounds isolated from Methnol Extract

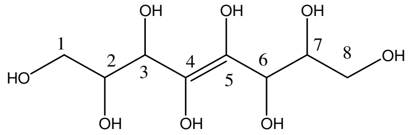
(I). Identification of EO-1 separated from fraction number 30 of methanol extract

The compound EO-1 was isolated as amorphous white powder. It was isolated from fraction number 30 eluted with chloroform: ethyl acetate in 6:4 ratio of methanol extract of *E. officinalis*. Compound EO-1 gives positive test for alcoholic group and unsaturation (3).

- **(i)** **U.V. visible spectrum**: The U.V. visible spectrum of this compound recorded in methanol showed λ_{max} at 224 and 213 nm for conjugate double bond.
- **(ii)** **I.R.**: I.R. (KBr cm^{-1}) of this compound reveals bands at 3423.2 ($OH_{stretch}$), 2974.0 cm^{-1}($C\text{-}H_{stretch}$), 1733.9 cm^{-1} (C=O), 1602.0(OH), 1249.5(C-O), and 1069 (C-C).
- **(iii)** **Mass Spectrum:** The mass spectrum of this compound has an M+1 peak at m/z 225 corresponding to the molecular formula $C_8H_{16}O_7$.
- **(iv)** **^{1}H- N.M.R.**: ^{1}H- N.M.R. of this compound recorded in MeOD (Figure 1) showed doublet at $\delta_H = 3.91$ ppm with coupling constant 2.68 Hz and multiplet at $\delta = 3.58$ to 3.70 ppm. The former was assigned to proton H-3 and H-6, while the later was observed due to broadening of splitting peaks of H-1, H-2, H-7 and H-8 and their merger caused by the virtual coupling of protons of –CH and -CH_2. This was expected because the value of coupling constants of protons of –CH_2 groups was greater than the difference in their δ values of H-1 and H-2, and H-7 and H-8.
- **(v)** **^{13}C-NMR:**The chemical shifts in ^{13}C-N.M.R. spectrum (Figure 2) of compound EO-1 gave four signals. The signal resonating at δ 150 was assigned to C-4 and C-5 which are quaternary in nature and attached with oxygen, δ 72.07 was assigned to two carbons C-2 and C-7 which were oxy-methine, δ 70.23 was assigned for C-3 and C-6 which were oxy-methine and two methylene carbons at 60.69 were assigned for C-1 and C-8.

Based on the spectral studies, compound EO-01 was characterized as (E) -oct-4-ene-1,2,3,4,5,6,7,8-octanol with molecular formula $C_8H_{16}O_7$ and molecular weigh 224.

To the best of our knowledge, this compound was reported for first time from the leaves of *E. officinalis*.



(E) -Oct-4-ene-1, 2, 3, 4, 5, 6, 7, 8-octanol (EO-1)

(II). Identification of EO-2 separated from fraction numbers 60-64 of methanol extract

The compound isolated was white powder and the melting point was 250°C, which is in good agreement with values reported in literature (10, 25). The compound EO-2 gave positive test for acidic and phenolic groups (1).

- **(i)** **U.V. visible spectrum**: U.V. visible spectrum of this compound gives λ_{max} at 270 and 224 nm signifying the presence of conjugation in compound.
- **(ii)** **I.R.**: The I.R. (in KBr, cm^{-1}) bands were observed at 3278.8 ($OH_{stretch}$) 3064 cm^{-1} ($C\text{-}H_{stretch}$) 1704.7 cm^{-1} (C=O), 1618.3 cm^{-1} (OH), 1540.4 cm^{-1} (C=C), 1245.1 cm^{-1} (C-O), and 1025 cm^{-1} (C-C).
- **(iii)** **Mass Spectrum:** The mass spectrum of this compound gives molecular ion peak of M+1 at m/z 171, which showed the molecular weight of compound EO-2 to be 170 corresponding to molecular formula C_7H_6O.

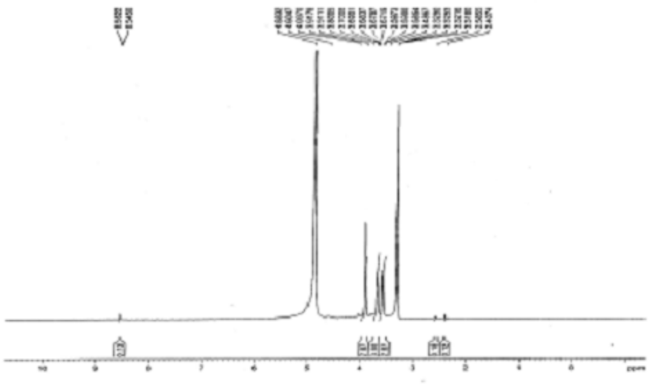


Figure 1. **H-N.M.R.** spectrum of EO-1 isolated from methanol extract of ***E. officinalis***

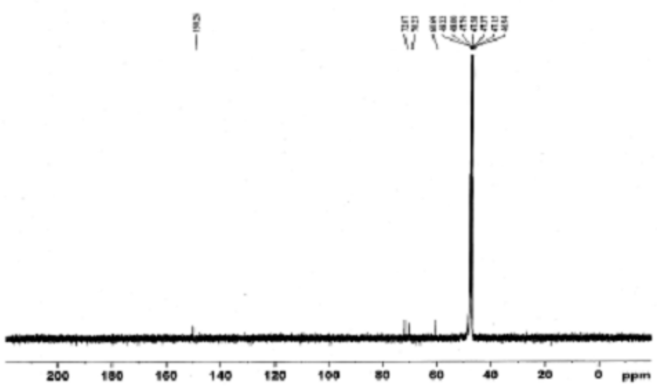


Figure 2. C-13 N.M.R. spectrum of compound EO-1 isolated from methanol extract of ***E. officinalis***

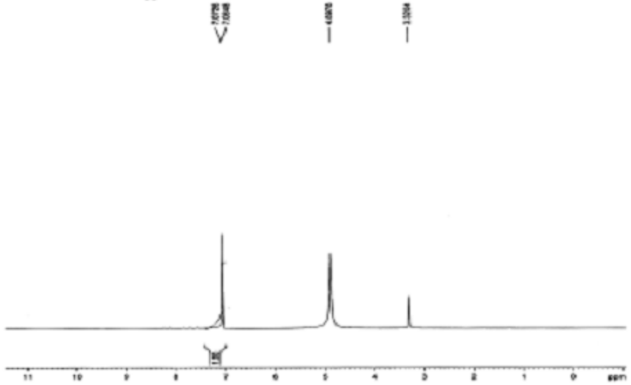


Figure 3. **H-N.M.R.** spectrum of compound EO-2 isolated from methanol extract of ***E. officinalis***

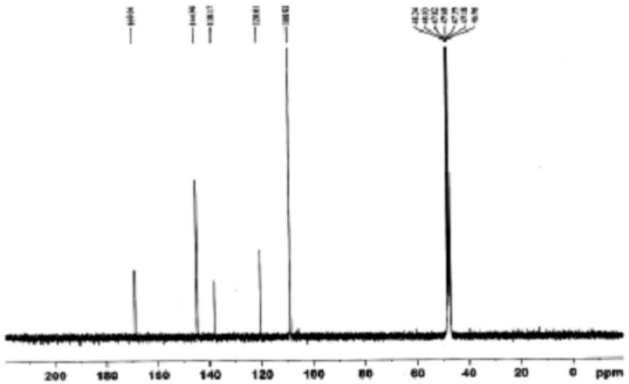
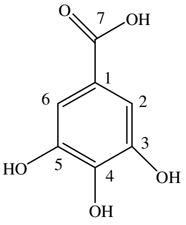


Figure **4**. C-13 N.M.R. spectrum of compound EO-2 isolated from methanol extract of ***E. officinalis***

(iv) **^{1}H- N.M.R.:** ^{1}H-N.M.R. spectrum of this compound in MeOD (Figure 3) has a peak at δ_H 7.06 indicating the presence of aromatic protons and was assigned for H-2, H-6 with coupling constant J=3.12 Hz, indicating meta coupling of these two protons which was in good agreement with the value reported in literature (25).

(v) **^{13}C- N.M.R.**: ^{13}C- N.M.R. of this compound (Figure 4) recorded in MeOD, showed presence of 7-carbons in molecule. Signal observed at δ_c 169.01 was assigned to carbonyl carbon (C7) one carbon signal at 120.61 was quaternary carbon assigned to C-1, signal at δ 108.93 assigned for C2, C6 carbons, two hydroxyl carbons at δ 144.98 was assigned for C3, C5 and one oxy-quaternary carbon at 138.17 was assigned for C-4.



3,4,5-trihydroxybenzoic acid (EO-2)

Based in the above spectral data and by comparing the spectral and physicochemical data with that reported in literature (25) the compound EO-2 was characterized as 3, 4, 5- trihydroxybenzoic acid. The 3,4,5 trihydroxybenzoic acid was major component of methanol extract of *E. officinalis*. It was present in fraction no 60-80 along with other constituents.

(III). Identification of compound EO-3 isolated from fraction-80 of methanol extract

Two chemical constituents namely 3, 4, 5- triyhydroxybenzoic acid (EO-2) and propyl 3, 4, 5-trihydroxybebzoate (EO-3) with molecular formula $C_7H_6O_5$ and $C_{10}H_{12}O_5$, respectively, could be isolated from fraction 80 based on following studies.

Compound EO-3 was isolated as creamy yellow crystals and its melting point was 150 °C, which was in good agreement with values reported in literature (33). The compound gives positive test for phenolic and acidic groups (1).

(i) **U.V. visible spectrum**: U.V. visible spectrum showed λ_{max} at 273 and 224 nm indicating the presence of aromatic ring and carbonyl group.

(ii) **I.R.**: The I.R. spectrum (in KBr, cm^{-1}) showed bands at (i) 3369.1 and 3287.7 (s, broad) which was characteristic of OH-stretching (ii) 2600 and 3100 (m-s), characteristic of C-H stretching (iii) 1700.3(s) characteristic of C=O of ester group (iv) 1450.3 (m) due to C-H deformation (v) 1336.6 and 1259.9 were the expected bands of ester group (vi) 1201.8 was a moderate band due to C-O stretching (vii) 868.6, 910.7, 957.8 and 791.7(w) were assigned to C-C stretching. Two strong bands appearing in the range of 1380-1050 cm^{-1} were characteristic of ester group.

(iii) **Mass Spectrum:** Mass spectrum showed M+1 peak at m/z=213, M-15 at 197 and M- 29 at 183 corresponding to the molecular formula $C_{10}H_{12}O_5$.

(iv) ^{1}H- N.M.R.: ^{1}H-N.M.R spectrum (Figures 5,6) of this component was recorded in MeOD solvent. The ^{1}H- N.M.R. signals were observed at δ_H 1.23 (t), 2.02, 4.11 and 7.07 ppm. The signal resonating at δ_H 1.23 ppm was assigned to methyl protons attached to C-10. ^{1}H-N.M.R. signals at 2.02 and 4.11 ppm were assigned to methylene protons attached to C-9 and C-8 respectively, while signals of aromatic protons attached to C-2 and C-6 were observed at 7.07 ppm. These signals were merged with the signals of aromatic protons of EO-2.

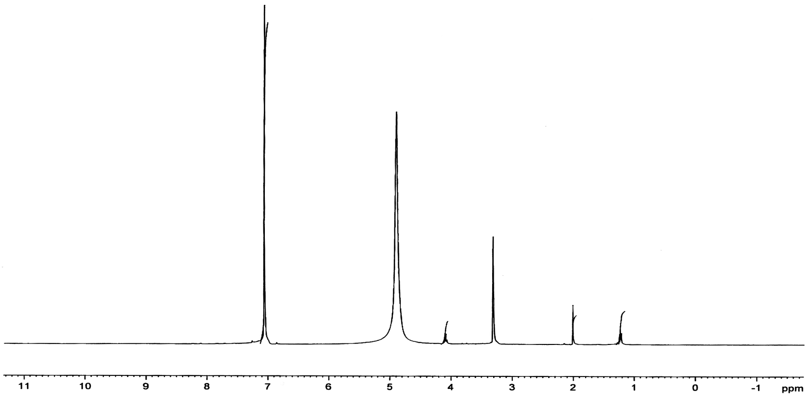


Figure 5. H-N.M.R. spectrum of mixture of EO-2 and EO-3 isolated from methanol extract of *E. officinalis.*

(v) ^{13}C- N.M.R.: C-13 N.M.R. of compound EO-03 (Figure 7) showed total eight signals. The signal appeared at δ 120 is assigned for C-1, the signal at 108.96 for two carbons, was assigned for C-2 and C-6, signal resonating at 144.98 was also for two carbons, assigned for C-3, C-5, signal appeared at 138.17 for C-4 and 169.01 for C-7. The signal appeared at δ 66.02 was of methylene carbon which is assigned for C-8. The 13C-NMR data also shows one peak at 22.02 for C-9 and one peak at 10.01 which was methyl proton assigned for C-10 (Table 3).

Table 3. NMR spectral data of EO-3

Position	$\delta_{H}(J_{HZ})$ (ppm)	δ_{C}(ppm)	HMBC(^{1}H→^{13}C)	HMQC(^{1}H→^{13}C)
1		120		
2	7.07	108.96	C-6,C-1, C-4, C-3, C-7	C-2
3		144.98		
4		138.17		
5		144.98		
6	7.07	108.96	C-2, C-1, C-4, C-5, C-7	C-6
7		169.01		
8	4.11	66.02		
9	2.02	22.02		
10	1.23	10.01		

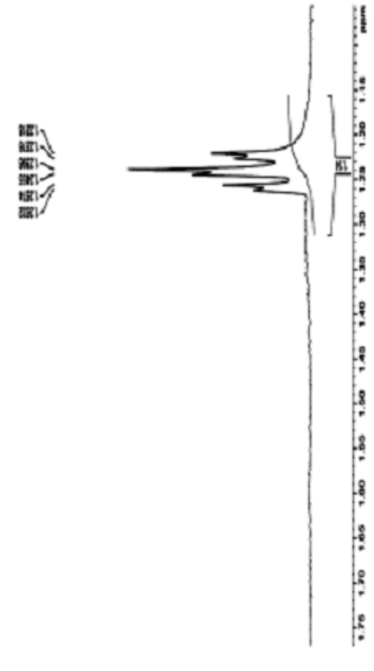


Figure 6. **H-N.M.R.** spectrum of compound EO-3 isolated from methanol extract of ***E. officinalis***

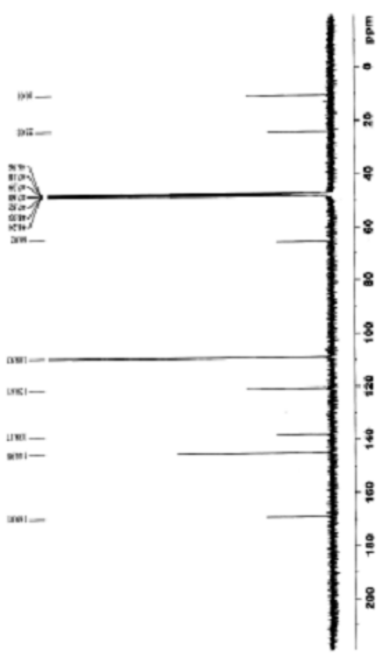


Figure 7. C-13 N.M.R. spectrum of compound EO-3 isolated from methanol extract of ***E. officinalis***

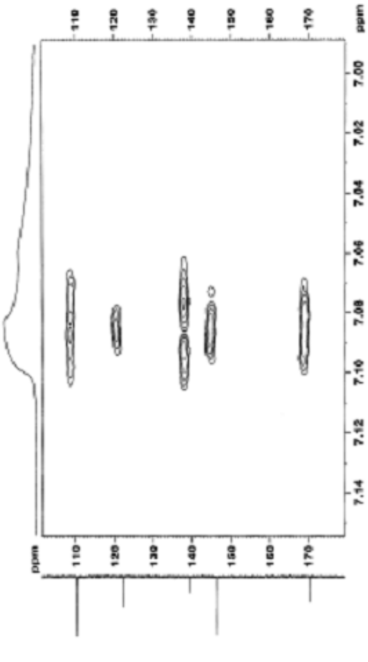


Figure 8. **HMBC** spectrum of compound EO-3 isolated from methanol extract of *E. officinalis*

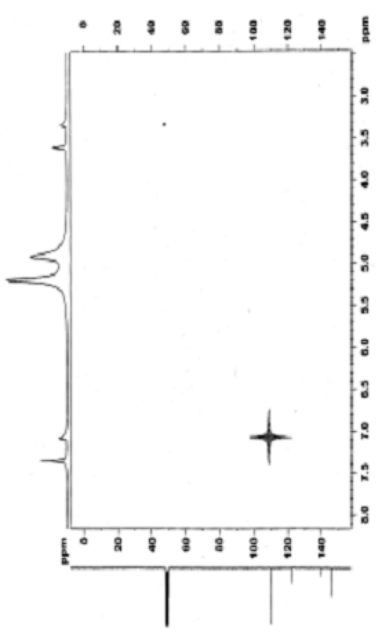
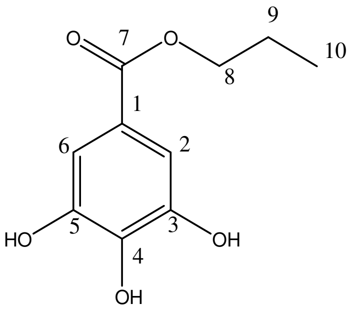


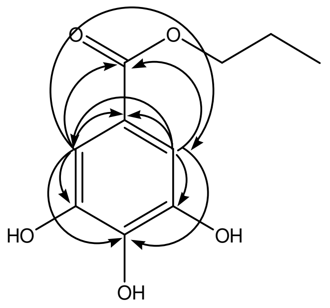
Figure 9. **HMQC** spectrum of compound EO-3 isolated from methanol extract of *E. officinalis*

(vi) **2D N.M.R.:** Proton attached to C-2 show three bond HMBC correlations (Figure 8) with C-4, C-6 and C-7 and two bond correlations with C-1 and C-3. Similarly proton attached to C-6 showed three bond correlations with C-2, C-4 and C-7; and two bond correlations with C-1 and C-5. In HMQC spectrum (Figure 9) proton attached to C-2 appeared at 7.07 ppm showing correlation with C-2 (108.96 ppm), while proton attached to C-6 (7.07 ppm) showed correlation with signal of C-6 appearing at 108.96 ppm.

Based on the above spectroscopic analysis, the structure of compound EO-3 was deduced as propyl 3,4,5-trihydroxybenzoate. To the best of our knowledge presence of propyl 3, 4, 5-trihydroxybenzoate was reported for the first time in leaves of *E. officinalis.* Based on the above observations the compound was assigned the following structure.



Propyl 3, 4, 5-trihydroxybenzoate (EO-3)



Key HMBC Correlations in EO-3

(IV). Identification of compound EO-4 isolated from fraction 87 of methanol extract

The compound (EO-4) isolated was brown coloured powder. It was eluted with solvent system ethyl acetate: acetone (2:8). Its melting point was 316°C, which was in good agreement with value reported in literature (9,25). The compound gives positive test for phenolic group (1).

(i) **U.V. visible spectrum**: λ_{max} of this compound was observed at 370 and 271 nm showed the presence of conjugation in compound and was in good agreement with values reported in literature (8).

(ii) **I.R.**: I.R. bands (KBr cm^{-1}) have been assigned as 3296 cm^{-1} (OH $_{stretch}$), 1657 (C=O), 1620 cm^{-1}, 1611 cm^{-1} (OH), 1559, 1514 cm^{-1} (C=C), 1363, 1319, 1250 cm^{-1} C-O), 1169(C=C).

(iii) **Mass Spectrum:** The ESI MS exhibited $[M+H]^+$ and [M+Na] peaks at m/z 303 and 325 respectively, corresponding to molecular formula $C_{15}H_{10}O_7$. Signals corresponding to [M-CO], $[M-CO_2]$ and $C_7H_8O_2$ were observed at 273, 257 and 151 respectively.

(iv) **^{1}H- N.M.R.:** ^{1}H N.M.R. spectrum of compound EO-04 (Table 4) showed signals at δ_H 5.97 (1H) and 6.18 (1H) for protons at C-6 and C-8 position respectively. Another signal of one proton at δ_H 7.52 assigned to proton at C-2' and a doublet of one protons at δ_H 7.42 with coupling constant 8.32 Hz and one multiplet at δ_H 6.68 with coupling constant 4.04 and 3.8 assigned to protons at C-6'and 5' respectively. Signal

Table 4. NMR spectral data of EO-4

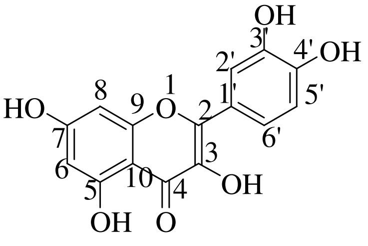
Position	δ $_H$(J $_{HZ}$) (ppm)	δ $_C$(ppm)	HMBC($^1H \rightarrow ^{13}C$)	HMQC ($^1H \rightarrow ^{13}C$)	COSY
2		146.57			
3		135.81			
4		175.90			
5		161.08			
6	5.97 (1H)	97.81	C-8,C-10, C-5, C-7	C-6	
7		164.14			
8	6.18 (1H)	92.99	C-6,C-10, C-9, C-7	C-8	
9		156.80			
10		103.10			
1’		122.73			
2’	7.52(1H)	114.58	C-2C-4’,C-6’	C-2’	
3’		144.80			
4’		147.35			
5’	6.68 (1H, dd)	114.81	C-1’,C-3’	C-5’	H5’→H6’
6’	7.42(d, J=8.32)	120.26	C-2,C-2’	C-6’	

at δ_H 7.42 showed COSY- correlation with the proton resonating at δ_H 6.68, so these two protons were ortho coupled. A singlet at δ_H 12.5 assigned to chelated hydroxy group at C-5. The absence of singlet around δ_H 6.30 indicated that the C-3 position was substituted.

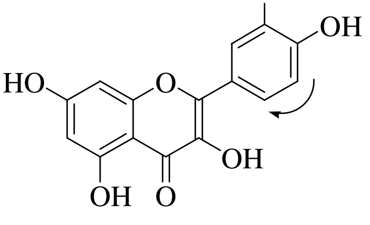
(v) **^{13}C- N.M.R.:** ^{13}C NMR spectrum (Table 4, Figure 10) showed ten quaternary carbons, signal at δ_C 175.90 for C-4 carbonyl group, at δ_C 122.73 and 103.10 ppm assigned to C-1' and C-10**;** at δ_C 146.57, 135.81, 161.08, 164.14, 156.80, 144.80 and 147.35 for hydroxylated carbons assigned to C-2, C-3, C-5, C-7, C-9, C-3' and C-4' respectively. This data indicated presence of *ortho* dihydroxy system at position C-3', 4'. Five aromatic methine at δ_C 97.81, 92.99, 114.58, 114.81 and 120.26 were assigned to C-6, C-8, C-2', C-5' and C-6' respectively.

(vi) **2D N.M.R.:** Key HMBC (figure-11) HMQC (Figure 12) and COSY correlations (figure-13) have been reported in Table 4.

Finally the structure of EO-4 was confirmed as 3, 5, 7, 3', 4'-penta-hydroxy flavone commonly known as quercetin by comparing the spectral and physicochemical data with that reported in literature (17, 32).



3, 5, 7, 3', 4'-penta-hydroxy flavone (EO-4)



COSY correlation of EO-4

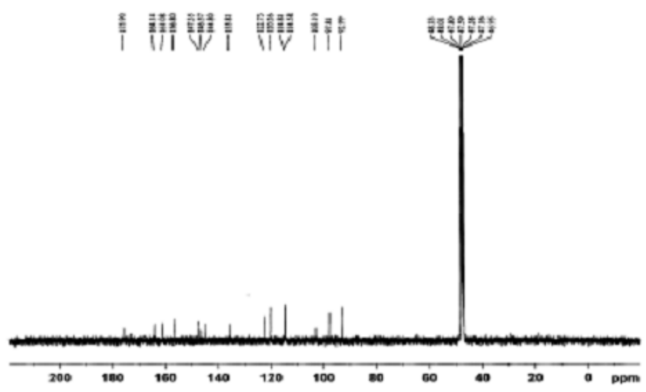


Figure 10. C-13 N.M.R. spectrum of compound EO-4 isolated from methanol extract of *E. officinalis*

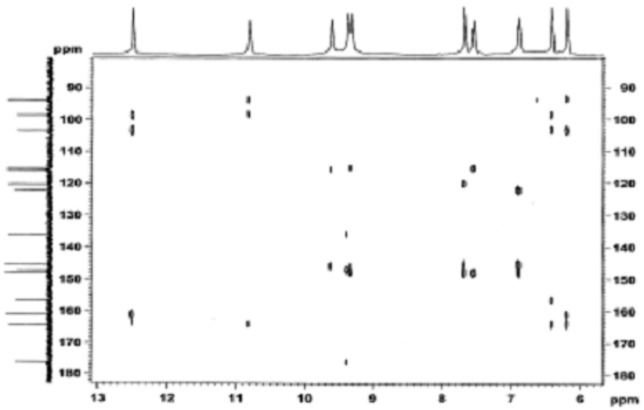


Figure 11. HMBC spectrum of compound EO-4 isolated from methanol extract of *E. officinalis*

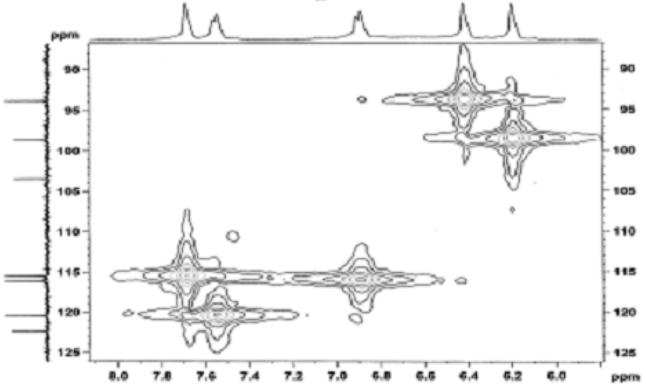


Figure 12. HMQC spectrum of compound EO-4 isolated from methanol extract of ***E. officinalis***

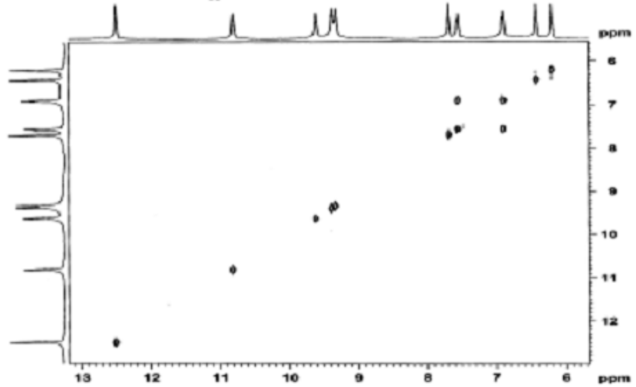


Figure 13. COSY spectrum of compound EO-4 isolated from methanol extract of ***E. officinalis***

(V). Identification of compound EO-5 isolated from fraction-106 of methanol extract

The compound EO-5 was isolated as dark brown coloured powder. It was eluted with solvent system acetone: methanol (6:4). Its melting point is 347 °C, it was in good agreement with reported values in literature (21). The compound EO-5 gave positive test for phenolic group (1).

(i) **U.V. visible spectrum**: λ_{max} was recorded at 370 and 271 nm in U.V visible spectrum of this compound indicating the presence of long conjugation in the compound.

(ii) **I.R.**: The I.R. spectrum (in KBr) showed bands at 3117 cm^{-1} (OH $_{stretch}$), 1669 cm^{-1}(C=O), 1620 cm^{-1} (C=C) and 1586, 1505, 1443, 1349(C-O).

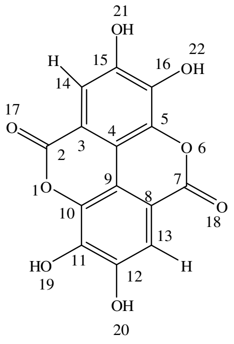
(iii) **Mass Spectrum:** The compound gave $[M+1]^+$ peak at m/z 303.4, and [M-28] at m/z 274.3.

(iv) **^{1}H- N.M.R.:** ^{1}HNMR spectra of compound EO-05 showed three peaks. One proton resonating at 7.46 was assigned for two aromatic protons and two protons at 7.78 and 8.58 were assigned for four hydroxy protons.

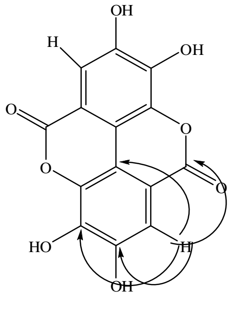
(v) **^{13}C- N.M.R.:** Total of 7 peaks appeared in ^{13}C-NMR spectrum of the compound. Signals resonating at δ_C 159.08, 148.07 and 139.50 were assigned for six oxygenated quaternary carbons, whereas δ_C 107.65, 112.27 and 136.35 were assigned to non-oxygenated quaternary carbons. One carbon resonating at 110.23 was assigned for aromatic methines.

(vi) **2D N.M.R.:** The final structure of the compound was assigned with the help of HMBC (Figure-14) and HMQC spectra (Figure 15).In HMQC spectrum the proton at δ 7.46 showed correlation with δ 110.23, which indicated that the proton resonating at δ 7.46 was attached with the carbon resonating at δ 110.23. The proton appeared at δ 7.46 exhibited HMBC-correlation with the carbon resonating at δ 159.08 (C-7), 112.27 (C-9), 139.50 (C-11), 148.07 (C-12). The mass of compound was 302 with molecular formula $C_{14}H_6O_8$ but 1HNMR showed only one peak and ^{13}C-N.M.R. showed only seven peaks indicating the dimeric nature of this compound. C-13 N.M.R., HMBC, HMQC and COSY assignments have been reported in Table 5.

Thus, analysis of Mass, COSY, HMQC and HMBC data confirmed that the compound separated from the leaves of *E. officinalis* was 2,3,7,8-tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione.



2,3,7,8-tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione (EO-5)



Key HMBC correlations

Table 5. NMR spectral data of EO-5

Position	δ_H(J $_{HZ}$) (ppm)	δ_C (ppm)	HMBC($^1H \rightarrow ^{13}C$)	HMQC($^1H \rightarrow ^{13}C$)
2		159.08		
3		107.65		
4		112.27		
5		124.17		
6				
7		159.08		
8		107.65		
9		112.27		
10		136.35		
11		139.50		
12		148.07		
13	7.46	110.23	C-9,C-11, C-12,C-7	C-13
14	7.46	110.23	C-4, C-16, C-15, C-2	C-14
15		148.07		
16		139.50		
17				
18				
19	8.58		C-10	
20	7.78			
21	7.78			
22	8.58		C-5	

(VI). Identification of compound EO-6 isolated from fraction 112 of methanol extract

Compound EO-6 was eluted with 100% methanol and was isolated in form of white powder, its melting point was 191 °C. The compound gives positive test for phenolic group (1).

(i) **U.V. visible spectrum**: U.V. visible spectrum of this compound showed λ_{max} at 245 which confirmed the presence of conjugation in compound and was in good agreement with reported values (22).

(ii) **I.R.**: IR (KBr cm^{-1}) showed signals at 3217-2915 cm^{-1} some overlapping signals from C-H stretching near the high frequency end the region such as the sharp peak at 2915 cm^{-1}, 1668-1656 cm^{-1}(C=C), 1457 cm^{-1} (CH_2) scissoring.

(iii) **Mass Spectrum:** The mass spectrum of this compound gave M+1 peak at m/z=177, M-28 Peaks at m/z=148 corresponding to the molecular formula $C_6H_8O_6$.

(iv) **^{1}H- N.M.R.:** ^{1}H-N.M.R. spectrum of this component recorded in D_2O solvent showed three signals at δ_H 3.89 (methine proton H-1'), δ 3.56 (methylene protons H-2'), δ 4.77 (H-5 J=1.76 Hz). All these protons were attached with oxygenated carbons. Signals corresponding to hydroxy protons could not be observed as the spectrum was recorded in D_2O.

(v) **^{13}C- N.M.R.:** ^{13}C- N.M.R. of EO-6, recorded in DMSO (Figure-16) showed six signals. Signal appearing at δ_C 62.40 was assigned to methylene carbon C-2', 68.82 for methine carbon C-1', 75.28 to C-5, 118.39 for C-3, 153.41 for C-4 and signal at δ_C 171.14 was assigned to carbonyl carbon C-2.

C-13 N.M.R. Based on the above observations the structure of EO-6 was as under.

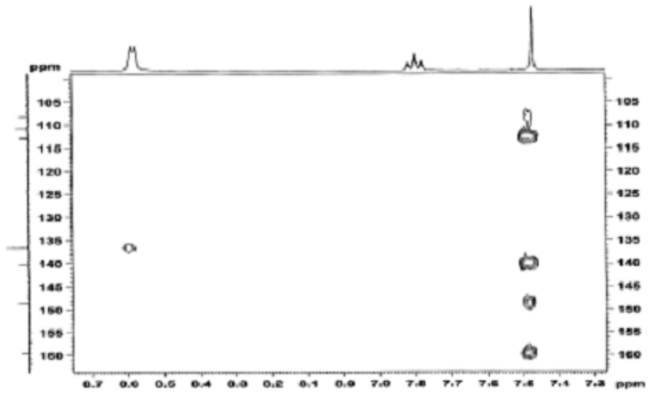


Figure 14. HMBC spectrum of compound EO-5 isolated from methanol extract of *E. officinalis*

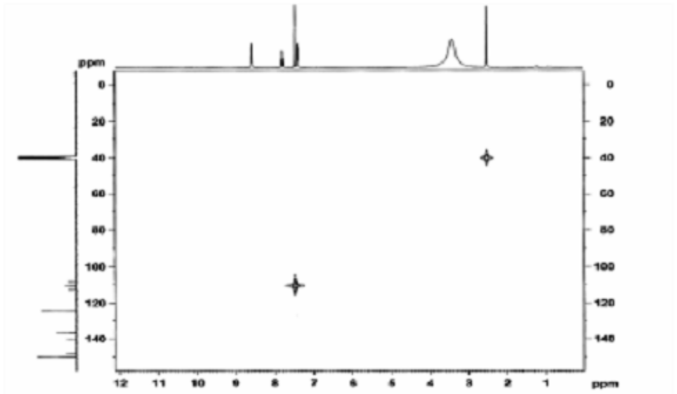


Figure 15. HMQC spectrum of compound EO-5 isolated from methanol extract of *E. officinalis*

Table 6. Antifungal activity of compound isolated **form** methanol extract of ***E. officinalis***

Fraction no.	Extract	Compound separated	*F. oxysporum*		*R. solani*	
			Inhibition (%) with fraction	Inhibition (%) with separated pure compound	Inhibition (%) with fraction	Inhibition (%) with separated pure compound
30	Methanol	(E)-oct-4-ene-1,2,3,4,5,6,7,8-octanol	45	NT	57	NT
60	Methanol	3,4,5-trihydroxybenzoic acid	64	59	66	52
80	Methanol	Propyl 3, 4, 5-trihydroxybenzoate	66	59	69	52
87	Methanol	3, 5, 7, 3', 4'-penta-hydroxy flavone	67	60	58	56
106	Methanol	2,3,7,8-tetrahydroxy-chromeno [5,4,3-cde]chromene-5,10-dione	74	65	77	69
112	Methanol	1,2-dihydroxyethyl]-3,4-dihydroxyfuran-2(5H)-one	75	68	69	59

NT: Not tested **due** to insufficient quantity

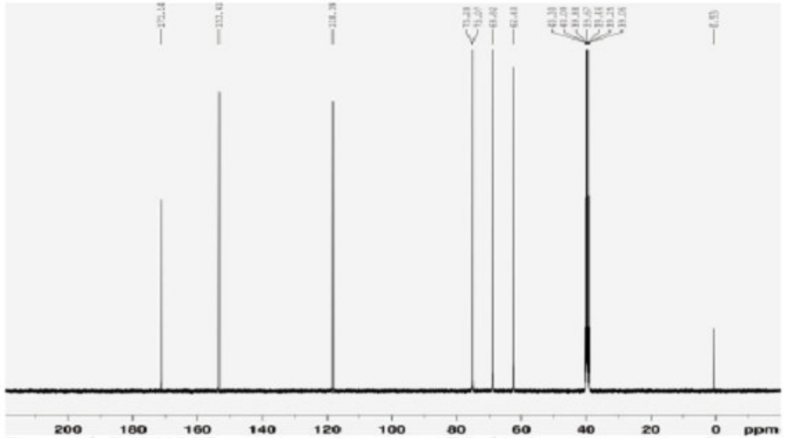


Figure 16. C-13 N.M.R. spectrum of compound EO-6 isolated from methanol extract of ***E. officinalis***

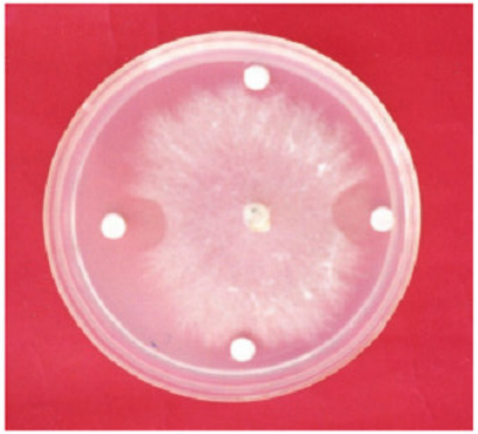


Figure 17. Effect of EO-2 isolated from fraction 60 of methanol extract of ***E. officinalis*** on growth of ***R. solani***

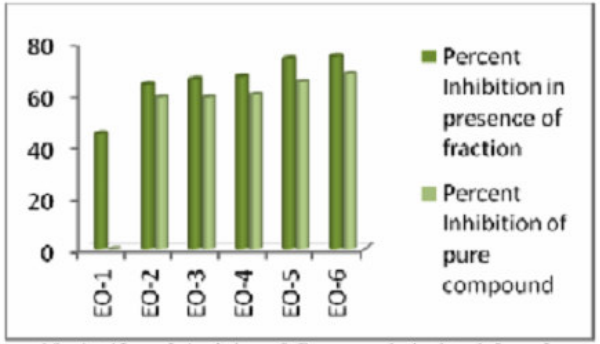


Figure 18. Antifungal Activity of Compounds isolated from leaves of *E. officinalis* against ***Fusarium oxysporum***

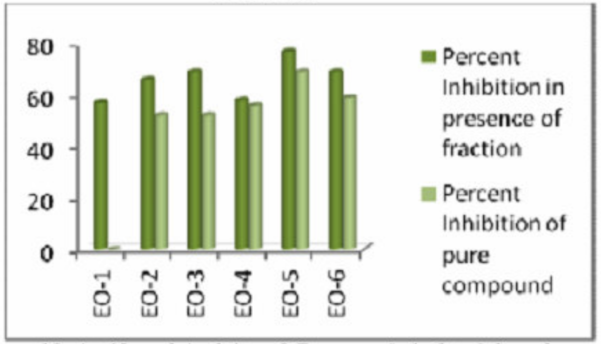
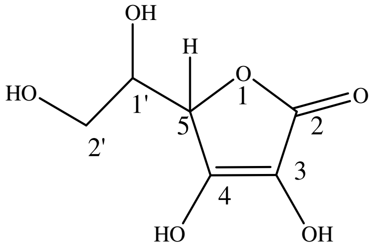


Figure 19. Antifungal Activity of Compounds isolated from leaves of *E. officinalis* against ***Rhizoctonia solani***



3,4-dihydroxy-5-[1,2-dihydroxyethyl]furan-2(5H)-one (EO-6)

Antifungal activity of pure compounds isolated from methanol extract

Activity of pure compound isolated from the fraction number 30, ((E)-oct-4-ene-1,2,3,4,5,6,7,8-octanol) were not determined due to insufficient quantity of isolated compound. The 3, 4, 5- trihydroxybenzoic acid isolated from fraction no 60 had shown 59 and 52% inhibition against test fungi *F. oxysporum* and *R. solani* (Figure 17) respectively, while 66 and 64% growth inhibition was recorded in presence of their respective fractions (Figure 18 and 19). Similar observations were observed for other compounds isolated from the plant. The whole fraction showed greater inhibition than pure constituent(s) isolated from the respective fraction(s). The probable reason for this observation may be the presence of other bioactive constituent(s) in the respective fraction alongwith the isolated major compound or synergistic effect of isolated bioactive constituent with other chemical constituents present in the fraction (Table 6, Figures 18 & 19). 2,3,7,8-Tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione and 1,2-dihydroxyethyl]-3,4-dihydroxyfuran-2(5H)-one (eluted from acetone: methanol 6:4 and methanol 100% respectively) isolated from fraction no 106 and 112 respectively have exhibited highest antifungal potential against the test pathogens.

CONCLUSIONS

Antifungal activity of methanol extract was higher than hexane extract. Six chemical constituents namely (E)-oct-4-ene-1,2,3,4,5,6,7,8-octanol; 3, 4, 5- trihydroxy benzoic acid; propyl 3,4,5-trihydroxybenzoate; 3,5,7,3’,4’-pentahydroxyflavon; 2,3,7,8-Tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione and 1,2-dihydroxyethyl]-3,4-dihydroxyfuran-2(5H)-one exhibiting significant antifungal potential were isolated from fractions of methanol extract of *E. officinalis*,. In this study, presence of (E)-oct-4-ene-1,2,3,4,5,6,7,8-octanol and has been reported for the first time in leaves of *E. officinalis*. All 6-constituents isolated from potential fractions, possessed hydroxy groups. 2,3,7,8-Tetrahy-droxy-chromeno[5,4,3-cde]chromene-5,10-dione and 1,2-dihydroxyethyl]-3,4-dihydroxyfuran-2(5H)-one (eluted from acetone: methanol 6:4 and methanol 100% respectively) isolated from the fraction no 106 and 112, respectively, showed highest antifungal potential against the test pathogens. These studies will help in development of plant based antifungal formulations for sustainable agriculture.

ACKNOWLEDGEMENTS

Authors are thankful to University Grants Commission, New Delhi for providing financial support F. No. 34-346/2008(SR) and Joint Director, Central Potato Research Institute, Modipuram, Meerut for providing fungal cultures.

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