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Phytochemical Investigation and Toxicological Studies of Lipid Constituents Isolated from *Leptadenia pyrotechnica*

[1]Amal M. Youssef Moustafa, [1]Ahmed I. Khodair and [2]Mahmoud A. Saleh
[1]Department of Chemistry, Faculty of Science,
Suez Canal University, BP 41522, Ismailia, Egypt
[2]Laboratory of Environmental Chemistry and Toxicology,
Texas Southern University, Houston, TX, USA

Abstract: Investigation of the chemical constituents of *Leptadenia pyrotechnica* (Asclepiadaceace family) let to isolate three terpenes; phytol, squalene and taraxerol, five sterols; cholesterol, campasterol, stigmasterol, β-sitosterol and fucosterol. Fifteen fatty acids were isolated (C_{14}-C_{25}), eleven n-alkanol (C_{29}-C_{39}), series of n-alkanes (C_{12}-C_{36}), one n-alkene; 3-tetradecene. Also, for the first time eighteen aromatic hydrocarbons were isolated. 5-phenyl-undecane and 6-phenyl-tridecane are the major constituents. The structures of these compounds were established by gas chromatography; GC-FID and GC-MS, spectroscopic techniques; Infra-red (IR) and comparison with the published data. The unsaponifiable matter was divided into two parts: The first part was directly subjected to GC/FID and GC/MS chromatographic analysis. While the second part, was subjected to column chromatographic fractionation. The isolated fractions were identified as fatty alcohols, hydrocarbons, terpenes and sterols. Adduction method was used to separate the straight-chain fatty acids. The acute toxicity study of the total lipid extract was examined on brine shrimp, the LC_{50} was 35.48 ppm. The extract is highly toxic.

Key words: *Leptadenia pyrotechnica*, Asclepiadaceace, brine shrimp, fatty alcohols, fatty acids, squalene, taraxerol

INTRODUCTION

The Asclepiadaceae family comprises many medicinal plants with a wide range of therapeutic activities. Many Asclepiadaceous constituents have been intensively investigated as possible anti-tumor agents and also as potential bioactive chemicals (Gopiesh and Kannabiran, 2007; Paulo and Houghton, 2003; Bazzaz and Haririzadeh, 2003; Atta and Mouneir, 2005). These active constituents are cardenolides, polyoxypregnane derivatives, alkaloids, flavonoids, sterols and triterpenes. Therefore, it was feasible to study one of the plants belonging to this family in a trial to find the active chemical principles as well as their biological activities. The plant chosen for this study was *Leptadenia pyrotechnica*.

The plant is a typical desert shrub (Dhawan and Singh, 1976) growing in different parts of Egypt especially in the eastern desert and Sinai Peninsula. It is known in the Arabic language as Markh, Assabay and Kalenba (Abu-Rabia, 2005; McLaughlin, 2006; Medina, 2003). Two species: *L. pyrotechnica* (Forssk) Decne and *L. heterophylla* (Del.) Decne are wildly growing in Egypt (Goyder and Singh, 1991; Täckholm, 1974).

Corresponding Author: Amal M. Youssef Moustafa, Department of Chemistry, Faculty of Science, Suez Canal University, BP 41522, Ismailia, Egypt Tel: 002066-3727487

Many medicinal uses of *Leptadenia pyrotechnica* are reported in traditional medicine, the branches of the plant are diuretic and the bedouins use the infusion of branches for treatment of retention of urine and to help expel uroliths (Aquino *et al*., 1996; Panwara and Tarafdarb, 2006). Also, the plant yields a fibre which is used in indigenous medicines as an antihistaminic and expectorant (Manavalan and Mithal, 1980; Farnsworth, 1996; Al-Yahya, 1986). Furthermore, it is used for treatment of gout and rheumatism (Cioffi *et al*., 2006).

To the best of our knowledge there are no concrete phytochemical studies on *Leptadenia pyrotechnica*, other than few studies which report the detection of some phenolic compounds, flavonoid; quercetin-3-*O*-galactoside, alkaloids, amino acids, sterols; β-sitosterol and triterpenoids; taraxerol, fernenol and leptadenol (Abd El-Ghani and Amer, 2003; El-Hassan *et al*., 2003; Noor *et al*., 1993; Panwara and Tarafdarb, 2006; Boulos, 1983).

A method, utilizing brine shrimp (*Artemia salina* LEACH) (Alluri *et al*., 2006), is proposed as a simple bioassay for determining LC_{50} values in μg mL^{-1} of petroleum ether, methanol and methanol after defatted extracts of *Leptadenia pyrotechnica* plant.

MATERIALS AND METHODS

Plant Material

Fresh aerial parts of *Leptadenia pyrotechnica* (Asclepiadaceae) were collected in September, during the flowering stage, from Wadi Khashab and Wadi Matzos, Sharm El-Sheikh to El-Tur road, Southern Sinai, Egypt. The identity was established by Dr. Samia Heneidak, Department of Botany, Faculty of Science, Suez Canal University. A voucher specimen (No. AMYM-1004) has been deposited in the Herbarium of Botany Department, Faculty of Science, Suez Canal University, Ismailia, Egypt.

General Methods

Melting points were determined on Büchi 535 melting point apparatus and are uncorrected. IR spectra were recorded on infra-red spectrophotometer, 1430 Ratio Recording, Perkin-Elmer; Infra-red data station Epson FX-86e, in KBr disks and Infra-red spectrophotometer, Perkin-Elmer system 2000 NIR FT-Raman, FT-IR. Gas-liquid chromatographic analysis (PYE UNICAM PU 4550 PACKED), using coiled glass column (2.8 m×0.4 mm ID), packed with Diatomite C (100-120 mesh) and coated with 1% OV-17, programmed at 10°C min from 70 to 270°C, then isothermally at 270°C for 30 min, injector temperature at 250°C, FID detector at 300°C and the nitrogen carrier gas at a flow rate of 30 mL min^{-1}. GC/MS data were collected on HP 5972 GC/MS. Ionization energy was 70 eV, ionization current 300 μA, detector voltage 1000V, fitted with a 30 m length column HP-5 (0.25 mm ID, 0.25 μm film thickness), coated with cross-linked 5% phenyl substituted methyl polysiloxane. Injection temperature at 200°C, detector temperature at 280°C and helium carrier gas with a flow rate of 1.0 mL min^{-1}, linear velocity 36.2 cm sec^{-1}, split less. The oven temperature program was set at 80 to 260°C at 10°C min^{-1}, then isothermally at 260°C for 30 min. For methyl esters of fatty acids; HP-1 MS capillary column (50 m length, 0.25 mm ID and 0.25 μm film thickness), was used and coated with Cross-linked 100% dimethyl polysiloxane. Also, PONA MS capillary column (50 m length, 0.15 mm ID and 0.50 μm film thickness) coated with polyimide coated fused silica; was used. The identification of the chemical constituents was based on comparisons of their relative retention times and mass spectra with those obtained from authentic sample and/or the NBS and Wiley libraries spectra (Massada, 1976) as well as by comparison of their retention indices with literature data (Aromdee and Sriubolmas, 2006; Viña and Murillo, 2003; Bisio *et al*., 1998). TLC was performed on Merck precoated silica gel 60 F_{254} aluminium foil plates and detection was achieved by vanillin/sulphuric acid (Macek, 1972). A glass column (CC) (80×2 cm) was used.

Extraction, Isolation and Characterization

About 2 kg of the aerial parts of *Leptadenia pyrotechnica* were shock-frozen with liquid nitrogen, lyophilized and pulverized then percolated with methanol, then filtered and evaporated under vacuum. The methanol extract yielded about 1100 g (55%) was defatted with petroleum ether (40-60°C). The methanol extracts after defatted yielded 950 g (47.5%) Petroleum ether extract yielded 75.3 g (3.77%) lipid fraction.

The phytochemical screening was performed in accordance with AOAC (1990).

Lipids

The lipid fraction (dark green oily residue) was treated with hot acetone to effort 9 g (30%) of acetone insoluble fraction and 19.5 g (65%) of acetone soluble fraction as oily material. Fifteen grams of the last fraction were saponified to yield 6.96 g (46.4%) of yellowish brown, semi-solid residue of unsaponifiable matter and 7.99 g (53.27%) of semi-solid residue of fatty acids.

The unsaponifiable matter was divided into two parts: The first part of unsaponifiable matter was subjected to GC/FID and GC/MS chromatographic analysis, using the same conditions. While the second part, was subjected to column chromatographic fractionation. About 4 g of the unsaponifiable matter (second part) was applied onto a glass column (80×2 cm) packed with silica gel in petroleum ether. Elution was performed first with petroleum ether followed by mixtures of petroleum ether/benzene in increasing ratios of the latter then with benzene-methanol (75:25). The course of the chromatographic fractionation was followed by Thin Layer Chromatography (TLC).

Investigation of Total Fatty Acids

Gas chromatographic analysis has been applied in the field of fatty acids by converting the acids to their methyl esters (Moustafa *et al*., 2007).

Straight-chain fatty acids were separated by urea using adduction method in accordance with Braga *et al*. (1996) and Majinda *et al*. (1997).

The ether extracts yielded 530 mg of ester (I) which represented 53% of crude methyl ester of saturated fatty acids. The mother liquor and the washing of the ethereal layer were combined and acidified with 2.5% sulphuric acid till (pH 2) yielded 250 mg of ester (II) which represented 25% of crude methyl ester of unsaturated fatty acids. Moreover, this method was repeated using thiourea.

Toxicity Test: Brine Shrimp

The brine shrimp, *Artemia salina* LEACH, is a crustacean belonging to the subclass Branchiopoda order Anostraca, it is found worldwide in bodies of water ranging from the brackish to the ultra saline (from 10-20 to 180-220 g L^{-1}).

The eggs of brine shrimp were obtained from San Francisco bay Brand, Inc., 8239 Enterprice Drive, Newark, USA. Eggs in desiccators should be stored in a refrigerator (5°C).

Preparation of Test Samples

Samples of *Leptadenia pyrotechnica* extracts, petroleum ether (lipid fraction), methanol, methanol after defatted, were tested.

In general, weighed amounts of plant extracts, were first dissolved in a small amount (50 mg to 5 mL) of methanol (Solution A). Solution B was prepared by diluting 0.5 mL of A to 10 mL with methanol.

Dissolution or emulsification could be assisted by ultrasound. Any insoluble material, especially fibers, must be removed by filtration. Fibers can entrap the swimming nauplii and kill them. Usually three concentrations were obtained for each series of tests. We had always attempted to start with a stock solution of at least 1000 ppm for an extract. Appropriate amounts of solution (100 μL B, 50 μL

A and 500 μL A for 10,100 and 1000 μL mL^{-1}, respectively) were transferred to 1.25 cm discs of filter paper (SCHLEICHER and SCHUELL, No. 740-E). The discs were dried in air, placed in 2 g vials and then dried further *in vacuo* for 1 h. Control discs were prepared using only methanol. Five replicates were prepared for each dose level. The negative control solution was simply the same saline solution used to prepare the stock test sample solution. The standard positive control made used of a heavy metal salt as a toxicant. The use of potassium dichromate was convenient.

Bioassay Procedure

The assay was begun after sowing of the cysts (i.e., with larvae that have 20 to 32 h old). Multiwelled culture plates could be used for the bioassay, although any clear glass container with flat bottoms (for example, small beakers or glass vials) could be used. Ten nauplii were collected, using disposable Pasteur pipette (Scientific products, diSPo pipette), from the hatching dish and were transferred to a well or to each sample vial, using the minimum amount of seawater. Two milliliters of the test solution were added then artificial sea water was added to make 5 mL. The nauplii could be counted macroscopically in the stem of the pipette against a lighted background. A drop of dry yeast suspension (Red Star) (3 mg in 5 mL artificial sea water) was added as food to each vial. The solutions were maintained under illumination and the time was noted. This was repeated for four additional wells, thereby requiring 50 nauplii for each concentration of test sample. A parallel series of tests with the standard potassium dichromate solution (1000, 100 and 10 ppm) and the blank control are always conducted.

To determine the acute LC_{50}, the numbers of dead nauplii were counted in each well after 6 h. Counting of the chronic LC_{50} was begun after initiation of the test (24 h). Nauplii were considered dead if they were lying immobile at the bottom of the well. A hand lens or a 3x magnifying glass was useful to check for inactivity of the appendages; the antennae, antennulae and the mandibles (Fig. 1).

LC_{50} Determinations

LC_{50}`s intervals were determined from the 24 h counts using the probit analysis method described by Wu *et al*. (2002).

Reed-Muench method was the procedure assumes that an animal that survived a given dose would also have survived any lower dose and conversely, that an animal that died with a certain dose would have also died at any other higher dose (Bowden and Garnett, 2000; Pisutthanana, 2004).

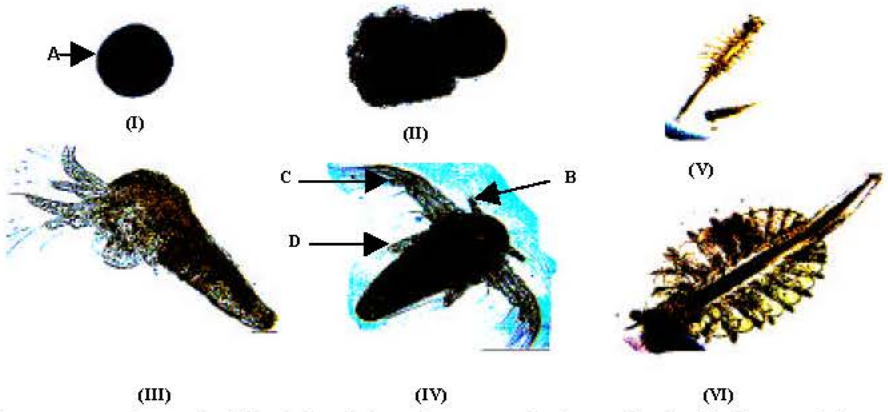

Fig. 1: Stages of growth of the brine shrimp (*Artemia salina*). Freshly hatched instar (IV) nauplius showing antennulae (B), antennae (C) and mandibles (D), Next to a hydrated Cyst (A). (VI) addult male brine shrimp (8-10 mm long) with the antennae transformed into a pair of graspers and showing the stalked lateral eyes and 11 pairs of thoracic legs

RESULTS

The results obtained from the preliminary phytochemical screening of *Leptadenia pyrotechnica* revealed the presence of cardiac glycosides, flavonoids, alkaloids, sterols and/or triterpenes, carbohydrates and/or glycosides, coumarins, saponins, catechol and pyrogallol tannins were present as major constituents. While, aerial parts are free from crystalline sublimate, volatile oils, volatile amines, cyanogenic glycosides, anthraquinons and iridoids. During the investigation of the lipid constituents of *Leptadenia pyrotechnica*, both of GC/FID and GC/MS were applied. However, GC/MS gave the best results which are more reliable and easier to be interpreted.

GC/MS analysis results of acetone insoluble fraction were represented in Fig. 2 and Table 1, revealed that the acetone insoluble fraction was a mixture of a series of fatty alcohols, which represented as n-alkanol (n-C_{29}- n-C_{39}) with pentatriacontanol as the major constituent (51.51%), while the relative percent of the other constituents ranged from 0.46 to 19.01%. IR-spectrum of total fatty alcohols fraction showed the O-H stretching vibrations of unbounded or free hydroxyl group which absorbed strongly in 3649-3566 cm^{-1} region. Intermolecular hydrogen bonding appeared at lower frequencies, 3454-3214 cm^{-1}. O-H in-plane bending vibration occurred in the region

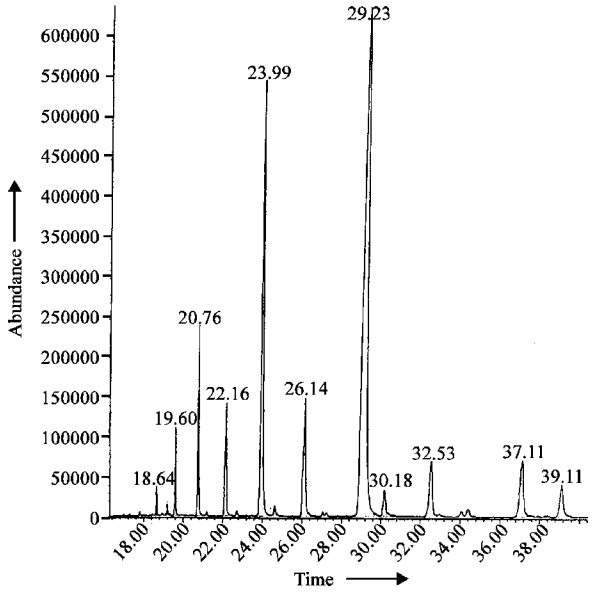

Fig. 2: GC/MS of acetone insoluble fraction

Table 1: GC/MS of acetone insoluble fraction

Fatty alcohol	Retention time (min)	Relative area (%)
Nonacosanol	18.64	0.46
Triacontanol	19.60	1.47
Hentriacontanol	20.76	3.95
Dotriacontanol	22.16	4.04
Tritriacontanol	23.99	19.01
Tetratriacontanol	26.14	6.39
Pentatriacontanol	29.23	51.51
Hexatriacontanol	30.18	1.54
Heptatriacontanol	32.53	4.25
Octatriacontanol	37.11	4.86
Nonatriacontanol	39.11	2.52

of 1417-1339 cm^{-1}, the second at 1330 cm^{-1}, beside, a broad absorption band in 765-722 cm^{-1}. This is due to of out-of-plane bending of the bonded O-H group. C-O stretching vibrations produced a strong band in 1160-1150 cm^{-1} region of the spectrum. Bands apparently resulted from interaction between O-H bending and C-O stretching showed at 1390-1330 cm^{-1}. C-H stretch occurs in the region 2956-2924 and 2856-2852 cm^{-1}. C-H bend appeared at δ_s CH_2 (1468 cm^{-1}) and δ_{as} CH_3 (1452 cm^{-1}). The CH_2 rock showed at 722 cm^{-1} and C = C stretch occurs at 1645 cm^{-1}.

The acetone soluble fraction (15 g) was saponified, yielded unsaponifiable matter as a yellowish brown, semisolid residue (6.96 g; 46.4%) and a semisolid residue of saponifiable material (7.99 g; 53.27%) of the fatty acids.

GC/MS analysis results of the first part of unsaponifiable matter (acetone soluble fraction) (Fig. 3a-c) revealed that it was a mixture of a series of n-alkanes; n-C_{13} to n-C_{32} (19.16%) beside n-alkenes (1.45%) and aromatic hydrocarbons (0.83%), mixture of diterpene; phytol (4.39%) and triterpenes; squalene (9.54%), taraxerol (4.65%) and lupeol (34.56%) and sterol; cholesterol (1.17%), campesterol (3.07%), stigmasterol (1.38%) and β-sitosterol (9.99%) as shown in Table 2.

The results obtained from column chromatographic fractionation of the second part showed that there are four fractions (A-D), were isolated which represented hydrocarbon, hydrocarbon and triterpenoid, triterpenoid and sterol respectively.

The hydrocarbon fraction A; (76 mg, R_f; 0.93, 0.82, toluene-acetone (90:10), S.G.), eluted with petroleum ether-benzene (90:10) gave upon crystallization from CH_2Cl_2/MeOH, white powdered, m.p. 64-66°C and gave a single spot on TLC.

GC/MS results (Fig. 4a-c and Table 3) revealed that the hydrocarbon fraction is a mixture of a series of n-alkanes; n-C_{12}- n-C_{36} (87.79%) with 9-butyl-docosane and nonacosane as the major constituents (33.75, 16.35%) respectively, beside n-alkenes (0.41%) and aromatic hydrocarbons which represented 11.8% with 1-butylheptyl-benzene and 1-pentyloctyl-benzene as the major constituents which represented 1.42 and 0.84%, respectively. IR-spectrum showed absorption bands for C-H stretching at frequency 3000-2850 cm^{-1}, beside two absorption bands appeared at 1462.98 cm^{-1} δ_s-CH_2 and 1378 cm^{-1} δ_s-CH_3 for C-H bending and 728.25cm^{-1} for -CH_2 rocking. These bands are characteristic for n-alkane hydrocarbons. C-C bending vibrations occurred at very low frequencies below 500 cm^{-1}. The bands assigned to C-C stretching vibrations are weak and appeared in the broad region of 1200-800 cm^{-1}. C = C stretching vibration appeared at 1642, 990.89 and 910.31 cm^{-1}. Methylene rocking appeared at 722 cm^{-1}, which are characteristic for n-alkene hydrocarbons. The most prominent in the spectra of aromatic compounds occur in the low-frequency ranged from 850 to 676 cm^{-1}.

Fraction B (45 mg, R_f; 0.82, 0.74, 0.66, toluene-acetone (90:10), silica gel (S.G.)), eluted with petroleum ether-benzene (50:50), gave positive *Lieberman burchardt* reaction. GC/MS results of fraction B (Fig. 5) revealed that it was a mixture of squalene and taraxerol (retention times; 29.20 and 37.40 min) as triterpenoids with relative percentages 36.28 and 35.03%, respectively, beside three hydrocarbons (retention times; 19.62, 20.77 and 23.99) which represented 6.7% as contaminants. IR-spectrum showed O-H stretching vibrations, unbounded or free hydroxyl group absorbed strongly in 3648-3589 cm^{-1} region. Intermolecular hydrogen bonding appeared at lower frequencies, 3287-3305 cm^{-1}. C-O stretching vibrations produced strong bands in 1188.97-1085 cm^{-1} region of the spectrum. Bands apparently resulted from interaction between O-H bending and C-O stretching showed at 1381.27-1331.27 cm^{-1} and 1257-1191 cm^{-1}. C-H stretch appeared at 2949.64 cm^{-1} ν_{as} CH_3, 2867cm^{-1} ν_s CH_3, 2931 cm^{-1} ν_{as} CH_2 and 2851 cm^{-1} ν_s CH_2. C-H bend occurred at 1467.94 cm^{-1} δ_s CH_2 and1450 cm^{-1} δ_{as} CH_3. CH_2 rock appeared at 741 cm^{-1} ρ CH_2 and the C = C stretch showed at 1638.90 cm^{-1}.

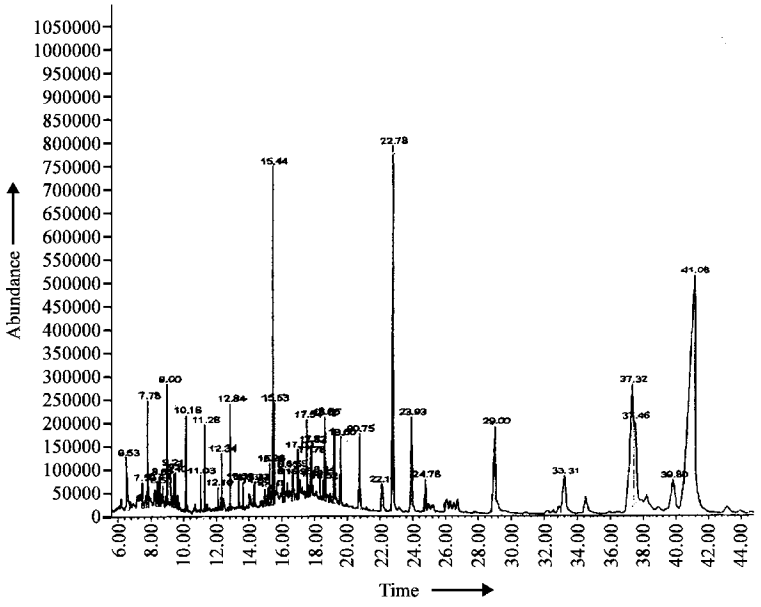

Fig. 3a: GC/MS results of the first part of total unsaponifiable matter

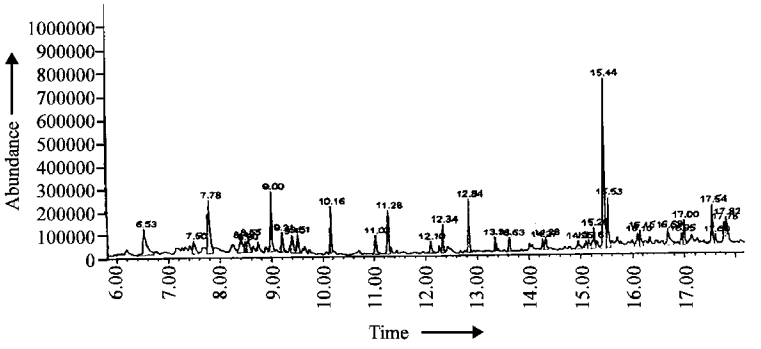

Fig. 3b: GC/MS results of the first part of total unsaponifiable matter (R_t fron 6 to 18 min)

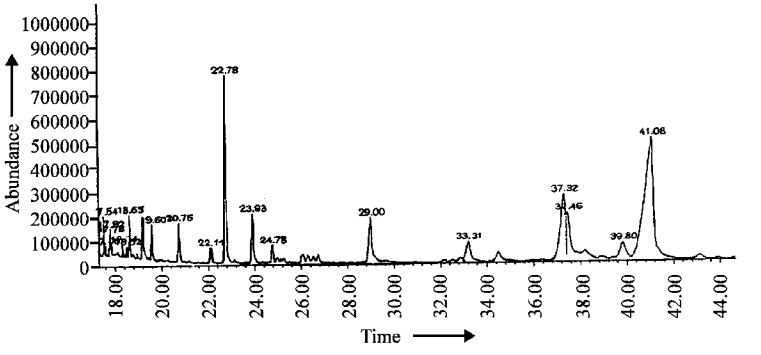

Fig. 3c: GC/MS results of the first part of total unsaponifiable matter (R_t from 18 to 44 min)

Table 2: GC/MS results of the first part of total unsaponifiable matter

Components	Retention time (min)	Relative area (%)
Tridecane	6.53	1.45
2,6,10-trimethyl-dodecane	7.49	0.47
Tetradecane	7.78	1.69
Unidentified	8.49	0.25
Unidentified	8.55	0.55
Pentadecane	9.00	1.41
Unidentified	9.21	0.47
Unidentified	9.40	0.53
Unidentified	9.51	0.54
Hexadecane	10.16	0.91
4-ethyl-1,2-dimethyl-benzene	11.03	0.49
Heptadecane	11.28	0.82
4-ethenyl-1,2-dimethyl-benzene	12.10	0.34
Octadecane	12.34	0.49
Unidentified	12.84	1.02
Nonadecane	13.36	0.23
Unidentified	13.64	0.37
1-nonadecene	14.27	0.21
Eicosane	14.33	0.29
1-nonadecanol	15.16	0.22
Hencosane	15.26	0.61
Phytol	15.44	4.39
3-eicosene	16.10	0.38
Docosane	16.15	0.43
5-eicosene	16.95	0.31
Tricosane	17.00	0.51
2-docosene	17.78	0.26
Tetracosane	17.82	0.29
1-docosene	18.34	0.29
Unidentified	19.60	1.28
Heptacosane	20.75	1.59
Squalene	22.78	9.54
Nonacosane	23.93	2.94
Triacontane	26.76	0.61
Dotriacontane	29.00	4.42
Cholesterol	33.21	1.17
Campesterol	33.31	3.07
Stigmasterol	34.58	1.38
β-sitosterol	37.32	9.99
Taraxerol	37.46	4.65
Lupeol	41.08	34.56
Unidentified	46.06	4.58

Fraction C (59 mg, R_f; 0.66, toluene-acetone (90:10), S.G.), eluted with petroleum ether-benzene (25:75), white crystalline needles (CH_2Cl_2/MeOH), m.p. 174-176°C and gave positive Lieberman burchardt reaction. GC/MS analysis results of triterpenoid fraction C (Fig. 6) revealed that it was a mixture of taraxerol and lupeol (retention times; 37.37 and 40.80 min) with 18.73 and 77.93%, respectively.

Fraction D (62 mg, R_f; 0.47 and 0.31, toluene-acetone (9:1), S.G.), eluted with 100% benzene, white crystalline needles (CH_2Cl_2/MeOH), m.p.133-135°C and gave positive Lieberman burchardt reaction.

GC/MS results (Fig. 7), revealed that the sterol fraction D, was a mixture of cholesterol, campesetrol, stigmasterol, β-sitosterol and fucosterol (retention times; 33.13, 33.34, 34.59, 37.69 and 38.15 min) with relative percentages 0.14, 17.32, 9.75, 69.62 and 3.17%, respectively. IR-spectrum showed the O-H stretching, unbounded or free hydroxyl group absorbed strongly in the

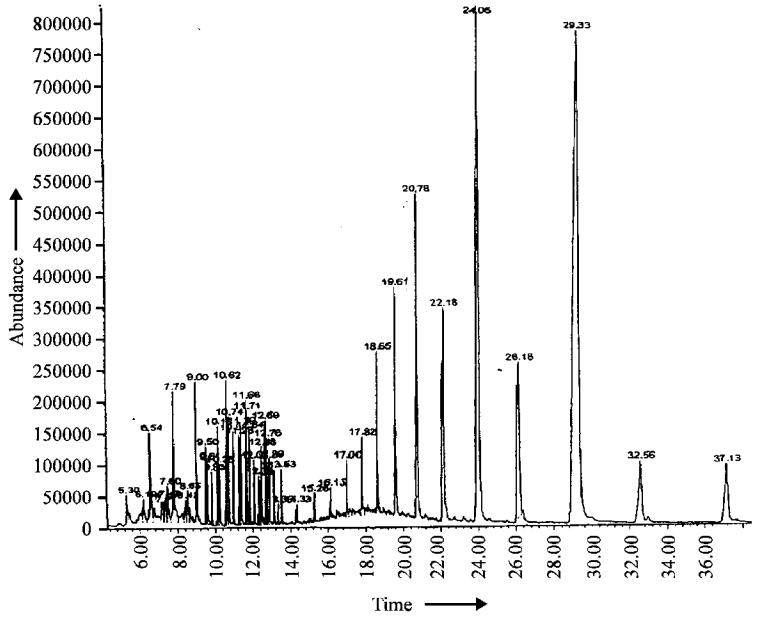

Fig. 4a: GC/MS results of hydrocarbon (Fraction A)

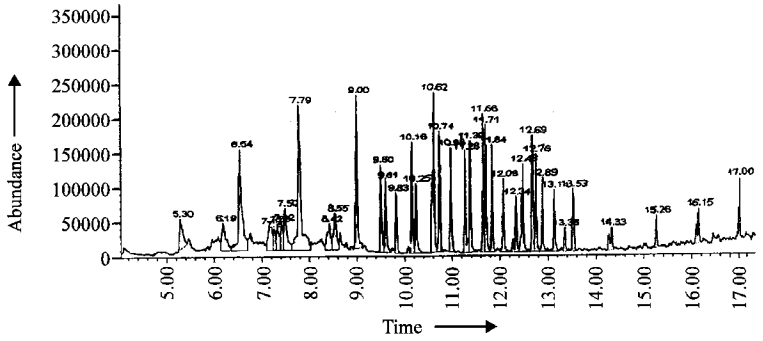

Fig. 4b: GC/MS results of hydrocarbon (Fraction A, R_t 4 to 17 min)

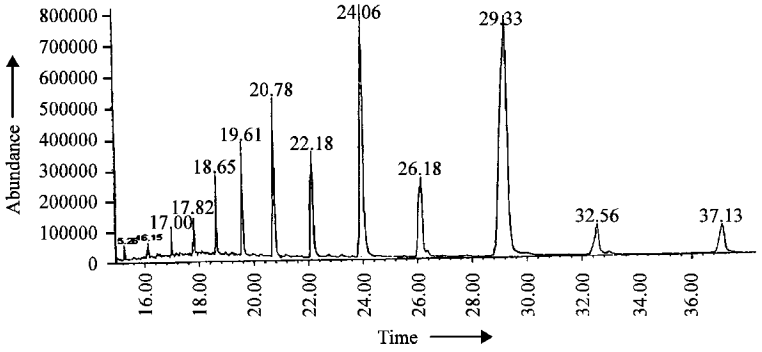

Fig. 4c: GC/MS results of hydrocarbon (Fraction A, R_t from 16 to 37.5 min)

Table 3: GC/MS results of hydrocarbon fraction

Components	Retention time (min)	Relative area (%)
Dodecane	5.30	0.42
2,6,11-trimethyl-dodecane	6.20	0.53
Tridecane	6.54	1.72
4,8-dimethyl-undecane	7.27	0.20
2,6,10-trimethyl-dodecane	7.50	0.56
Tetradecane	7.79	2.24
3-tetradecene	8.42	0.41
Pentadecane	9.00	1.19
1-butylhexyl-benzene	9.51	0.61
1-propylnonyl-benzene	9.61	0.55
1-ethyloctyl-benzene	9.83	0.44
Hexadecane	10.16	0.72
1-methylnonyl-benzene	10.24	0.51
1-butylheptyl-benzene	10.62	1.42
1-propyloctyl-benzene	10.74	0.78
1-ethylnonyl-benzene	10.98	0.62
Heptadecane	11.28	0.65
1-methyldecyl-benzene	11.39	0.72
1-pentylheptyl-benzene	11.65	0.77
1-butyloctyl-benzene	11.71	0.79
1-propylnonyl-benzene	11.84	0.59
1-ethyldecyl-benzene	12.08	0.55
Octadecane	12.34	0.42
1-methylundecyl-benzene	12.48	0.69
1-pentyloctyl-benzene	12.69	0.84
1-butylnonyl-benzene	12.76	0.58
1-propyldecyl-benzene	12.90	0.47
1-ethylundecyl-benzene	13.14	0.42
Nonadecane	13.36	0.16
1-methyldodecyl-benzene	13.53	0.45
Eicosane	14.33	0.27
Hencosane	15.26	0.21
Docosane	16.15	0.34
Tricosane	17.00	0.54
Tetracosane	17.82	1.01
Pentacosane	18.65	1.62
Hexacosane	19.61	2.88
Heptacosane	20.78	5.24
Unidentified	22.18	5.47
Nonacosane	24.06	16.35
Dotriacontane	26.18	5.88
9-butyl-docosane	29.33	33.75
Pentatriacontane	32.56	2.65
Hexatriacontane	37.13	2.77

3650-3584 cm^{-1} region and intermolecular hydrogen bonding appeared at lower frequencies (3306.82 cm^{-1}). C-O stretching vibrations produced a strong band in 1133.36-1022.55 cm^{-1} region of the spectrum. The bands apparently resulted from interaction between O-H bending and C-O stretching appeared at 1380.61 and 1133.36 cm^{-1}. O-H in plane bending vibration occurred in 1380.61 cm^{-1}. C = C stretch appeared at 1642.01 cm^{-1} and absorption bands characteristic for $-CH_3$ showed in 1380.61-802.68 cm^{-1} region.

GC/MS results of methyl esters of total fatty acids (Fig. 8a and Table 4), revealed they were mixture of n-C_{13} to n-C_{25}. The saturated fatty acids represented 65.09% of the total fatty acids. Stearic and palmitic were the major constituents (21.49 and 19.84%, respectively). Unsaturated fatty acids represented 34.91% and the major constituents were oleic, linolenic and linoleic which represented 12.19, 10.76 and 10.01, respectively from the total fatty acids.

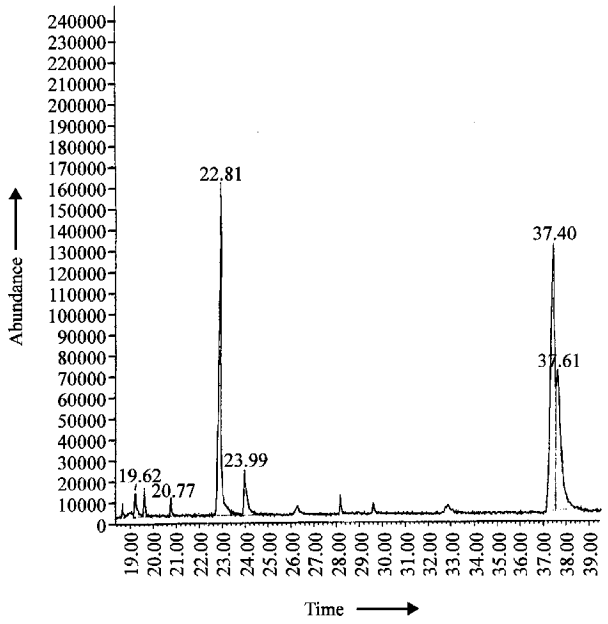

Fig. 5: GC/MS results of fraction B

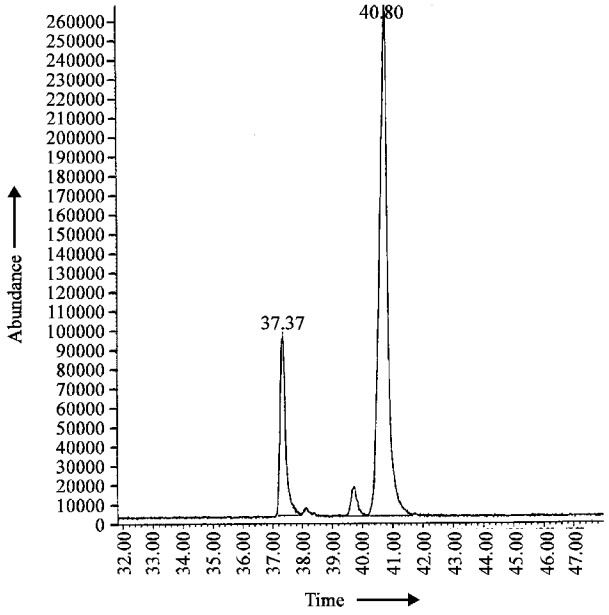

Fig. 6: GC/MS results of fraction C

GC/MS results of methyl esters of fatty acids (I) (Fig. 8b), revealed that it was a mixture of saturated fatty acids; lauric, myristic, pentadecanoic, palmitic, heptadecanoic, stearic, arachidic and behenic (retention times, 19.02, 24.37, 30.16, 33.79, 40.71, 50.85, 53.85 and 59.67 min), with relative area percentage 0.35, 9.68, 9.47, 38.76, 2.34, 27.39, 4.16 and 7.85%, respectively. Palmitic and stearic

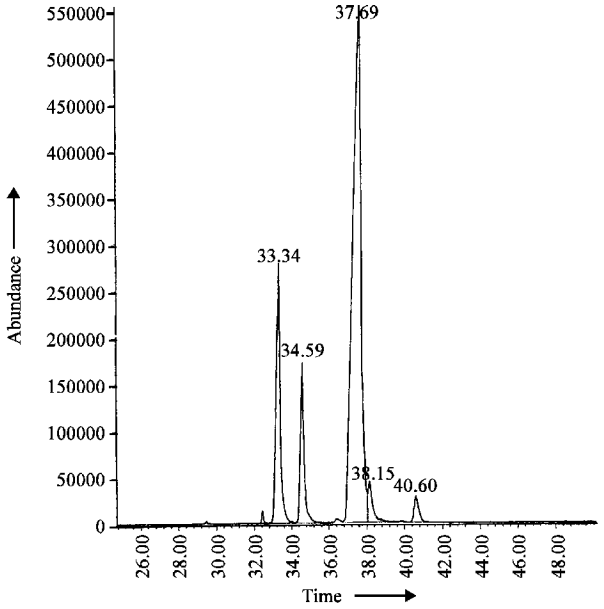

Fig. 7: GC/MS results of fraction D

Table 4: GC/MS results of methyl esters of total fatty acids

Components	Retention time (min)	Relative area (%)
Lauric	20.68	0.33
Myristic	24.98	7.99
Pentadecanoic	27.34	0.41
Palmitic	28.95	19.84
7-hexadecenoic	29.21	0.31
Heptadecanoic	30.69	1.78
Stearic	32.58	21.49
Oleic	32.85	12.19
Linoleic	33.59	10.01
Linolenic	34.63	10.76
Arachidic	35.82	4.62
Behenic	38.92	3.34
Tricosanoic	39.78	2.60
Lignoceric	41.83	2.69
11,14,17-eicosatrienoic	42.13	1.64

represented the major constituents (38.76 and 27.39%), respectively. Methyl esters of fatty acids (II) (Fig. 8c), contained the unsaturated fatty acids. Oleic and linoleic represented the major constituents (18.04 and 66.34%), respectively with retention times 24.95 and 25.09 min. Also, two saturated fatty acids; pentadecanoic and heptadecanoic which represented the relative area percentages 10.03 and 5.59%, respectively, with retention times 19.96 and 22.47 min were isolated.

The logarithms of retention times of methyl esters of fatty acids (I) which contained a mixture of straight saturated fatty acids from $-C_{13}$ to $-C_{23}$ were plotted against the number of carbon atoms. A straight line was obtained. This suggested that, all these acids belong to one homologous series.

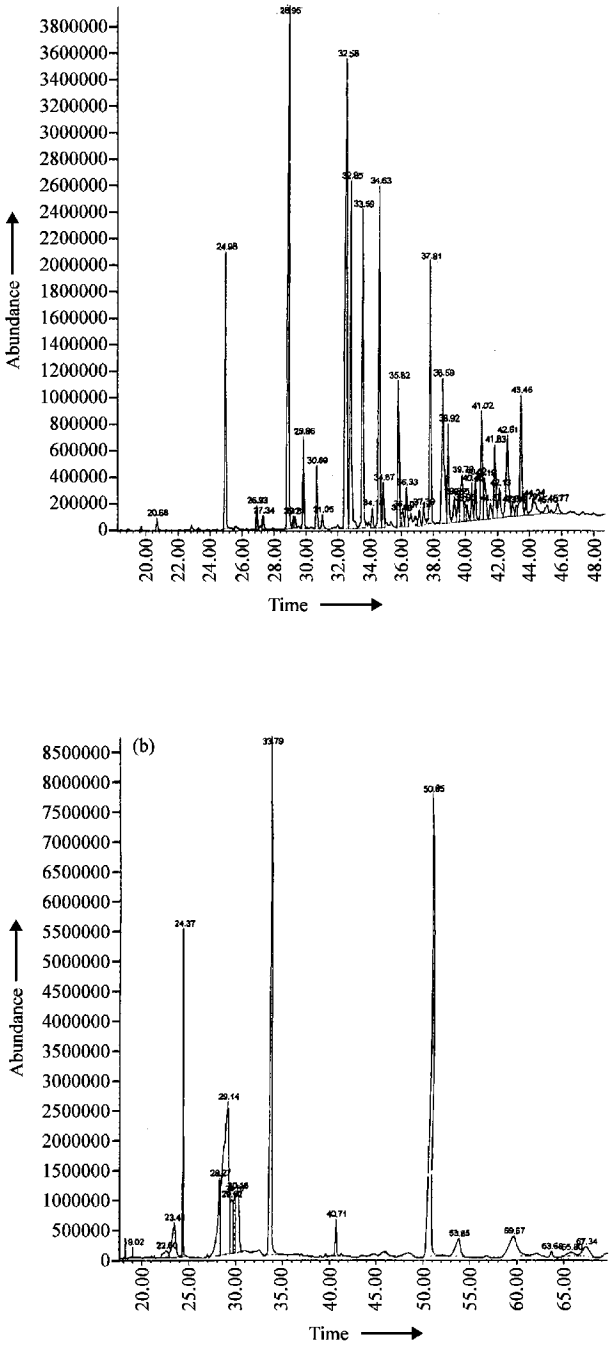

Fig. 8a and b: GC/MS results of metyle eslers of (a) total (b) staturated

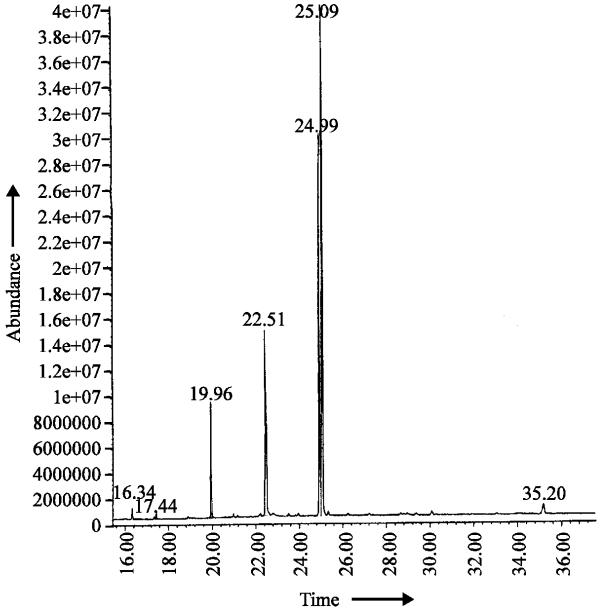

Fig. 8c: GC/MS results of metyle esters of unsaturated fatty acids

DISCUSSION

Leptadenia pyrotechnica contains unusual aromatic hydrocarbons which are not usually detected before in plants. The presence of aromatic hydrocarbons in this plant, may such be due to the marked changes in temperature and water stress in desert environment. Therefore, it is probable that, the detected aromatic hydrocarbons may be formed as intermediates from the shikimic acid pathway (Anand and Heberlein, 1977) that is responsible for aromatic amino acid biosynthesis. Moreover, it has been reported that the aromatic hydrocarbons are endogenically synthesized by bacteria, fresh water algae and land plants (Webster *et al*., 2003; Kirso *et al*., 2001; Spriggs *et al*., 2005; Alexandre and Débora, 2003). It is suggested that, the presence of these aromatic hydrocarbons may be involved in the synthesis of sterols, flavonoids, alkaloids in plants, etc. which they are detected as secondary metabolites in several plants. It is worthy to mention that the building blocks of aromatic amino acids and sterol are phoshoenol pyruvate and squalene respectively (Anand and Heberlein, 1977). Moreover, the progress and development of the analytical tools of GC/MS analysis have interpreted new structures of many compounds that could have not been elucidated by previous investigators.

Present study revealed the presence of taraxerol as previously reported (Manavalan and Mithal, 1980). Lupeol, squalene and phytol were not isolated before from the same plant. However, the previously reported fernenol (Manavalan and Mithal, 1980) is not detected in present study.

The polyunsaturated fatty acids are essential fatty acids and have biological activity that the human body needs. It is known that, the essential fatty acids can not be biosynthesized by the body. Urea fractionation method was adopted for the separation of the two oil fractions, namely, saturated and unsaturated. The unsaturated fraction is considered as a concentrate of polyunsaturated fatty acids which is very useful as source of essential polyconcentrated fatty acids.

CONCLUSIONS

The data of mortality rates, point out that, with concentration 1000 ppm, the extracts; methanol, methanol after defatted and petroleum ether, represented 100.00, 98.91 and 97.00%, respectively, with

Table 5: Mortality of brine shrimp at various concentration of the different extracts of *Leptadenia pyrotechnica*

Plant extract	Dose (ppm)	Dosage (log dose)	Dead	Alive	Accum. dead	Accum. alive	Ratio dead: total	Mortality (%)	LC_{50} (ppm)
Methanol	1000	3	50	0	94	0	94/94	100.00	
extract	100	2	44	6	44	6	44/50	88.00	–
	10	1	–	–	–	–	–	–	
Methanol	1000	3	49	1	91	1	91/92	98.91	
extract	100	2	42	8	42	9	42/51	82.35	–
after defatted	10	1	–	–	–	–	–	–	
Petroleum	1000	3	47	3	97	3	97/100	97.00	35.48
ether extract	100	2	35	15	50	18	50/68	73.53	
	10	1	15	35	15	53	15/68	22.10	

concentration 100 ppm, the extracts methanol, methanol after defatted and petroleum ether exhibited the high mortality; and represented 88.00, 82.35 and 73.53%, respectively, On the other hand, with concentration 10 ppm; petroleum ether extract exhibited the higher mortality which represented 22.10%. The results obtained (Table 5), revealed that the higher toxicity was exhibited in petroleum ether extract, which represented 35.48 ppm.

Moreover, the 95% confidence limits of the previous extracts were calculated, the results obtained revealed that, the estimated LC_{50} and its confidence limits for petroleum ether extract were 35.48 (19.89-63.29 ppm). Present study revealed the presence of beta-sitosterol as previously reported (Manavalan and Mithal, 1980). Stigmasterol, campsterol and cholesterol, were not isolated before from the same plant.

So, by comparing the obtained results of the investigation unsaponifiable constituents before and after chromatographic fractionation, we can conclude that, it is urgent to analyze the unsaponifiable matter as a total before fractionation to characterize which types and relative percentages of the major components and to focus on component which we need to isolate. Moreover, some compounds may be not detected after fractionation like phytol which was detected in unsaponifiable matter before fractionation only. However, the method of fractionation was useful to separate each component or fraction to facilitate further investigations. Also, to simplify the detection of each component through the detection (to make more resolution) like what happened with sterol fraction, fucosterol was detected through fractionation. Also, the percentage and numbers of aromatic hydrocarbons were increased after chromatographic fractionation.

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