Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2013, 5(7):80-87

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Phytochemical investigation of the roots of*Grewia microcos*Linn.

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ABSTRACT

The present study reports the phytochemical investigation of the roots of Grewia microcos linn. Seven Phytoconstituents have been reported namely 9,12- octadecadienoic acid; Ursolic acid; Stigmasterol; 6,4-dihydroxy-3-propen chalcone; Dioctyl phthalate; N-methyl-6-β-(1',3',5'-trienyl)-3-β-methoxyl-3-β-methyl piperidine; and Dibutyl phthalate have been reported for the first time from the roots of Grewia microcos.

Keywords: *Grewia microcos,* Phytoconstituents, Ursolic acid, Dibutyl phthalate.

INTRODUCTION

Grewia microcos is a small semi-deciduous tree, sometimes shrubby up to 50 feet high and 5 feet girth, belonging to the family Tiliaceae[1].The plant is also known as *Microcos paniculata* [2]. It is found in North eastern parts of India, Western Ghats and Andaman islands. Flowers are in terminal panicles and auxiliary towards the apex, is the distinguishing character of this plant[3]. Boiled leaves along with turmeric and shell of snail are taken for the treatment of Jaundice. Traditionally it is used to improve digestion and is also for used for cold, hepatitis, diarrhea, heat stroke, dyspepsia, typhoid fever, syphilitic ulceration of the mouth, small pox, eczema and itches [4,5].

Literature survey revealed that, the stem bark of *Grewia microcos* contained a new alkaloid, N-methyl-6-β-(deca-1',3',5'-trienyl)-3-β-methoxy-2-methylpiperidine which showed good insecticidal activity against Aedes aegypti second instar larve[6,7]. Two new piperidine alkaloids microcosamine A and B were isolated from the leaves, showed significant larvicidal activity againat Culex quinquefasiatus[8]. A new triterpene named methyl -3-o-p-hydroxy-cinnamoyloxy-2-∝, 23-dihydroxyolean-12-en-28-oate, epicatechin, 3-trans-feruloyl masilinic acid and masilinic acid were identified from the stem bark[9]. Analgesic and cytotoxic activity of leaves extract were also reported[10].

EXPERIMENTAL SECTION

All the melting points were recorded in Bio Technics India, Model No. BT2-38 melting point apparatus and were uncorrected. IR spectra of the compounds were recorded using KBr pellet method on Bruker α-T Spectrophotometer, at National Facility for Clinical Trials, ISISM Chennai. ^{1}HNMR spectra and LC MS spectra of the compounds were taken on Bruker 500 MHz PMR Spectrophotometer using $CDCl_3$ as solvent and LC-MS Shimadzu LC 2020 at National Facility for Clinical Trials, ISISM Chennai. ESI-MS spectra were recorded using ESI-MS Expression CMS Advion at SynZeal Research Laboratory, Ahmedabad. TLC was carried out using Aluchrosep Silica Gel 60/UV_{254} from S. D. Fine Chemicals Pvt. Ltd, Mumbai. Column Chromatography was carried out using glass column with stopcock, 30 x 600 mm from Merck Specialities Pvt. Ltd, Mumbai packed with Silica Gel (200-400 mesh) from Molychem, Mumbai. All the Chemicals and Reagents used were obtained in high purity either from S. D. Fine Chemicals Pvt. Ltd, Molychem and Chemport Pvt. Ltd, Mumbai.

Authentication and Collection of the Plant Material
The roots of Grewia microcos was collected from Dhavali, Ponda –Goa during the month of October 2012. It was authenticated by Prof. G.I. Hukkeri, Department of Botany, Dhempe College of Arts and Science, Miramar-Goa [3].

Preparation of Ethanolic extract
The roots were collected, washed and dried in shade. The dried roots were then powdered (500gm) and exhaustively extracted by maceration with ethanol (95%) for three days. After three days, the ethanolic layer was decanted off. The process was repeated thrice. The solvent from the total extract was distilled off using Rotary vacuum evaporator (Superfit) and then evaporated to dryness (45g) [11].

Preliminary Phytochemical analysis
A preliminary phytochemical screening was carried out using the ethanolic extract of the roots by employing the standard procedures[12, 13].

1. Alkaloids

- *Dragendroff 's Test:*

To 2mg of the methanolic extract, 5ml of distilled water was added, 2M Hydrochloric acid was added until an acid reaction occurs .To this 1ml of Dragendroff's reagent was added. Formation of orange or orange-red precipitate indicated the presence of alkaloids.

- *Mayer's Test:*

To 2mg of the methanolic extract, a few drops of Mayer's reagent was added. Formation of white or pale yellow precipitate indicated the presence of alkaloids

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- Wagner's Test:

To 2mg of the methanolic extract, 1ml of Hydrochloric acid was added along with few drops of Wagner's reagent. A yellow or brown precipitate indicated the presence of alkaloids.

- *Hager's Test:*

To 2mg of the methanolic extract, was taken in a test tube, a few drops of Hager's reagent was added. Formation of yellow precipitate confirmed the presence of alkaloids.

2. Carbohydrates

- *Molish's Test*

In a test tube containing 2ml of the extract, 2 drops of freshly prepared 20% alcoholic solution of ∝-naphthol was added. 2ml of conc. Sulphuric acid was added so as to form a layer below the mixture. Red –violet ring appeared, indicating the presence of carbohydrates, which disappeared on the addition of excess of alkali.

- *Benedict's Test*

To 0.5ml of the extract, 5ml of Benedict's solution was added and boiled for 5 minutes. Formation of the brick red colored precipitate indicated the presence of carbohydrates.

- *Fehling's Test*

To 2ml of the extract, 1ml mixture of equal parts of Fehling's solution A and B were added and boiled for few minutes. Formation of the brick red colored precipitate indicated the presence of carbohydrates.

3. Flavanoids

- *Shinoda Test*

In a test tube containing 0.5ml of the extract, 10 drops of Hydrochloric acid followed by a small piece of magnesium were added. Formation of pink, reddish, or brown color indicated the presence of flavonoids.

- *Lead acetate Test*

To 2ml of plant extract add 1ml of Lead acetate solution. Formation of yellow precipitate indicates the presence of flavonoids.

- *Vanillin HCl Test*

Vanillin HCl was added to the alcoholic solution of drug, formation of pink color shows the presence of flavonoids.

4. Triterpenoids & Steroids

· *Libermann –Burchard's*

2mg of dry extract was dissolved in acetic anhydride, heated to boiling, cooled and then 1ml of concentrated sulphuric acid was added along the sides of the test tube. A brown colored ring is formed at the junction of two layers and the upper layer turns green which shows the presence indicated the presence of steroids and formation of deep red color indicates presence of triterpenoids.

· *Salkowshi Test*

Treat the extract with few drops of conc. Sulphuric acid, red color in the lower layer indicates presence of steroids and formation of yellow colored lower layer the indicates the presence of triterpenoids.

5. Tannins and Phenolic compounds

· To 1-2ml of the extract, few drops of 5%w/v $FeCl_3$ solution were added. A green color indicated the presence of gallotannins, white brown color indicated the presence of pseudotannins.

· To 1-2ml of the extract add lead acetate was added. White precipitate indicated the presence of tannins and phenolic compounds.

6. Resins

1ml of the extract was dissolved in acetone and the solution was poured in distilled water. Turbidity indicated the presence of resins.

7. Proteins

· *Biuret's Test*

To 1ml of hot extract, 5-8 drops of 10%w/v of sodium hydroxide solution, followed by 1 to 2 drops of 3%w/v of copper sulphate solution were added. Formation of violet red color indicated the presence of proteins.

· *Millon's Test*

1ml of the extract was dissolved in 1ml of distilled water and 5-6 drops of millon's reagent were added. Formation of white precipitate, which turns red on heating, indicated the presence of proteins.

8. Glycosides

Determine free sugar content of the extract. Hydrolyze the extract with mineral acids (dil. HCL/dil. H_2SO_4). Again determine the total sugar content of hydrolyzed extract. Increase in sugar content indicates the presence of glycosides in the extract.

· Test for cardiac glycosides

i. Baljet's test

A thick section shows yellow to orange color with sodium picrate.

ii. Legal's Test

To aqueous or alcoholic extract, add 1ml pyridine and 1ml sodium nitropruside. Pink to red color appears.

iii. Test for deoxysugars(killer –killani test)

To 2ml extract add glacial acetic acid, one drop 5% $FeCl_3$ and conc. H_2SO_4. Reddish brown color appears at the junction of the two liquid layers and upper layer appears bluish green.

iv. Libermann's test (test for bufadienoloids)

Mix 3ml extract with 3ml acetic anhydride. Heat and cool. Add few drops conc. H_2SO_4. Blue color appears.

· Test For Anthraquinone Glycosides

i. Borntrager's test for Anthraquinone glycosides

To 3ml extract add 5ml 5% dil. H2SO4. Boil and filter. To cold filtrate add equal volume of benzene or chloroform. Shake well. Separate the organic solvent. Add ammonia. Ammonical layer turns pink or red.

ii. Modified Borntrager's test for C-glycosides

To 3ml extract add 5ml of 5% dil. HCl. Heat for 5minutes in boiling water and cool. To cold filtrate add equal volume of benzene or organic solvent. Shake well. Separate the organic solvent. Add ammonia. Ammonical layer turns pink or red.

· Test For Saponins

i. Foam test

Shake the drug extract or dry powder vigorously with water. Persistent foam observed.

- Test For Coumarin Glycosides

i. Fluorescence test

Take moistened powder in a test tube. Cover test tube with filter paper soaked in dilute NaOH. Keep in water bath. After some time expose filter paper to UV light. It shows yellowish-green fluorescence.

ii. $FeCl_3$ Test

To the concentrated alcoholic extract of drug few drops of alcoholic $FeCl_3$ solution was added. Formation of deep green color, which turned yellow on addition of conc. HNO_3 indicates the presence of coumarins.

9. Starch

0.01gms of Iodine and 0.075gms of KI were dissolved in 5ml of distilled water and 2-3ml of extract was added. Formation of blue color indicated the presence of starch.

Isolation of constituents

The ethanolic extract (12 gms) was mixed with silica gel (2 gms). After mixing, the sample was loaded on column packed with 160 gms of silica –gel (240-400 Mesh) prepared in Pet. Ether. The column was subjected to different solvent systems, starting with 100 % Pet. Ether (60-80^0 C) followed by graded mixtures of Pet. Ether (60-80^{0}C) : $CHCl_3$ (95:5, 90:10, 80:20, 70:30, 60:40, 50:50); $CHCl_3$ 100% followed by graded mixtures of $CHCl_3$: EtOAc. (95:5, 90:10, 80:20, 70:30, 60:40, 50:50); EtOAc. (100%) and finally with graded mixtures of EtOAc. : Methanol (99:1, 98:2, 97:3, 96:4, 95:5) [11].

The Elutions were monitored by TLC (Silica gel-G; visualization by UV 254nm, 366nm and Vanillin-Sulphuric acid spraying reagent heated at 110^{0}C). Each time 10 ml elutes were collected and identical elutes were combined (TLC monitored) and concentrated to 5ml and kept aside. Elutions carried out with graded mixture of Pet. Ether(60-80^{0}C) : $CHCl_3$ (80:20) resulted a single component on TLC (Pet. Ether(60-80^{0}C) : $CHCl_3$ 80:20). After removing the solvent yellow oily liquid resulted, which was designated as Compound I (160mg).Elutions carried out with $CHCl_3$ 100% , resulted a single component on on TLC ($CHCl_3$ 100%). After removing the solvent yellow powder resulted, which was designated as Compound II (100 mg).Elutions carried out with $CHCl_3$: EtOAc. (80:20) resulted a single component on TLC ($CHCl_3$: EtOAc. 80:20). After removing the solvent pale yellow crystallinepowder resulted, which was designated as Compound III (110 mg).Elutions carried out with $CHCl_3$: EtOAc. (50:50) resulted a single component on TLC ($CHCl_3$: EtOAc. 50:50). After removing the solvent white powder resulted, which was designated as Compound IV (95 mg).Elutions carried out with EtOAc. : Methanol(99:1) resulted a single component on TLC (EtOAc. : Methanol 99:1). After removing the solvent yellow liqiud resulted, which was designated as Compound V (125 mg). Elutions carried out with EtOAc. : Methanol(98:2) resulted a single component on TLC (EtOAc. : Methanol 98:2). After removing the solvent viscous brown liqiud resulted, which was designated as CompoundVI (130 mg). Elutions carried out with EtOAc. : Methanol(95:5) resulted a single component on TLC (EtOAc. : Methanol 95:5). After removing the solvent pale yellow liqiud resulted, which was designated as Compound VII (140 mg).Elutions carried out with other graded mixtures resulted in brown resinous mass which were not processed further[11] .

RESULTS AND DISCUSSION

Phytochemical screening of the ethanolic extract of *G.microcos* led to the presence of phytoconstituents like alkaloids, carbohydrates, flavonoids, steroids, triterpenoids, tannins, and saponins.

The chemical investigation led to the isolation of seven compounds from the ethanolic extract of the roots of *G. microcos*. The isolated compounds are 9,12- Octadecadienoic acid; Ursolic acid; Stigmasterol; 6,4-dihydroxy-3-propen chalcone; Dioctyl phthalate; N-methyl-6-β-(1',3',5'-trienyl)-3-β-methoxyl-3-β-methyl piperidine; &Dibutyl phthalate.

Compound I (9, 12- Octadecadienoic Acid): b.p. 229^{0}C (lit. 229-230^{0}C);IR (KBr): 3369.69 cm^{-1} (br, OH), 2926.13 cm^{-1} (C-H str. inCH_3), 2858.69 cm^{-1} (C-H str. in CH_2), 1727.47 cm^{-1} (C=O str. ofCOOH), 1458.23 cm^{-1}(C-H def. in CH_3). ^{1}H-NMR ($CDCl_3$) δ 0.866 - δ 0.896 (m, 3H, H-18) terminal methyl group, δ 0.907 - δ 0.960 (m, 14H, H-4, 5, 6, 7, 15, 16, 17), δ 1.687 (s, 2H, H-3) , δ 2.287 (s, 4H, H-8, 14) δ 2.301 (s, 2H, H-2), δ 2.316 (s, 2H, H-11), δ 5.348 (d, 4H, H-9, 10, 12, 13) Vinylic proton. The ESI-MS spectrum displayed the molecular ion peak at m/z 280.5 corresponding to the molecular formula $C_{18}H_{32}O_2$.

Table no. 1: Result of the qualitative tests for Phyto-constituents of the ethanolic extract of the roots of *G. microcos.*

Sr. No.	Tests	Inference
1.	**ALKALOIDS**	
	a) Dragendroff's Test	+ve
	b) Hayer's Test	+ve
	c) Wagner's Test	+ve
	d) Mayer's Test	+ve
2.	**GLYCOSIDES**	
	Test for Cardiac Glycosides	
	a) Baljet Test	-ve
	b) Legal Test	-ve
	c) Keller-Killani's Test	-ve
	Test for Anthraquinone Glycosides	
	a) Borntrager's Test	-ve
	b) Modified Borntrager's Test	-ve
3.	**CARBOHYDRATES**	
	a) Molish's Test	+ve
	b) Fehlings Test	+ve
	c) Benedicts Test	+ve
4.	**FLAVONOIDS**	
	a) Shinoda's Test	+ve
	b) Vanillin HCl Test	+ve
5.	**PROTEINS**	
	a) Biuret's Test	-ve
	b) Millon's Test	- ve
6.	**TANNINS**	+ve
7.	**RESINS**	-ve
8.	**SAPONINS**	+ve
9.	**TRITERPENOIDS**	
	a) Libermann-Burchard's Test	+ve
10.	**STEROIDS**	
	a) Libermann-Burchard's Test	+ve
	b) Salkowshi Reaction	+ve
11.	**STARCH**	-ve

+ = Present , - = Absent

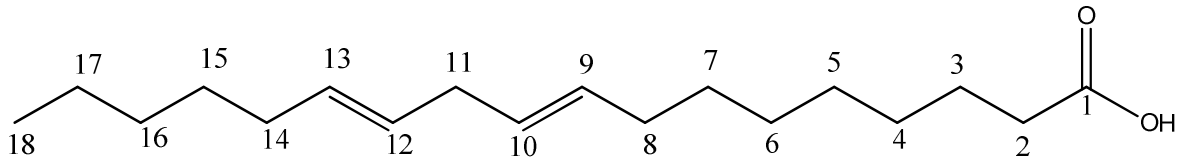

Fig. 1: 1 9, 12-Octadecadienoic acid

Compound II (Ursolic Acid): m.p. 287^{0}C. IR (KBr): 3429.35 cm^{-1} (br, OH), 2926.03 cm^{-1} (C-H str. in CH_3), 2859.85 cm^{-1} (C-H str. in CH_2), 1728.33 cm^{-1} (C=O str. ofCOOH), 1604.24 cm^{-1} (C=Cstr), 1457.64 cm^{-1} (C-H def. in CH_3), 1379.11 cm^{-1} (C-H def. in gem dimethyl).^{1}H-NMR ($CDCl_3$) **:**δ 0.856 - δ 0.894 (m, 21H, 7x CH_3), δ1.259 - δ 1.705 (m, 18H, 9x CH_2), δ 2.144 - δ 2.193 (m, 1H, OH), δ 2.301 - δ 2.377 (m, 1H, H-18), δ 2435 - δ 2.594 (m, 4H, methine protons), δ 3.545 - δ 3.574 (m, 1H, H-3), δ 5.341 - (s, 1H, Vinylic proton). The LC-MS spectral data of the compound showed the molecular ion peak at m/z 456.20[M^+] and the base peak m/z 248.10 along with a strong absorption peak at m/z 203.5 due to Retro–Diel-Alder fragmentation, typical for Δ^{12}-oleanene or ursine triterpene with molecular formula $C_{30}H_{48}O_3$[14].

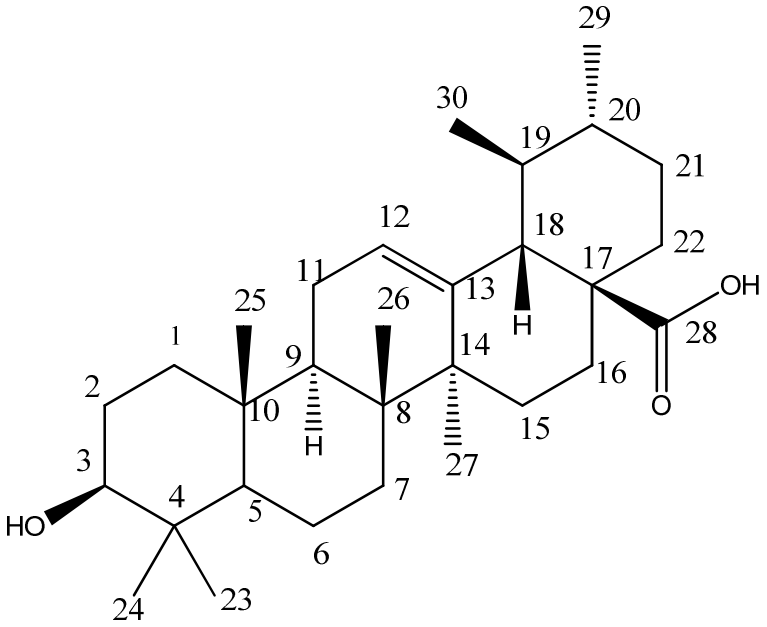

Fig. 2 Ursolic acid

Compound III (Stigmasterol): m.p. 167^{0}C.IR (KBr): 3429.35 cm^{-1} (br, OH), 2926.03 (C-H str. in CH_2), 1604.24 cm^{-1} (C=Cstr.), 1457.64 cm^{-1} (C-H def. in CH_3), 1072.76 cm^{-1} (C-O str. in 2^0 alc.). ^{1}H-NMR ($CDCl_3$) δ 0.831- δ 0.848 (t, 9H, H-18, 26, 27), δ 0.854 (s, 3H, H-29), δ 0.940 - δ 0.943 (d, 6H, H-19, 21), δ1.254 -δ 1.728 (s, 18H, 9 x CH_2 ,H- 1, 2, 4, 7, 11, 12, 15, 16, 28 and 7H, H-6, 8, 9, 14, 17, 20, 24, 25 methine protons), δ 2.014-δ 2.039 (m, 1H, OH-3), δ 3.657 (s, 1H, H-3), δ 5.338-δ 5.340 (t, 3H, H-6, 22, 23 Vinylic proton). The LC-MS spectrum showed the molecular ion peak at m/z 413.25 [M+H$^+$] which was consistent with the molecular formula $C_{29}H_{48}O$ [15].

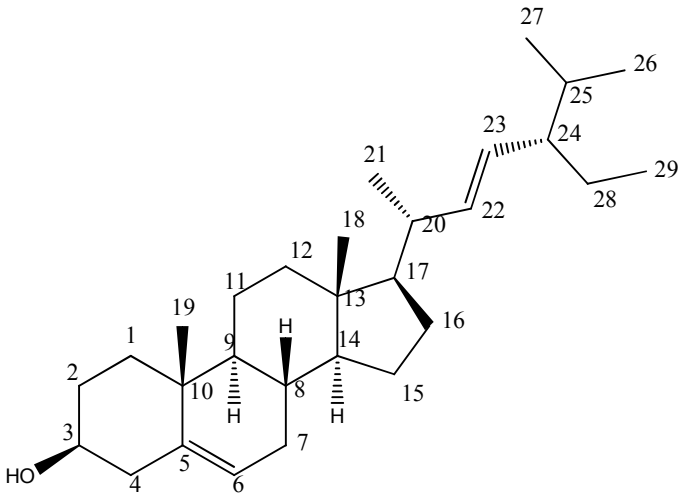

Fig. 3 Stigmasterol

Compound IV (6, 4-dihydroxy-3-propen chalcone): IR (KBr): 3408.15 cm $^{-1}$ (br, OH), 2926.21 cm $^{-1}$ (C-H str. Of CH_3)**,** 1728.06 cm $^{-1}$ (C=O str.)**,** 1600.33 cm $^{-1}$ (C=C str.)**,** 1072.23 cm $^{-1}$ (C-O str.). ^{1}H-NMR ($CDCl_3$)**:** δ1.201 - δ 1.296 (m, 3H, H-9')**,** δ 4.289 (s, 1H, H-8')**,** δ 4.316 (s, 1H, H-7')**,** δ 5.007 (d, 2H, OH-4',6)**,** δ 6.683 (t, 1H, H-5)**,** δ 6.712 (t, 1H, H-4)**,** δ 7.161 – δ 7.193 (m, 2H, H-3, H-5')**,** δ 7.505 – δ 7.519 (m, 2H, H-2', 6')**,** δ 7.692(s, 1H, H-2)**,** δ 7.703 (t, 2H, H-∝,β). The ESI-MS spectrum of the compound showed the presence of molecular ion peak at m/z 280.2 [M$^+$] which was consistent with the molecular formula $C_{18}H_{16}O_1$. The peak obtained at 279.2 was due to proton migration. The compound under goes ∝- cleavage from carbonyl to produce a base peak at 149.1 ($C_9H_9O_2$)[16].

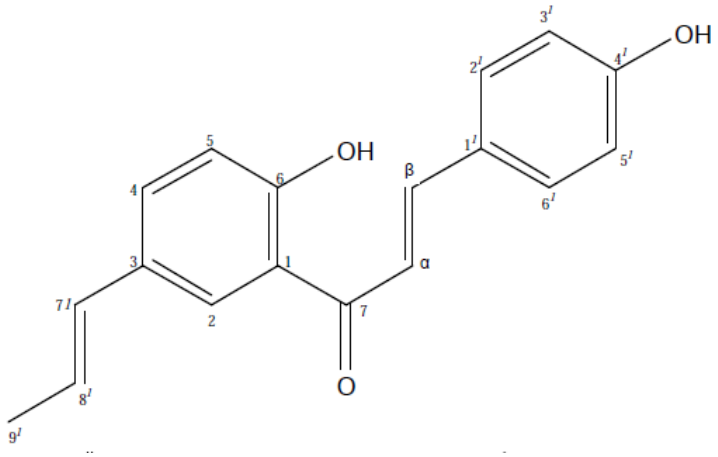

Fig. 4: 6, 4-dihyroxy-3-propen chalcone

Compound V (Di-n-octyl phthalate): b.p. 381^{0}C. IR (KBr) : 2926.21 cm^{-1} (C-H str. in CH_3), 2859.46 cm^{-1} (C-H str. in CH_2), 1728.33 cm^{-1} (C=O str.), 1600.33 cm^{-1} (C=Cstr.), 1455.77 cm^{-1} (C-H def. in CH_3), 744.88 cm^{-1} (C-H def. in aromatic ring). ^{1}H-NMR ($CDCl_3$) :δ 0.827 - δ 0.997 (m, 6H, H-8', H-8"), δ 1.258 (s, 12H, H-4', 4", 5', 5", 6', 6"), δ 1.397 (s, 4H, H-7', 7"), δ 1.411 - δ 1.464 (m, 4H, H-3', 3"), δ 1.674- δ 1.733 (m, 4H, H-2', 2"), δ 4.196 - δ 4.319(m, 4H, H-1', 1"), δ 7.516 - δ 7.541(m, 2H, H-4, 5), δ 7.676 - δ 7.730(m, 2H, H-3, 6). The ESI-MS spectra showed molecular ion peak at m/z 391.1 $[M+H]^+$ in positive ion mode which was consistent with the molecular formula $C_{24}H_{38}O_4$. Fragment ion peak at 279.2 and 163.1 exhibited in the mass spectrum were characteristic of alkyl phthalates and the base peak 149.0 was due to the protonated phthalic anhydride ($C_8H_5O_3$) [17].

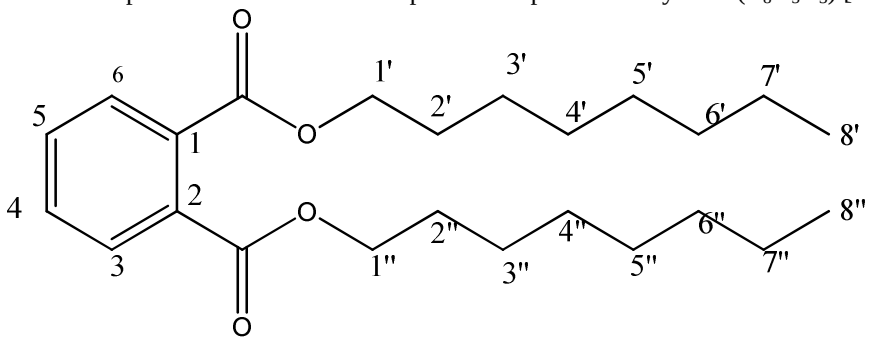

Fig. 5: Di-n-octyl phthalate

Compound VI [N-methyl-6-β-(1',3',5'-trienyl)-3-β-methoxyl-3-β-methyl piperidine] : m.p. 52^{0}C. IR (KBr): 3083.40 cm^{-1} (C-H str. in aromatic ring), 2924.30 cm^{-1} (C-H str. in CH_3), 2857.02 cm^{-1} (C-H str. in CH_2), 1601.37 cm^{-1} (C=Cstr.), 1456.18 cm^{-1} (C-H def. in CH_3), 1278.73 cm^{-1} (C-O str.), 742.00 cm^{-1} (C-H def. in aromatic ring). ^{1}H-NMR ($CDCl_3$):δ 0.866 -δ 0.895 (m, 3H, H-10'), δ 1.201 - δ 1.208 (d, 3H, 2 eq- CH_3), δ 1.273 - δ 1.445 (m, 6H, H-5ax, H-4ax, H-8', H-9'), δ 1.684 - δ 1.728 (dd, 1H, H-5eq), δ 2.006 (s, 1H, H-4eq) ,δ 2.144 - δ 2.193 (m, 6H, H-2ax, H-7', N-CH_3), δ 2.596 (s, 1H, H-6ax), δ 3.319 (s, 3H, O-CH_3), δ 3.545 - δ 3.574 (m, 1H, H-3ax), δ 5.770 - δ 5.797 (m, 1H, H-1'), δ 5.804 - δ 5.831 (m, 1H, H-6'),δ 6.683 (t, 4H, H-2' to H-5'). The ESI-MS spectra showed molecular ion peak at m/z 278.2 $[M+H]^+$ in positive ion mode which was consistent with the molecular formula $C_{18}H_{31}NO$. The other peaks appeared at m/z 264.5, 248.3, 234.5, 205.3, 163.2, and 108.2[6].

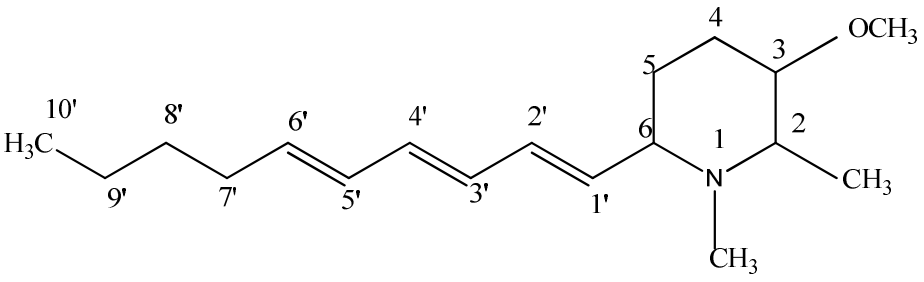

Fig. 6: N-methyl-6-β-(1',3',5'-trienyl)-3-β-methoxyl-3-β-methyl piperidine

Compound AS VII (Dibutyl phthalate): b.p. 337^{0}C. IR (KBr): 2926.13 cm^{-1} (C-H str. in CH_3), 2858.69 cm^{-1} (C-H str. in CH_2), 1727.47 cm^{-1} (C=O str.), 1604.24 cm^{-1} (C=Cstr.), 1073.09 cm^{-1} (C-O str.), 744.44 cm^{-1} (C-H def . in aromatic ring).^{1}H-NMR ($CDCl_3$)δ0.866 - δ 0.960 (m, 6H, H-4', H-4"), δ 1.258 (s, 4H, H-3', H-3"), δ 1.718 (s, 4H, H-2', H-2"), δ 4.306 (t, 4H, H-1', H-1"), δ 7.514 –δ 7.525 (t, 2H, H-4, H-5), δ 7.704 – δ 7.772 (dd, 2H, H-3,H-6). The ESI-MS spectra showed molecular ion peak at m/z 279.4 $[M+H]^+$ in positive ion mode which was consistent with the molecular formula $C_{16}H_{22}O_4$. The mass spectrum showed other fragments at 167.2 and 149.2 which are considered characteristic of alkyl phthalates. The fragment ion at 223.4 suggested that the alkyl phthalate is Dibutyl phthalate. The base peak at 149.0 is due to the protonated phthalic anhydride ($C_8H_5O_3$) [18].

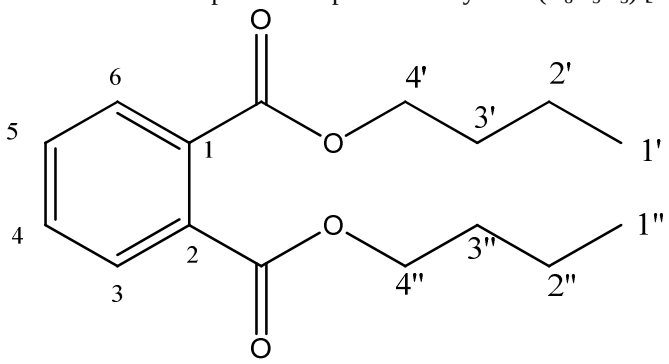

Fig. 7: Dibutyl phthalate

CONCLUSION

The chemical investigation led to the isolation of seven compounds from the ethanolic extract of the roots of *G. microcos*. The isolated compounds are 9,12- Octadecadienoic acid; Ursolic acid; Stigmasterol; 6,4-dihydroxy-3-propen chalcone; Dioctyl phthalate; N-methyl-6-β-(1',3',5'-trienyl)-3-β-methoxyl-3-β-methyl piperidine; & Dibutyl phthalate. All the compounds named above are isolated for the first time from the roots of *G. microcos.*

Aknowledgement
The authors are grateful to the authorities of Government of Goa and Principal for immense support and also providing laboratory facilities. Authors are also thankful to National Facility for Clinical Trials, ISISM, SRM University, Chennai and SynZeal Research Laboratory, Ahmedabad for providing spectras. We also thank Prof. G.I. Hukkeri, Associate Professor, Department of Botany, Dhempe College of Arts and Science, Miramar-Goa for authenticating the plant.

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American Journal of Advanced Drug Delivery

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Original Article

TERMINALIA TOMENTOSA ROXB (ex DC) WIGHT & ARN: PHYTOCHEMICAL INVESTIGATIO

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Date of Receipt- 06/07/2013
Date of Revision- 19/07/2013
Date of Acceptance- 24/07/2013

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ABSTRACT

The present study was undertaken to carry out phytochemical investigation of the ethanolic extract of the stem bark of Terminalia tomentosa Roxb (ex DC) Wight & Arnbelonging to the family Combreteaceae. The plant is known in Sanskrit as Asana, in English as Black murdah, in Hindi as Asan, Saj, Sain and in Marathi as Ain.The plant has been known to possess various pharmacological activities like antifungal, antioxidant, antihyperglycaemic, antidiarrhoeal, antileucorrheal etc. The bark of the plant is astringent& useful in ulcers, vata, fractures, haemorrhages, bronchitis, diarrhoea etc.Preliminary phytochemical screening of the ethanolic extract of stem bark revealed the presence of carbohydrates, flavonoids, triterpenoids,steroids, tannins and saponins. The chemical entities isolated and characterised includes 4 – methy l - 4 - hydroxymethylene - 6β - (10 - methyl octanyl) cyclohexane (Arjuna homoses quiterpenol), di-n-octyl phthalate, di isobutyl phthalate and dibutyl phthalate. All these phytoconstituents are reported for the first time from the ethanolic extract of stem bark of T.tomentosa.

Keywords: Terminalia tomentosa,Combreteaceae, Di-n-octyl phthalate, Triterpenoids.

INTRODUCTION

Terminalia tomentosa Roxb (ex DC) Wight & Arn (Synonyms: *Terminalia alata* Heyne ex Roth, *Terminalia crenulata Roth, Terminalia ellipticaWilld.)* is a large deciduous tree, 20-35m high & 1m in diameterbelonging to family Combretaceae[1,2,3,4]. The bark is rough, dark grey to black in colour with deep vertical fissures & transverse cracks [5]. Leaves are simple, sub-opposite or the uppermost alternate, thick coriaceous, ovate-oblong or elliptic-oblong, rarely obovate, softly tomentose when young ;becoming more or less glabrous when mature, with 1-2 glands (

which are often turbinate or long stalked) usually on the midrib but sometimes absent. Flowers are hermaphrodite and in axillary fulvous-pubescent spikes or terminal panicles. Fruits are $1^1/2$ -2 inches long and ¾ inch wide with 5 broad, coriaceous, brown, glabrous wings striated with numerous straight lines running horizontally from the axis to the edges[6]. The plant is common in the forests, especially in the humid regions of India, including the sub-Himalayan tracts of North West provinces, Nepal & Sikkim, also Southwards throughout the Peninsula [7].It is a prominent part of both dry and moist deciduous forests in southern India up to 1000 m. The bark is bitter & stypic, useful in vitiated conditions of pitta, ulcers, vata, fractures, haemorrhages, bronchitis cardiopathy, strangury, wounds, haemoptysis, dysentery, cough, verminosis ,leucorrhoea, gonorrhoea & burning sensation (Ayurveda)[8,9]. Phytoconstituents such as tannins like arjunic acid, arjunolic acid, arjunetin, ellagic acid, gallic acid, and triterpenoids like oleanolic acid, betulinic acid and steroid like β-sitosterol have been reported to be present in *T.tomentosa*[10, 11, 12, 13, 14]. The plant is known to possess many pharmacological properties like antifungal [15], antioxidant [16], anti-hyperglycaemic[17], anti-diarrhoeal& anti leucorrheal[18]. From the literature survey, it was learnt that no substantial work has been carried out on the stem bark of *T.tomentosa* .Hence an attempt was made to investigate the phytoconstituents from ethanolic extract of *T.tomentosa*stem bark.

MATERIALS AND METHODS

All the melting points were recorded in Bio Technics India, Model no.BT2-38 melting point apparatus & were uncorrected. IR spectra of the compounds were recorded using the KBr pellet method on a Bruker α-T Spectrophotometer, at National facility for Clinical Trial, ISISM Chennai.[1]HNMR & LC-MS spectra of compounds were taken on Bruker 500 MHz PMR Spectrophotometer using $CDCL_3$ as solvent & Shimadzu LC 2020 at National facility for Clinical Trial, ISISM Chennai. ESI-MS spectra were recorded using ESI-MS Expression CMS Advion at SynZeal Research Laboratory, Ahmedabad. TLC was carried out using Aluchrosep Silica gel 60/UV254 from S.D. Fine Chemicals Pvt. Ltd, Mumbai. Column chromatography was carried out using glass column with a glass stop cock, 30×600mm from Merck Specialities Pvt. Ltd, Mumbai, packed with silica gel (200-400 mesh) from Molychem, Mumbai. All the chemicals & reagents used were obtained in high purity from S.D. Fine chemicals Pvt. Ltd, Bombay, Molychem & Chemport Pvt. Ltd, Mumbai.

Plant Material[6]

The stem bark of *T. tomentosa* was collected from Darbandora, Ponda- Goa during October 2012. It was authenticated by Prof G. I. Hukkeri, Dept. of Botany, Dhempe College of Arts & Science, Miramar-Goa.

The stem bark was then washed thoroughly to remove the soil and adhering materials and dried in shade. The dried stem bark was powdered and used for the preparation of ethanolic extract.

Preparation of ethanolic extract[19]

The dried stem barkpowder (500g) was extracted by maceration with ethanol (95%) for 3 days. After 3 days ethanolic layer was decanted off. The process was repeated thrice. The solvent from the total extract was distilled off using Rotary vacuum evaporator (Superfit) and the concentrate was evaporated to a syrupy consistency and then evaporated to dryness (80g)

.

Preliminary Phytochemical Screening (Qualitative Analysis)[20,21]

The preliminary phytochemical studies were performed for testing the different phytoconstituents present in the ethanolic extract of the stem bark of *T.tomentosa*as per the standard procedures. The results are tabulated in table 1.

Isolation of Compounds from Ethanol Soluble Fraction [19]

The ethanol soluble fraction (10g) was mixed with silica gel (2g).The sample was then loaded on columnpreviously packed with 150g of Silica gel (Molychem, 200-400mesh) prepared in petroleum ether (60-80^{o}C).The column was subjected to different solvent systems, starting first with petroleum ether 100% followed by petroleum ether:chloroform graded mixtures (95:5,90:10,80:20,70:30,60:40,50:50) then with chloroform 100% followed by graded mixtures of chloroform : ethyl acetate (95:5,90:10,80:20,70:30,60:40,50:50) & finally with ethyl acetate 100% & graded mixtures of ethyl acetate: methanol (99:1,98:2,97:3,96:4,95:5).The elutions were monitored by TLC (Silica gel-G), visualization by UV 254,366nm & vanillin–sulphuric acid spraying reagent heated at 110°C.Each time 10ml elutes were collected & identical elutes were combined (TLC monitored) & concentrated to 5ml & kept aside.

Elutions carried out with graded mixture of petroleum ether (60-80^{o}C): (80:20) chloroform, resulted a single component on TLC (petroleum ether (60-80^{o}C): chloroform, 80:20). After removal of the solvent, an off white powder was obtained, which was designated as **Compound AM 1** (70mg).

Elutions carried out withethyl acetate:methanol (99:1) resulted into a single component on TLC (ethyl acetate:methanol, 99:1). After removing solvent yellow liquid resulted, this was designated as **Compound AM 2** (62mg).

Elutions carried out with ethyl acetate:methanol (97:3) resulted into a single component on TLC (ethyl acetate:methanol, 97:3). After removing solvent yellow viscous liquid resulted, this was designated as **Compound AM 3** (55 mg).

Elutions carried out with ethyl acetate:methanol (95:5) resultedinto a single component on TLC (ethyl acetate:methanol, 95:5). After removing solvent pale yellow viscous liquid resulted, this was designated as **Compound AM 4** (45 mg).

Elutions carried out with other graded mixtures of solvents resulted in resinous mass which was not processed further.

RESULTS AND DISCUSSION

The preliminary phytochemical screening of the ethanolic extract of stem bark of T.tomentosa was performed and it was found to contain carbohydrates, flavonoids,triterpenoids, steroids, tannins and saponins. The chemical investigation led to the isolation of four compounds from the ethanolic extract of the stem bark of T.tomentosa. The isolated compounds were 4 – methyl – 4 – hydroxymethylene - 6β - (10 - methyl octanyl) cyclohexane (Arjunahomosesquiterpenol), di-n-octyl phthalate, di isobutyl phthalateand dibutyl phthalate.

Compound 1 (Arjuna homoses quiterpenol): m.p.152oC; IR (KBr): 3437.39 cm^{-1} (br, OH), 2925.14 cm^{-1} (C-H str. in CH3), 2858.08 cm^{-1} (C-H str. in CH2), 1458.11 cm^{-1} (C-H deformation in CH3), 1279.29 cm-1 (C-O str.); 1HNMR (CDCl3):δ 0.779 – δ 0.956 (m, 3H, H-14), δ0.977 (s,3H , H-17), δ 1.078 (brs, 3H, H-16), δ 1.258 (s, 12H, 6xCH2), δ 1.511-δ 1.550 (m, 4H, H-2, H-5 of the ring), δ 1.557

(s,4H, H-1, H-3 of the ring) ,δ 2.314-δ 2.393 (m,1H, H-10α), δ 2.413-δ 2.592 (m, 1H, H-6α) ,δ 3.730-δ 3.815 (m,1H, H2-15b) ,δ 3.828 (s,1H,OH), δ4.035-δ4.089 (m,1H,H2-15a);In the LCMS spectrum of Arjuna homoses quiterpenol, it exhibited molecular ion peak at m/z 255.10 $[M+H]^+$ which was consistent with molecular formula of C17H34O. Fragment ion peaks at m/z 239.20 $[M-Me]^+$, 225.10 $[M-C2H5]^+$, 223.25 $[M-CH2OH]^+$, 211.45 $[M-C3H7]^+$, 197.10 [M-C4H7], 169.45 $[M-C6H13]^+$, 155.30 $[M-C7H15]^+$, 141.25 $[M-C8H17]^+$, 127.25 $[M-C9H19]^+$ suggested that the molecule possessed a C9-side chain attached to a hydroxyl substituted dimethyl cyclohexane ring)[22]. (Figure 1).

Compound 2 (Di-n-octyl phthalate): b.p. 3790C; IR (KBr): 2927.17 cm^{-1} (C-H str. in CH3), 2860.76 cm-1 (C-H str. in CH2), 1727.64 cm^{-1} (C=O str.), 1628.74 cm-1 (C=C str.), 1459.24 cm^{-1} (C-H deformation in CH3), 744.07 cm^{-1} (C-H bending of aromatic ring); 1HNMR (CDCl3):δ 0.866- δ 0.997 (m, 10H, H-7', 7", 8', 8"),δ 1.364- δ 1.439 (m, 12H, H-4',4", 5', 5",6',6"),δ 1.674- δ 1.747 (m, 8H, H-2', 2", 3', 3"),δ 4.184- δ 4.256 (m, 4H, H-1', 1"),δ 7.510- δ 7.535 (m, 2H, H-4,5), δ 7.697- δ 7.723 (m, 2H, H-3,6); The ESI-MS spectrum showed molecular ion peak at m/z 391.4 $[M+H]^+$ in the positive ion mode which was consistent with molecular formula of C24H38O4.Fragment ion peaks at m/z 279.2, m/z167.1 exhibited in the mass spectrum were characteristic of alkyl phthalates & the base peak at m/z 149 is due to the protonated phthalic anhydride (C8H5O3)[23].(Figure 2).

Compound 3 (Di isobutyl phthalate): b.p. 319^{o}C; IR (KBr): 2928.50cm^{-1} (C-H str. in CH_3), 2861.96cm^{-1} (C-H str. in CH_2),1727.99cm^{-1} (C=O str.),1603.24cm^{-1} (C=C str.),1457.21cm^{-1} (C-H deformation in CH_3),1279.29cm^{-1} (C-O str.),1125.66cm^{-1} (C-H deformation in CH_2), 830.36cm^{-1} (C-H bending of aromatic ring); ^{1}HNMR ($CDCl_3$):δ 0.863-δ 1.684 (m, 12H, H-3', 3", 4', 4"),δ 3.574- δ3.621 (m, 2H,H-2',2"), δ 4.202- δ4.319 (m, 4H, H- 1',1"), δ 7.513-δ7.537 (m, 2H,H-3,4), δ 7.693- δ7.723 (m, 2H,H-2,5); The ESI-MS spectra showed molecular ion peak at m/z 278.3 $[M]^+$ which was consistent with molecular formula of $C_{16}H_{22}O_4$. The mass spectrum showed fragment ion peaks at m/z 167.1 & m/z 149 which are considered characteristic of alkyl phthalates & m/z 223.2 suggested that the alkyl phthalate is Di isobutyl phthalate. The ion peak at m/z 149 is due to the protonated phthalic anhydride ($C_8H_5O_3$)[24]. (Figure 3).

Compound 4 (Dibutyl phthalate): b.p.337^{o}C; IR (KBr):2925.79 cm^{-1} (C-H str. in CH_3),2858.25 cm^{-1} (C-H str.in CH_2),1728.23 cm^{-1} (C=O str.), 1637.65 cm^{-1} (C=C str.), 1458.18 cm^{-1} (C-H deformation in CH_3), 1073.39 cm^{-1} (C-O str.), 742.94 cm^{-1} (C-H bending of aromatic ring); ^{1}HNMR ($CDCl_3$):δ 0.896- δ 0.998 (m, 6H, H-4', 4"), δ 1.257- δ 1.424 (m, 4H, H-3',3"), δ 1.719 (s,4H, H-2',2"), δ 4.184- δ 4.320 (m,4H,H-1',1"), δ 7.505- δ 7.543 (m, 2H, H-4,5),δ 7.689- δ 7.723 (m,2H,H-3,6); The ESI-MS spectrum showed molecular ion peak at m/z 279.2 $[M+H]^+$ which was consistent with molecular formula $C_{16}H_{22}O_4$. The mass spectrum showed fragment ion peaks at m/z 167.1&m/z 149which are considered characteristic of alkyl phthalates & used in their characterization. The fragment ions at m/z 205.4 & 223.2 suggested that the alkyl phthalate is Dibutyl phthalate. The base peak at m/z 149 is due to the protonated phthalic anhydride ($C_8H_50_3$) [24]. (Figure 4).

CONCLUSION

The chemical investigation led to the isolation of four compounds from the ethanolic extract of the stem bark of *T.tomentosa*, which includes 4-methyl – 4 – hydroxymethylene - 6β - (10 -methyl octanyl) cyclohexane (Arjunahomosesquiterpenol), di-

n-octyl phthalate, di isobutyl phthalateand dibutyl phthalate. The isolated and characterised constituents can be categorised under the class of sesquiterpenoids and phthalate derivatives. The above compounds were isolated for the first time from theethanolic extract ofstem bark of *T.tomentosa*.

ACKNOWLEDGEMENTS

The authors are grateful to the Authorities of Government of Goa and the Principal, Goa College of Pharmacy for their immense support and providing the laboratory facilities. Authors are also thankful tothe Principal and staff members**,** National Facility for Clinical Trial, Interdisciplinary School of Indian System of Medicine, Chennai; and SynZeal Research Laboratory, Ahmedabad for providing the spectras. We are also grateful to Prof. G.I. Hukkeri, Dept. of Botany, Dhempe College of Arts and Science, Miramar-Goa for authenticating the plant material.

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Table 1. Result of Qualitative analysis for phyto-constituents isolated from the ethanolic extract of the stem bark of *T.tomentosa* W& A.

SR.NO	CHEMICAL TEST	INFERENCE
1.	Alkaloids	-
2.	Carbohydrates	+
3.	Flavonoids	+
4.	Triterpenoids	+
5.	Steroids	+
6.	Tannins	+
7.	Resins	-
8.	Proteins	-
9.	Glycosides	-
10.	Starch	-

Present = + Absent = -

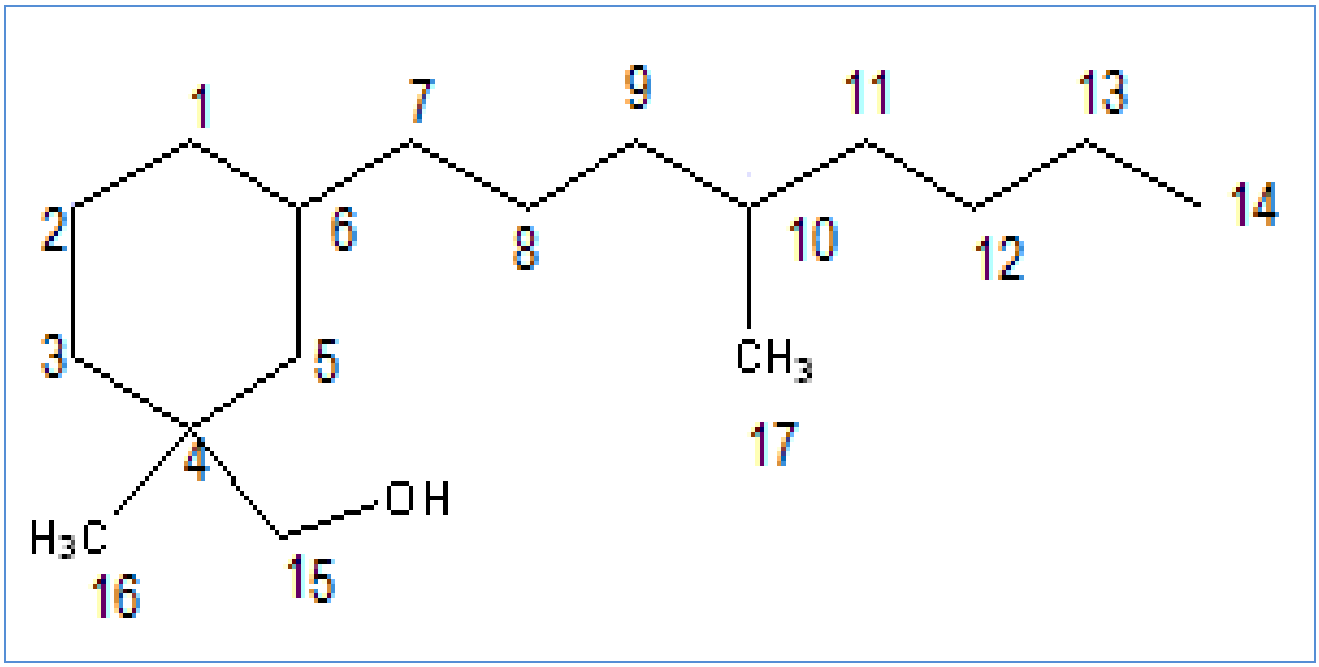

Figure.1. 4-methyl-4-hydroxymethylene-6β-(10-methyl octanyl) cyclohexane (Arjuna homosesquiterpenol)

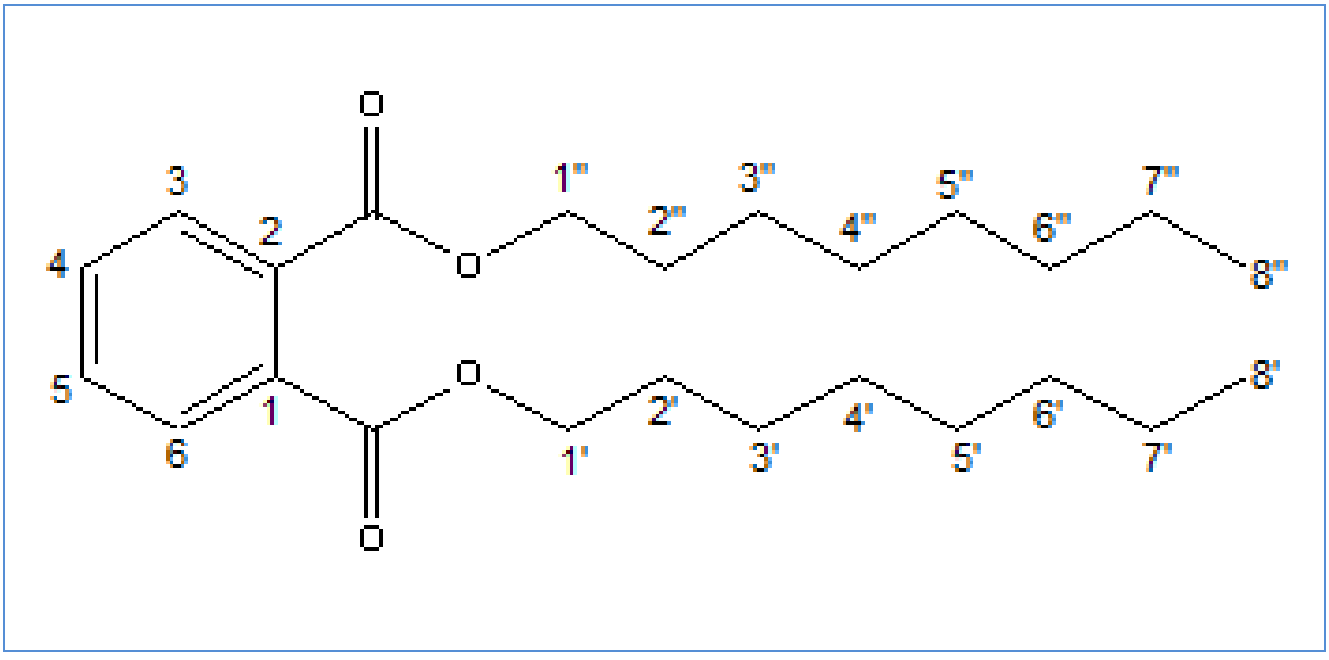

Figure.2. Di-n-octyl phthalate

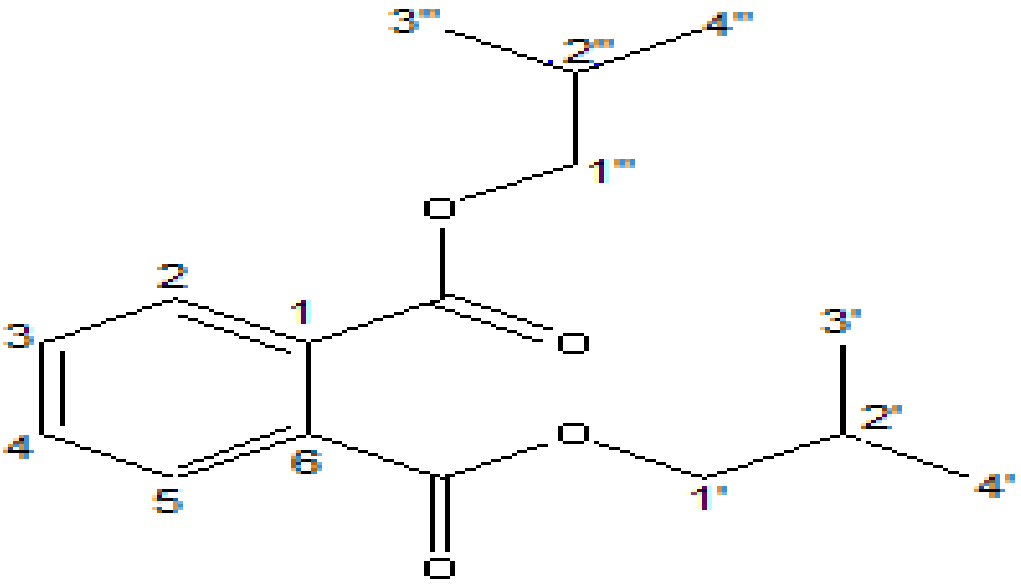

Figure.3. Di isobutyl phthalate

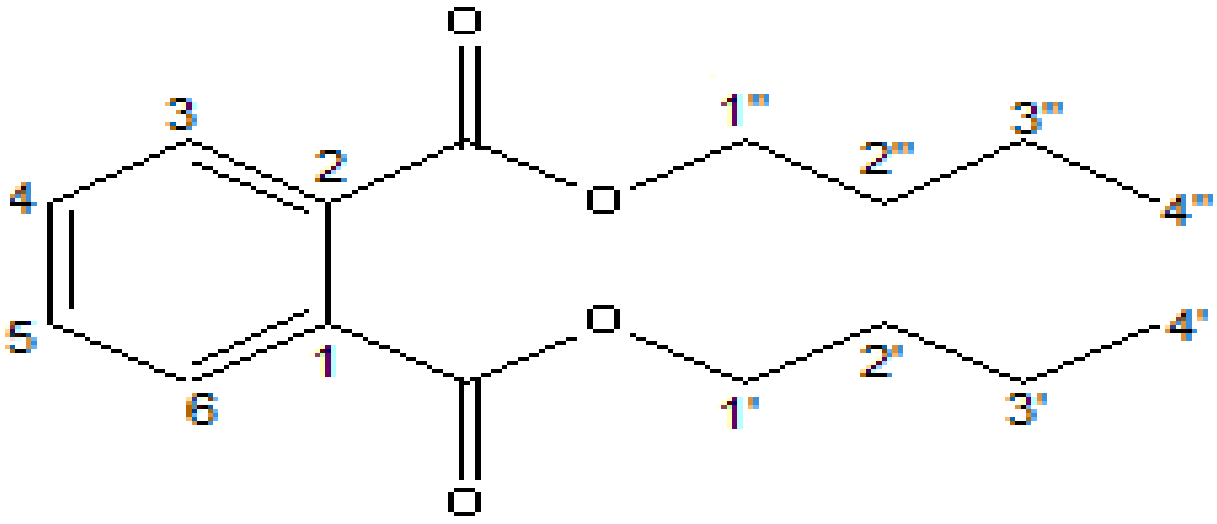

Figure.4. Dibutyl phthalate