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Isolation and bioactivity of diacetyltetritol from *Merremia emarginata* (Burm.f)

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ABSTRACT

Diacetyltetritol (1), a new tetritol derivative was isolated from the ethyl acetate extractives of the whole plant, *Merremia emarginata (Burm.f).* Scopoletin (2), tetritol (3) and cyanarin (4) were obtained from the methanol extractives. Compounds 1-4 were screened for cytotoxicity, anti-oxidant, anti-inflammatory activities. Compound 2 showed potent antiinflammatory activity (IC50=2.15 µg/ml). Compound 4 showed potent anti-oxidant activity (IC_{50}: 3.70µg/ml) and moderate cytotoxic activity (ED_{50}: 39.57 µg/ml). Compounds 1 and 3 showed moderate brine shrimp lethality and anti-inflammatory activity. The simultaneous estimation of scopoletin and cyanarin in the methanol extract of the total plant was also carried out by using HPLC and was found that 1.02% of scopoletin and 1.12% of cynarin were present.

KEYWORDS

Anti-inflammatory activitity;
Brine shrimp lethality;
Cyanarin;
Diacetyltetritol;
Merremia emarginata;
Scopoletin.

INTRODUCTION

The *Merremia* genus (fam: Convolvulaceae) consists of number of medicinal plants. The decoction of the whole plant of *Merremia emarginata (Burm.f)* used traditionally for various ailments like antibacterial (krimighna, kushthara), urinal disease (mootra rogahara), kaphaghna, and also used for headache, neuralgia and rheumatism[1]. The powdered leaves of the *M.emarginata* was used as snuff during epileptic seizures, juice act as purgative, given internally for headache and migraine, as an ear drop in cases of abscess. The powdered roots and leaves mixed with flour and water applied externally to swellings by the Ayurvedic physicians[2]. The genus *Merremia* contains simple pyrrolidines and propylhygrines as the alkaloids. Pyrrolidines *from M. aurea* and propylhygrines from *M.aegyptia* are the main alkaloids. *N*-methylpyrrolidinylhygrines are the principal alkaloids of *M. hederacea*[3]. Tropane alkaloids, nicotinoids, coumarins, flavonoids are the other ingredients isolated from this genus. In view of lack of detailed phytochemical studies and of the various medicinal importances on *Merremia emarginata (Burm.f),* we have undertaken the present study. Moreover a study on the biological activities on the crude extracts reported[4,5] by us also prompted for the present work.

EXPERIMENTAL

Melting points were determined by using open capillaries on meltemp apparatus and ^{1}H NMR spectra was recorded on Bruker Avance (400 MHz MHz) NMR spectrometer using DMSO-d_6 as solvent and TMS as

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internal standard and the ^{13}C NMR was recorded on Bruker Avance, 100 MH_Z instrument. The mass spectra were recorded on Elegant LC-1100 series instrument.

Plant material

The plant material *M. emarginata* was collected in Acharya Nagarjuna University campus, Guntur and in some places around Guntur district at the end of August in the year 2009. This whole plant was authenticated by NISCAIR (ref NISCAIR/RHMD/Consult/-2010-11/475/73). The total plant without flowers was shade dried and powdered.

Extraction and isolation

The dried and powdered whole plant material 5 kg was extracted with methanol under heating. The methanol residue was portioned with hexane, ethyl acetate and methanol by column chromatography. The hexane, ethyl acetate and methanol fractions were concentrated and residues were collected and weighed. The weights of the extracts were given in TABLE 1.

TABLE 1 : Extracts from *M. emarginata*

S.No	Solvent	Wt. of the Extract (g)
1.	Hexane	11.95
2.	Ethyl acetate	15.25
3.	Methanol	27.38

Column chromatography

To isolate the compounds from plant material extractives on gradient method, column (3.5" dia x 100 cm length) run over silica gel (100-200 mesh) with hexane, ethyl acetate, chloroform and methanol (all are L.R's), by changing the polarity. To purify or separate the mixture of compounds in small amounts different columns (which are appropriate) were used. These were run over silica gel and neutral alumina. Fractions were concentrated by simple distillation and collected with acetone and dichloromethane.

Thin layer chromatography

For separation of the mixtures and testing the homogeneity, commercially available TLC plates (silica gel coated over aluminum sheets) were used. TLC plates are visualized by UV chamber, Iodine, 10% H_2SO_4 in methanol spray and heated to 110°C.

Ethyl acetate extractives

Compound 1

The ethyl acetate residue was dissolved in ethyl acetate and eluted with hexane and ethyl acetate by varying the polarity (100% hexane, 9:1, 8:2, …., 2:8, 1:9, 100% ethyl acetate). Finally column washed with methanol. Among this 80%, 90%, 100% fractions, showed one UV inactive spot. So these portions were combined and again chromatographed with chloroform and methanol 100%, 2%, 5%, 10%, 15%, 20%. From the above obtained fractions the 2% and 5% fractions are mixed and concentrated. The concentrate was eluted with 1:1 hexane/ethyl acetate with about 400 ml (in 100ml portions). In these portions, the second portion was showed a spot with small impurities. To obtain the pure compound the second portion was concentrated and eluted with hexane/ethyl acetate in 30%, 40% and 50% concentrations. In order to isolate the pure compound the above said three fractions were again concentrated and rechromatographed with $CHCl_3/Me_2CO$ system (from 1 to 25% concentration). The TLC of these fractions showed the presence of the pure compound in 5% and 10% fractions. Finally, the 5 and 10% fractions after concentrating the elutants given an oily compound (% of purity=97%; R_f = 0.40; Wt. of the sample = 100 mg, TLC system: $CHCl_3$: Me_2CO = 8:2). Spectral data given in TABLE 2 & 3.

TABLE 2 : ^1H NMR, ^{13}C NMR and2D NMR spectral data of diacetyltetritol.

S.NO	^1H NMR	^{13}C NMR	^{13}C NMR (HSQC)	HMBC
1	3.904(2H,s)	68.63	68.63	170.46,71.04
2	4.754(1H,s)	71.68		
3	5.113(1H, d, J=5.6Hz)	71.04		
	3.625(1H,m)		71.04	
4	4.241(1H, dd, J=2.0,11.2Hz)	65.43	65.43	
	3.925(1H, dd, J= 9.2,11.2 Hz)		65.43	
5	1.007(3H, s)	18.77	18.77	71.68, 71.04, 68.63
1^I and 1^{II}		170.46,170.29		
2^I and 2^{II}	2.021(6H, s)	20.78, 20.73	20.73, 20.78	170.46

TABLE 3 : DEPT- 45, 90, 135 of compound-1(diacetyl tetritol)

Quertcarbans	Methyne carbon	Methylene carbon	Methyl carbon
170.46			20.73
170.29	71.04	68.63	20.78
71.68		65.43	18.77

Methanol extractives

The methanol residue was dissolved in methanol, adsorbed on silica gel and chromatographed over a column of silica gel. The column was eluted successively with a mixture of chloroform and methanol by varying the polarity [100% $CHCl_3$, 9:1, up to 1:9 MeOH, 100% MeOH] by continuous monitoring of TLC. It was observed that each fraction has closely moving spots. However, the fractions 10% and 30% which are having some concentrated spots were selected for isolation first.

Compound 2

The 10%, fraction was further eluted with 30%, 40%, and 50% of hexane and ethylacetate. The 50% fraction showed one UV long active spot (blue florescence) with R_f = 0.52 in mobile phase 1:1 hexane, ethyl acetate. These fractions were concentrated and allowed for crystallization with dichloromethane. These crystals were filtered, washed with hexane and dried, white crystals were obtained. (% of purity= 90 Chromatogram-1; wt. of the sample= 40mg; TLC system: hexane/ EtOAc: 1:1). The analysis and comparison of the spectral data with literature values confirmed that the compound is scopoletin. The percentage of purity and total Scopoletin available in *M.emarginata* was estimated by HPLC.

Compound 2 spectral data

^{1}H NMR (400MHz, $CDCl_3$) : δ (ppm) = 6.264 (1H, d, J = 9.6 Hz, 3-H), 7.592 (1H, d, J = 9.2 Hz, 4-H), 6.96 (1H, s), 6.845 (1H, s, 8-H), 6.163 (1H, br.s, 7-OH), 3.955 (3H, s, 6-OMe) ^{13}C NMR ($CDCl_3$/ DMSO): δ (ppm) 160.01 (C-2), 102.8 (C-3), 149.51(C-4), 111.54 (C-5), 143.17 (C-6), 144.82 (C-7), 108.15 (C-8), 150.70(C-9), 110.28(C-10), 55.76(OCH_3). LC-MS: (M^+) =191.2. So the molar mass of the compound 2 is192.2

Compound 3

The 30% crude from main column was rechromotographed with chloroform and methanol 2%, 5%, 10%, 15%, 20%. The fractions obtained at 20% of the mobile phase showed UV inactive spot. So these 20% fractions concentrated and rechromotographed with ethyl acetate and methanol to further purification. The 10% portion showed only single UV inactive spot. In methanol acid spray spot was observed (brown colour) with R_f = 0.31% in mobile phase $CHCl_3$, MeOH 8:2 which was concentrated and allowed for crystallization the yellow crystals washed with dichloromethane. (% of purity=97% Chromotogram-2; R_f = 0.31; wt. of the sample = 1.2g). TLC system: $CHCl_3$: Me_2CO = 8:2. State: Light yellow crystals. The analysis and comparison of the spectral data with available literature and confirmed that the compound is tetritol.

Compound 3 spectral data

^{1}H NMR (400MHz, $CDCl_3$): δ (ppm) = 3.46 (1H, d, j = 11.2, 1-H), 3.43 (1H, d, j = 11.2, 2-H), 1.2 (3H, S, 3-H), 3.705 (1H, dd, J = 2.0, 9.6, 4-H), 3.48 (1H, dd, J = 2.0, 11.6, 5-H), 3.5 (1H, dd, J = 2.0, 11.6, C-6). ^{13}C NMR ($CDCl_3$/DMSO): 75.01(C-1),76.26(C-3), 19.86(C-3),68.54(C-4), 63 (C-5) LC-MS: M^+Na=159.2. So the molar mass of the compound 3 is 136.2.

Compound 4

The 50% and 60%, crudes in main column of methanol extractives were again subjected to coloumn chromatopgraphy with $CHCl_3$, MeOH, 2 drops acetic acid in each portion and portioned (100% $CHCl_3$, 10%, 15%,, 20%... up to 50% MeOH). The 20% fraction of the above elutants showed two spots. The 20% frac-

TABLE 4 : ^{1}H NMR, ^{13}C NMR and 2D NMR spectral data of compound-4

S.NO	^{1}HNMR	^{13}CNMR	HMBC
1	7.62 (1H),d	146.78	169.10,123.08,115.37
2	7.59(1H,d,j=15.6hz)	147.10	168.94,123.08,115.37
3	7.106(1H)	115.37	149.5,147.1,123.08
4	7.066(1H)	115.49	149.5,147.1,123.08
5	6.97(1H)	123.08	149.5,115.37
6	6.95(1H)	123.08	149.5,115.37
7	6.80(1H)	116.6	149.5,147.1,146.78,127.9
8	6.79(1H)	116.6	149.5,147.1,146.78,127.9
9	6.43(1H,d,j=14.0hz)	115.92	127.9,128.10
10	6.296(1H,d,j=16.0hz)	115.49	127.9,128.10
11	5.52(1H,m)	72.14	
12	5.442(1H,S)	74.02	
13	3.995(1H)	72.70	
14	2.291(2SH)	39.90,37.37	
15	2.188(2H)	39.90,37.37	

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tion concentrated and recoloumn with EtOAc and acetone and 2 drops of acetic acid 200 ml portions were eluted among these in the portions 3 and 4 are having a UV active spot with small streaking. These portions were concentrated and recolumn with $CHCl_3$, MeOH and 2 drops acetic Acid. Among 15% one single spot appeared. TLC: EtOAc: Acetone (2 drops acetic acid)-7:3 Rf= 0.87, UV active, brown solid, M.P % of purity= 77.7% Wt. of the sample 80mg. Spectral data were given TABLE 4.

HPLC method for analysis of compound 2 and 4

For determination of purity and quantity of compounds phenomenex Luna colomnC18, 5µ, (4.6x250mm) was used at 300nm wave length with a flow rate 1.0 ml/minute at 30°C in gradient system. Volume of injection 20µl, MeOH used as a solvent and mobile phase 0.1% v/v phosphoric acid in water: Acetonitrile was used with a retention time 22.6min. Because of commercial and biological importance Compound-2, Compound-4 was estimated in the total plant extarction in the solvent methanol with pure references of the compound-2 (Scopoletin), compound-4 (cynarin). Purity of Compounds 2, 4 was estimated given in TABLE 5.

TABLE 5 : Gradient program

S.No	Time	Flow	% A	% B
1	0.01	1.00	90.0	10.0
2	10.00	1.00	70.0	30.0
3	20.00	1.00	0.0	100.0
4	24.00	1.00	0.0	100.0
5	26.00	1.00	90.0	10.0
6	35.00	1.00	90.0	10.0

Standard preparation

Weigh accurately about 5.0 mg of references standard in to a 50 ml volumetric flask, dissolve and make up to volume with solvent (MeOH)

Sample preparation

Weigh accurately about 100.0 mg of Crude Sample into a 25 ml volumetric flask, dissolve and make up to volume with solvent.

Procedure

Filter both standard and sample solutions through 0.45µ memberane fileter and inject

$$\text{Calculations} = \frac{\text{Peak area of sample} \times \text{conc. of standard} \times \text{purity of std.}}{\text{Peak area of std} \times \text{conc. of sample}}$$

The above compounds 1, 2, 3 and 4 obtained by isolation were screened for their following biological activities.

Super oxide scavenging activity

Superoxide scavenging activity of the test substance was determined by the method[6] of Mc Cord & Fridvich), modified[7] by Kuttan et al., which depends on the light induced superoxide generation by riboflavin and the corresponding reduction of NBT. The assay mixture contained different conc. of the test substances and EDTA (6mM containing 3µg NaCN), NBT (50µM) riboflavin (2µM) and phosphate buffer 58mM, pH 7.8) in a total vol. of 3ml. The tubes received uniform illumination for 15min and thereafter optical density was measured at 560nm.

% of inhibition = [(control- sample)/control] x 100

An Ic_{50} value was determined as the conc. that elicited the half maximal response.

Stastical analysis

The data were analyzed by (single factor) for multiple groups and the significance level was chosen as $p < 0.05$. Data is expressed as the mean + or – SEM with a min of three experiments performed per each variable.

DPPH free radical scavenging activity

When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in colour from deep violet to light yellow were measured at 517 nm on a UV/ visible light spectrophotometer.

DPPH (1, 1-diphenyl-2picryl-hydrazyl) free radical scavenging activity of the test compounds was determined by the method[8] of Lamaison et. al., which depends on scavenging of colored free radical (DPPH) in methanol solution by the test drugs. The reaction mixture contains DPPH and test drug in a final concentration of 3 ml. Absorption of DPPH at its adsorption maximum 516 nm is inversely proportional to the concentration of the scavenger (test drug). The activity was expressed as inhibitory concentration 50 (IC_{50}) i.e., the concentration of the test solution required to give 50% reduction in absorbance of the test solution as compared to that of blank solution.

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IC_{50} = [(OD of control- (OD of test - OD test blank)) ÷ OD of control] X 100

Brine shrimp method

Brine Shrimp lethality assay was used according to method[9] of Meyer *et al*. Brine Shrimp (Artemia salina) nauplii were hatched in sterile brine solution (prepares using sea salt 38 g/l and adjusted the pH to 8.5 using 1N NaOH). Under constant aeration for 38 hrs after hatching 10 nauplii were placed in each vial and added various concentrations of drug solutions in a final volume of 5 ml, maintained at 37°C for 24 hrs under the light of incandescent lamps and surviving larvae were counted[10] according to the method of Krishnaraju, *et al*. Each experiment was conducted along with control (vehicle treated), at various concentrations of the test substances. Percentage lethality was determined by comparing the mean surviving larvae of test and control tubes. The ED_{50} values were obtained using fenny probed analysis software. The result for test compound was compared with the positive control podophllotoxin. Calculated the ED_{50} using probed analysis at 95% confidence limits from observed data. Replicas maintained to get accurate results.

5-lipoxygenase inhibition

The assay mixture contained 80 mM linoleic acid and sufficient amount of potato 5-lipoxygenase enzyme in 50 mM phosphate buffer (pH 6.3). The reaction was initiated by the addition of enzyme buffer mix to the substrate (linoleic acid) and the enzyme activity was monitored as an increase in absorbance at 234 nm. The reaction was monitored for 120 sec using UV-Kinetic mode on Varian Cary-50 UV-Vis spectrophotometer. In the inhibition studies the activities were measured by incubating various concentrations of test substances with enzyme buffer mix for two minutes before addition of the substrate. All assays were performed in triplicate and mean values were used for the calculation. Percentage inhibition was calculated by comparing slope or increase in absorbance of test substances with that of control enzyme activity. The activity[15] of 5-lipoxygenase extracts was compared with the standard positive control LI01020.

RESULTS AND DISCUSSION

Compound 1

Compound 1 was isolated as viscous oil and exhibited $[\alpha]_D$= +11.28 (at C = 1 at t = 25°C in methanol). The interpretation of spectral data (IR, ^{1}HNMR, ^{13}C NMR, Mass, and 2D NMR, DEPT) revealed that it is diacetyltetritol (Figure 1).

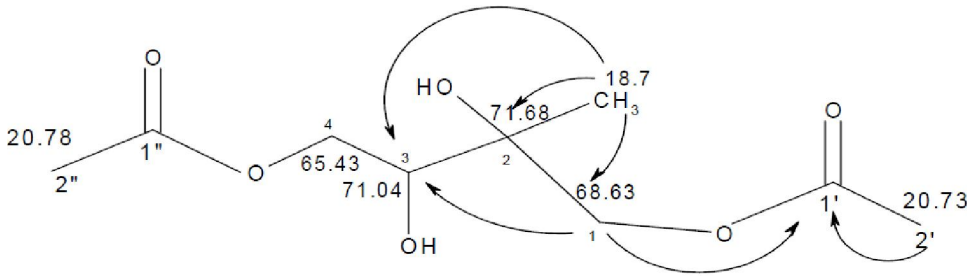

Figure 1 : Diacetyl tetritol

Compound 2

Compound 2 was identified as Scopoletin (Figure 2) based on interpretation of spectral data and comparison with literature values[11]. Scopoletin was previously isolated in this genus from *Ipomoea batatas* (L.) Lam[12].

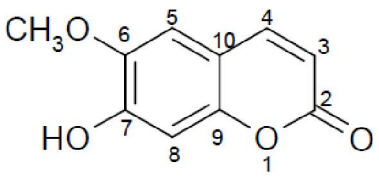

Figure 2 : Scopoletin

Compound 3

Compound 3 was identified as tetritol (Figure 3) based on interpretation of spectral data and comparison with literature[13] values. Tetritol shown $[\alpha]_D$ = +11.40 (at C=1 at T=25°C in methanol) confirming its D- configuration.

Compound 4

Compound 4 was identified as cynarin (Figure 4) based on interpretation of spectral data and comparison with literature[14] values.

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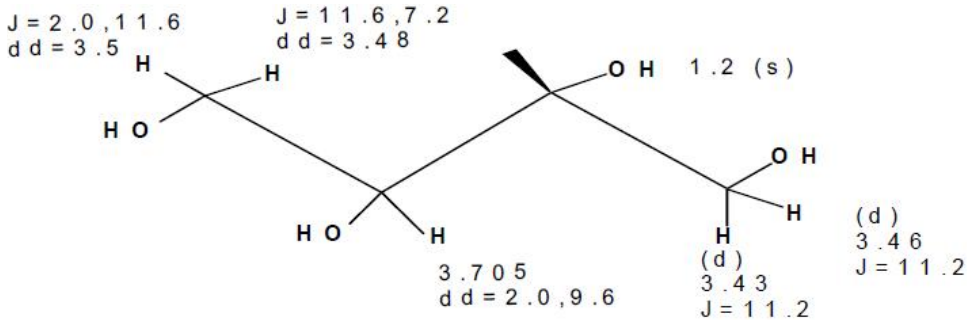

Figure 3 : Tetritol

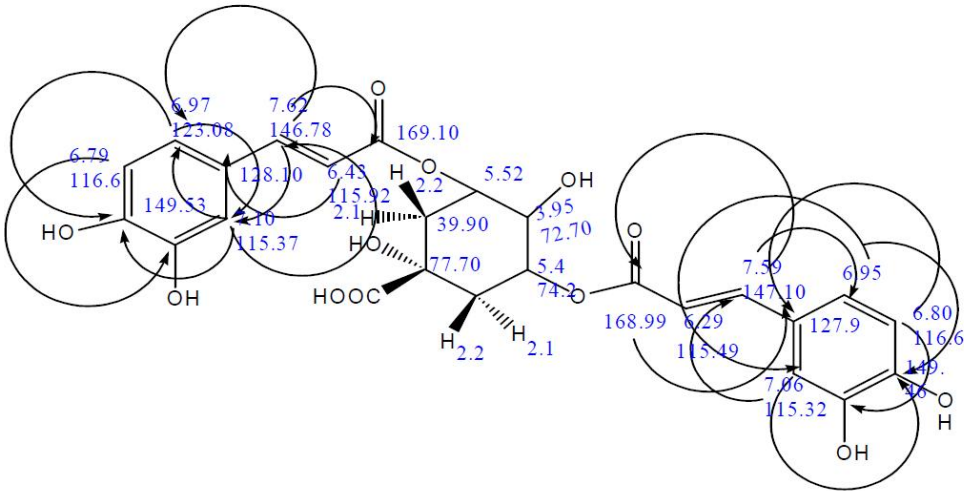

Figure 4 : Cynarin (1,3-dicaffeoylquinic acid)

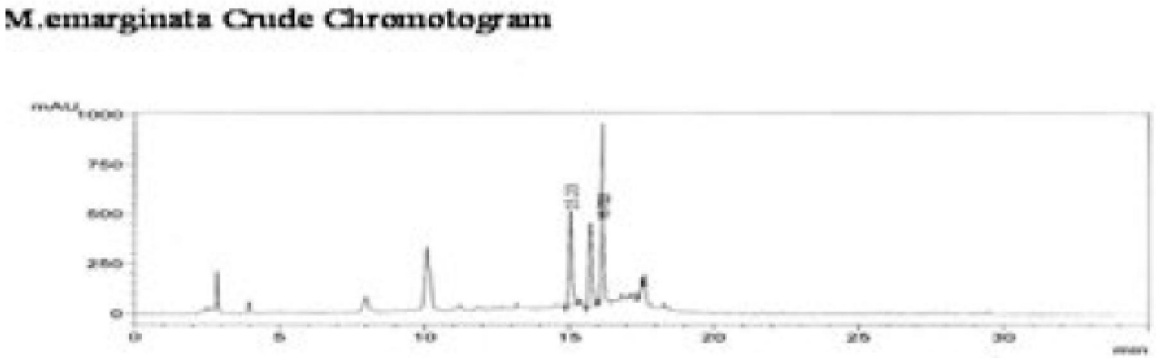

Figure 5

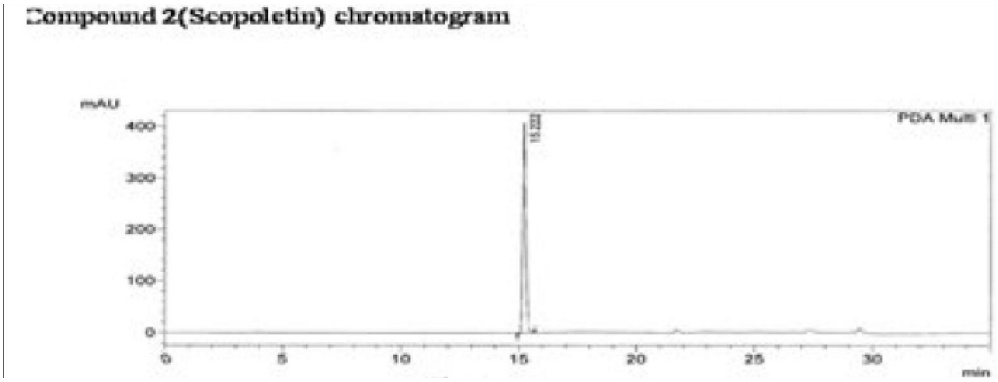

Figure 6

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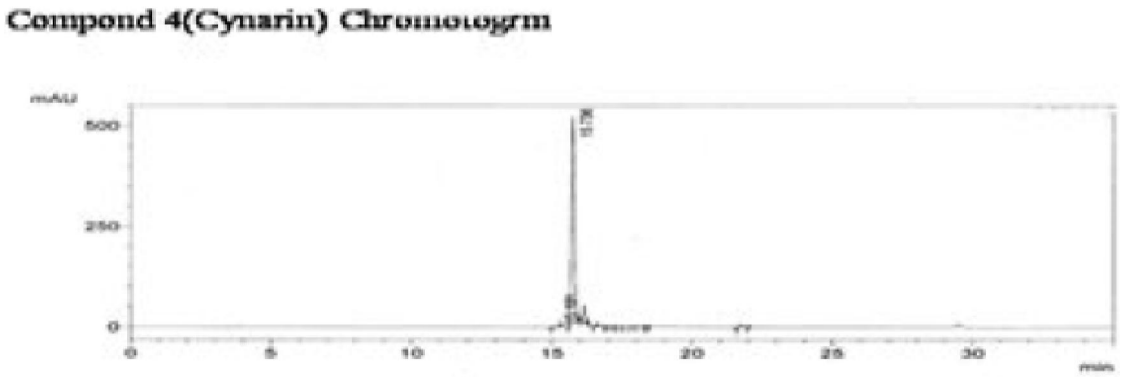

Figure 7

The HPLC analytical study on the methanol extract of whole plant of *M.emarginata* has shown that 1.02% of scopoletin and1.12% of cynarin was presented. These results were calculated by comparing the standard chromatograms of scopoletin and cynarin (Figure 6 and Figure 7) with the chromatogram of *M.emarginata* crude (Figure 5).

Bioactivites of isolated compounds

A preliminary evaluation was carried out by us on different extracts (hexane, ethyl acetate, methanol solubles) of *M.emarginata* for their bioactivity[8,9]. The study had showed that the ethyl acetate extract has potential inflammatory activity (IC_{50} µg/ml 5.9), anti-oxidant activity (IC_{50} µg/ml 21.50) and brine shrimp lethality (IC_{50} µg/ml 34.29). Methanol extract showed strong anti-oxidant activity (IC_{50} µg/ml 8.59), anti-inflammatory activity (IC_{50} µg/ml 36.4). Based on these results ethyl acetate extract and methanol extract were subjected to column chromatography to Isolated the potential pure compounds and to evaluate their activity.

Compounds 1, 2, 3 and 4 were studied for their bioactitivity by in-vitro methods (TABLE 2).

Compound 2 (Scopoletin) showed potent anti-inflammatory activity (IC_{50}:2.15 µg/ml) by the inhibition of pro-inflammatory cytokine. Compound 4 showed potent antioxidant activity (IC_{50}: 3.70 µg/ml) and moderate cytotoxic activity, Compound 3 (Tetritol) showed moderate brine shrimp lethality (ED_{50}: 41.66 µg/ml) and moderate anti-inflammatory (IC_{50}: 17.36 µg/ml). The compound 1 (Diacetyltetritol) showed moderate anti-inflammatory activity (IC_{50}: 25.38 µg/ml) against 5-lipoxygense and brine shrimp lethality (ED_{50}: 89.77 µg/ml). the results were given in TABLE 6.

TABLE 6 : Bioactivities of isolated compounds

Compound	Antioxidant activity (IC_{50}, µg/ml)		Cytotoxic activity (ED_{50}, µg/ml)	Antiinflammatory activity (IC_{50}, µg/ml)
	Super-oxide method	DPPH-freeradical method	Brine Shrimp method	5-lipoxygenase method
Diacetyl tetritol (1)	-	-	89.77	25.38
Scopoletin (2)	-	89.2	76.4	2.15
Tetritol (3)	7.17	25.14	41.66	17.36
Cynarin (4)		7.7	39.57	-
Standard	Vitamin-c 3.71	Gallic Acid 0.56	Podophyllotoxin 3.61	LI01020 4.55

CONCLUSIONS

In summary, Diacetyltetritol, a new tetritol derivative was isolated naturally for the first time from *M.emarginata* plant species. The compounds scopoletin, tetritol and cynarin were also first time isolated from this plant. Among these four compounds, the compound 4 showed potent antioxidant and moderate cytotoxic activity. Compound 2 showed potent anti-inflammatory activity and compounds 1 and 3 showed moderate brine shrimp lethality and anti-inflammatory activity. These results supported our previous investigation of biological activities of crude extracts of *M.emarginata*.

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