

Detection of Secondary Metabolites Using HPTLC and GC-MS Analysis and Assessment of Pharmacological Activities of *Phoenix loureiroi* Kunth (Arecaceae) Ethanolic Leaves Extract in the Management of Pyrexia, Pain and Inflammation



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ABSTRACT

The present research work was carried out the High Performance Thin Layer Chromatography (HPTLC) and Gas Chromatography-Mass Spectrometry (GC-MS) analysis and assessment of pharmacological activities of *Phoenix loureiroi* Kunth (Arecaceae) ethanolic leaves extract at doses of 200, 400 and 600 mg/kg, body weight, *per os*. Preliminary phytochemical screening, HPTLC and GC-MS studies were carried out according to the standard methods. The acute toxicity studies were conducted on Swiss albino mice as per Organization for Economic Cooperation and Development (OECD) guidelines 420. For the screening of analgesic activity, writhing test was conducted for peripheral analgesic activity whereas tail immersion test was conducted for central analgesic activity. Antipyretic activity was performed by using the yeast induced hyperpyrexia method and for the screening of anti-inflammatory activity

carrageenan-induced rat paw edema method was used. Preliminary phytochemical screening of the ethanol extract of *Phoenix loureiroi* leaves (EEPLL) contains sterols, flavonoids, saponins, proteins, reducing sugar, tannins, and phenolic compounds. The HPTLC analysis method employed in this work resulted in good peak shape and enabled good resolution of quercetin present in the extract and GC-MS analysis showed a total of 25 peaks and led to the identification of 22 different phytoconstituents in the ethanolic extract. Lethal Dose 50 (LD₅₀) was above 2,000 mg/kg and no death was recorded. The prevailing study demonstrated that EEPLL possesses widespread analgesic, antipyretic and anti-inflammatory effects in dose dependent manner. It can be concluded that the ethanolic extract from *Phoenix loureiroi* leaves possesses promising analgesic, antipyretic and anti-inflammatory activities.

Keywords: *Phoenix loureiroi*, HPTLC finger print, GC-MS analysis, Acute toxicity, Brewer's yeast, Carrageenan, Aspirin, Ibuprofen, Paracetamol, Pentazocine.

INTRODUCTION

Phoenix loureiroi Kunth is a very common mountain dates palm which belongs to the family Arecaceae. The family arecaceae is one of the largest families in monocotyledons that consist of 217 genera and more than 2,500 species and are distributed throughout the tropical regions around the world. In India, it represented 25 genera and more than 225 species.¹ This plant contains solitary and clustering plants with trunks from 1-4 m high and 25 cm in width, usually covered in old leaf bases. The leaves are 2 m in length with leaflets which vary to some degree but wide at the base and sharply pointed apices. The leaflets emerge from the rachis at varying angles creating a stiff, plumose leaf. The fruit is a single seeded drupe, bluish-black when ripe, produced on erect, yellow inflorescences, usually hidden within the leaf crown.² The species is noted for its variability indifferent habitats.³ Date

fruits have a great significant for both nutritional and therapeutic point of view.^{4,5} It contains high amount of sugars, vitamins, minerals and fibers. In some varieties, the sugar content reaches up to 88%, and such fruits are considered a high energy yield food.⁶ Moreover, these fruits possess antioxidant and anticancer properties.^{7,8} It attributes high level polyphenolic compounds and also vitamins.⁹ Dates extracts have the antibacterial and antifungal properties.¹⁰⁻¹² Regular intake of *Phoenix* species traditionally used for cough, rheumatism, burning sensation, nephropathy, gastropathy, bronchitis and sexual debility.¹³ It can be prescribed for gastroenteritis, cough, respiratory diseases, asthma, chest complaints, fevers, high blood pressure and fatigue.^{13,14} Several *Phoenix* species have a number of pharmacological activities like Anti-mutagenic,¹⁵ Antioxidant, Anti-diarrheal,¹⁶

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Analgesic,¹⁷ Anti-inflammatory.¹⁸ Different classes of compounds were isolated and identified from genus *Phoenix* that contains lipids, phenolic acids, flavonoids, procyanidines, triterpenes, carbohydrates, vitamins, inorganic ions etc.¹⁹

The literature available from all possible scientific sources showed very little research work on this species, whereas tribes claim that *P. loureiroi* was used in the treatment of various diseases and ailments, although there is no inbuilt scientific proof in support of the utility of this species as an analgesic, anti-inflammatory and antipyretics agent. So, the present study explored the analgesic, antipyretics and anti-inflammatory activities of ethanol extract from *Phoenix loureiroi* leaves (EEPLL) and detection of polyphenolic compounds present in the extract by HPTLC fingerprinting analysis and exploration of bioactive compounds in ethanolic extract by using Gas chromatography and mass spectrometry.

MATERIALS AND METHODS

Collection and authentication of plant material

The leaves of *Phoenix loureiroi* was collected during June 2019 from in and around East Godavari dist., Andhra Pradesh, India and authenticated by Dr. K. Madhava Chetty, Professor, Dept. of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India. A voucher specimen has been kept in our research laboratory for further reference (Voucher No. SVU/2019/GIP-SM-12). The collected materials were washed with water and shade dried for one week and then the dried leaves were pulverized by using a mechanical grinder to obtain coarse powder.

Preparation of extract

The powdered plant material (500 g) was extracted with ethanol (90% v/v) for 48 hrs using Soxhlet extractor. The extract obtained was evaporated under vacuum to remove the solvent completely and concentrated to obtain a dark greenish residue (9.89 g).

Preliminary phytochemical tests

A plant may be considered as a biosynthetic laboratory, not only for the chemical compounds such as carbohydrates, proteins and lipids that are utilized as food by man, but also for a multitude of a compounds like glycosides, alkaloids, volatile oils, saponins, etc., that exert a physiological effect.²⁰ The compounds that are responsible for therapeutic effects are usually the secondary metabolites. A systematic study of a crude drug

embraces through consideration of both primary and secondary metabolites derived as a result of plant metabolism.²¹ The ethanol extract of *Phoenix loureiroi* leaves (EEPLL) subjected to preliminary phytochemical screening for the detection of various Phytoconstituents using standard procedures.²²

HPTLC finger print profile

HPTLC studies were carried out according to the standard methods.^{22,23} The HPTLC fingerprint technique was used to detect possible polyphenolic compounds from EEPLL.

Sample preparation and stationary phase:

Ethanolic extract of *Phoenix loureiroi* was dissolved in methanol which was used for sample. Silica gel 60 F 254 HPTLC plates (E. MERCK KGaA) of size (10.0 × 10.0 cm) were used as the stationary phase.

Sample application:

Different applied volume of reference standards quercetin such as 1 µL, 2 µL, 3 µL and 4 µL and 5 µL were used for the detection of flavonoids. The reference standard along with 10 µL of the extract was applied as a band on the plates using syringe (100 µL), with inert gas flow providing a delivery speed of 150 nL per second. The syringe was mounted on a CAMAG linomat V sample applicator attached to CAMAG HPTLC system and was programmed through WIN CATS software. The length of the applied band was kept at 8 mm.

Development of chromatogram:

The plate for flavonoids were developed in toluene: ethyl acetate: formic acid (7:3:1) and 10 mL of mobile phase was used per chromatography run. The linear ascending development was carried out in a (20 cm × 10 cm) twin trough glass chamber saturated with the mobile phase.

Detection of spots and photo-documentation:

The developed plate was dried by hot air with the help of a hair dryer at a temp of 60°C to evaporate the solvent from the plate. The plate was scanned using D2 lamp at 275 nm in CAMAG TLC Scanner 3. The R_f values and finger print data were recorded by WIN CATS software.

Metabolite profiling using GC-MS analysis

The GC-MS analysis of ethanol extract from *Phoenix loureiroi* leaves (EEPLL) was carried out using Thermo GC-Trace Ultra Ver: 5.0, Thermo MS DSQ II. The column DB 35 - MS Capillary Standard Non-Polar Column [30 m × 0.25 mm inner diameters (i.d.), film thickness: 0.25 µm] was used for separation of compounds.²⁴ The column oven temperature was programmed from 70°C (hold for 2 mins) to 260°C at a rate of 60C/min with a final hold time of 10 mins. The injector temperature

was maintained at 250°C. The carrier gas used was Helium (He) at a flow rate of 1.0 mL/min. GC was performed in a split less mode. For mass spectrometric detection, the electron ionization mode with ionization energy of 70 eV was used, with a mass range at 50–650 m/z. The ion source of the mass spectrometer was operated at 220°C and the MS transfer line was maintained at a temperature of 280°C. An injection volume of 1 µL of sample was used. The phyto-constituents present in the ethanol crude extract were expressed as peak area percentage. The detection and endorsement of phytochemical compounds in the EEPLL was based on GC retention time. The mass spectra were matched with those of standards available in mass spectrum libraries such as Wiley 9.

Animals Used

Swiss albino mice (20–25 g) and Wistar albino rats (150–250 g) of either sex were maintained in the animal house at GITAM institute of pharmacy, GITAM University, Visakhapatnam, Andhra Pradesh under standard environmental conditions of temperature (25°C) and light/dark cycles (12/12h). All experimental protocols were approved by the Institutional Animal Ethics committee of GITAM Institute of Pharmacy, Visakhapatnam, Andhra Pradesh, India (Regd. No.1287/ac/09/CPCSEA). Experiments were performed according to the guide for the care and use of laboratory animals. All standard drugs and EEPLL were suspended in normal saline solution using sodium carboxy methyl cellulose (0.5% w/v) for pharmacological studies. All control groups' animals received 0.5% w/v sodium CMC in normal saline as vehicle (3 ml/kg body weight, *per os*) through oral route.

Acute toxicity studies

The acute toxicity experiments were performed over Wistar albino rats as per guidelines of the Organization for Economic Cooperation and Development (OECD).^{25,26} The selected male and female rats were then assigned to standard control and remedy groups (5/sex/ group). The research group rats obtained EEPLL once orally as a test sample at doses of 1000, 1500, and 2000mg / kg, body weight, which was prepared by suspending EEPLL in tween 20 solutions (1%v/v) and blended thoroughly. Tween 20 solution (1% v/v) was obtained as a vehicle by the control group rats. Both rats were weighed before the experiment started, marked for identification, and fasted overnight but were given free access to water. After dosing, the rats fasted further for 4 h and for any mortality and irregular changes observations were reported continuously for each individual rat in their respective groups during the first 4 h and then 24 h after

drug treatment. They were then observed two times each day for a duration of 14 days to find out any toxic effect *viz.*, modifications in the attention, eye, hair and skin color changes, consumption of food and water, tremors, seizures, salivation, diarrhoea, lethargy, respiration sleep and coma.

Screening for antinociceptive activity of *P. loureiroi* leaves

Pain is a symptom of many diseases requiring treatment with analgesics. It is a common phenomenon in all animals, at least in vertebral animals, similar to that felt by man. Analgesic effects in animals are comparable with the therapeutic effects in man.²⁷

Peripheral analgesic activity (Writhing tests):

Writhing was induced in mice by single intraperitoneal injection (10 ml/kg) of 0.6% acetic acid. The number of writhing's counted over a 20 min period. Different groups of animals were treated with Aspirin (100 mg/kg, p.o.) or ethanol extract of *Phoenix loureiroi* leaves (EEPLL) (200, 400 or 600 mg/kg) through oral route just 30 min prior to injection of acetic acid. The control group received only vehicle (3 ml/kg, p.o.). The writhing effect indicated by stretching of abdomen with simultaneous stretching of at least one hind limb. The percentage inhibition was calculated.^{28,29}

Central analgesic activity (Tail immersion test):

The tail immersion test was carried out as described by Mondal *et al.*, 2016.²⁹ The animals (Swiss albino mice) were and had the last 3.5 cm of their tail immersed in hot water thermo-statistically maintained at 55±0.5°C, a procedure that caused them to rapidly withdraw their tail. Five groups of animals were held in position in a suitable restrainer with the tail extending out. The latency to withdraw the tail was recorded with a stopwatch, and a cut-off maximum latency of 10 sec was established in order to prevent tissue damage. Group I served as control, which received only vehicle (3 ml/kg, p.o.). Other groups of animals received one of the following in a similar manner: Pentazocine (30 mg/kg, p.o.) or EEPLL (200, 400 or 600 mg/kg, p.o.). The initial reading was taken immediately before administration of test samples and then at 15, 30, 45 and 60 min after the administration.

Screening for anti-inflammatory activity of *P. loureiroi* leaves (Carrageenan-induced rat paw oedema test)

Carrageenan-induced rat paw oedema is the most commonly used technique for the screening of anti-inflammatory drugs. The test is based upon the ability of the drug to inhibit the acute edema produced in the hind paw of the rat after injection of a phlogistic agent such as carrageenan, dextran, histamine or serotonin.³⁰

Five groups of rats, six in each group were selected for the study. Different groups were treated with saline; ibuprofen (10 mg/kg, p.o.) and EEPLL at doses of 200, 400 and 600 mg/kg, body weight, *per os*. The animals were treated with the extract 1.0 h before the administration of carrageenan. Acute inflammation was produced by the sub-plantar administration of 0.1 ml of 1% carrageenan in normal saline in the right hind paw of the rats. The paw volume was measured at 0, 1, 2 and 3 hrs after Carrageenan injection using plethysmometer.³¹

The anti-inflammatory effect was calculated by the following equation:

$$\% \text{ inhibition} = \left(1 - \frac{V_t}{V_c}\right) \times 100$$

Where, Vc represents oedema volume in control and Vt the oedema volume in the group treated with the tested EEPLL or ibuprofen.³²

Evaluation of antipyretic activity of *P. loureiroi* leaves

In Wistar albino rats the antipyretic activity was screened using the yeast induced hyperpyrexia method. Fever was induced by injecting 10 ml/kg of 20% aqueous suspension of Brewer’s yeast in normal saline below the neck’s nape subcutaneously. Each rat’s rectal temperature was measured seventeen hours after the injection using a digital thermometer. Only rats were chosen for the study which showed an increase in temperature of at least 0.7°C, and animals were divided into five groups. Group one that is the control group acquired vehicle (3 ml/kg, p.o.) via oral route, group two received paracetamol (150 mg/kg, p.o.) used as a reference standard, group three, four and five received EEPLL (200, 400 and 600 mg/kg, p.o.) respectively. After

treatment the rectal temperature of each rat was measured at 1, 2 and 6 hrs.²⁹

Statistical Analysis

The data obtained in the studies were subjected to one way of analysis of variance (ANOVA) for determining the significant difference. The inter group significance was analyzed using Dunnet’s t-test. A *p*-value <0.05 was considered to be significant. All the values were expressed as mean ± SEM.

RESULTS

Preliminary phytochemical tests

Preliminary phytochemical screening of the ethanol extract of *Phoenix loureiroi* leaves (EEPLL) contains sterols, flavonoids, saponins, proteins, reducing sugar, tannins, triterpenoids, and phenolic compounds (Table 1).

HPTLC fingerprinting analysis

HPTLC fingerprinting of the ethanol extract from *Phoenix loureiroi* leaves revealed several well distinguished peaks under a wavelength of 275 nm. HPTLC fingerprinting of the reference standard quercetin revealed seven peaks whereas ten peaks were detected in the test extract solution. The result showed (Figure 1) that one peak of the test extract has similar R_f value with that of standard quercetin. Thus quercetin was detected in *Phoenix loureiroi* ethanol extract.

Metabolite profiling using GC-MS analysis

The GC-MS assessments of the *P. loureiroi* leaf ethanol extract showed a total of 25 peaks and contributed to the identification of 22 specific phyto-constituents and three unknown compounds (Figure 2). The details of the identified phytochemical compounds are tabulated in Table 2, and the chemical structures of these compounds are shown in Figure 3. The compounds identified in the ethanol extract mainly comprised of phytosterols, saponin, acyclic olefins, saturated hydrocarbons, fatty alcohol, saturated fatty acid, fatty acid esters, phthalate ester, benzenedicarboxylic acid and benzene-propanoic acid mimic compound, along with organosilicon, ethanoanthracene and oxaspiro-lactone-enone cyclic ketone derivative compounds. The ethanol extract comprises of two compounds of organosilicon, namely Cyclodecasiloxane eicosamethyl and Eicosamethylcyclodecasiloxane with a retention time of 29.859 and 28.760 minutes and an area percentage of 0.976% and 13.035% respectively. Likewise, Spirost-8-en-11-one, 3-hydroxy-, (3β,5α,14β,20β,22β,25R) was the most abundant phyto-constituent (saponin) found in

Table 1 Preliminary phytochemical screening of ethanol extract from *P. loureiroi* leaves (EEPLL)

Phytoconstituents	EEPLL
Alkaloids	-
Carbohydrates (Reducing Sugar)	+
Gums and mucilages	+
Proteins and amino acids	+
Tannins and phenolic compounds	+
Phlobatannins	-
Steroids and sterols	+
Triterpenoids	+
Saponins	+
Flavonoids	+

(+) Present; (-) Absent

Table 2 GC-MS analysis of ethanol extract of *P. loureiroi* leaf

Sl. No.	Category	Compound	Synonyms	Retention time (mins)	Molecular Formula	Molecular weight (g/mol)	% Area
01	Acyclic olefins	1-Tetradecene	n-Tetradec-1-ene	5.412	C ₁₄ H ₂₈	196.37	7.435
02	Aliphatic hydrocarbon	Tetradecane	n-Tetradecane	5.534	C ₁₄ H ₃₀	198.39	2.793
03	Aliphatic hydrocarbon	Tetradecane, 2,6,10-trimethyl	2,6,10-trimethyltridecane	7.994	C ₁₇ H ₃₆	240.5	1.179
04	Fatty alcohol	1-Hexadecanol	Cetyl alcohol	8.946	C ₁₆ H ₃₄ O	242.4	9.925
05	Aliphatic hydrocarbon	Pentatriacont-9-ene	Pentatriacontane	9.094	C ₃₅ H ₇₀	490.93	3.046
06	Ethanoanthracene	9,10-Ethanoanthracene, 9,10-dihydro-11,12-diacetyl	-	12.109	C ₂₀ H ₁₈ O ₂	290.4	1.630
07	Acyclic olefins	1-Nonadecene	Nonadecene	12.381	C ₁₉ H ₃₈	266.5	1.30
08	Oxaspiro compound, a lactone, an enone and a cyclic ketone	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	1-Oxaspiro[4.5]deca-6,9-diene-2,8-dione, 7,9-bis(1,1-dimethylethyl)	12.859	C ₁₇ H ₂₄ O ₃	276.4	5.899
09	Fatty acid esters	Hexadecanoic acid methyl ester	Methyl palmitate	15.189	C ₁₇ H ₃₄ O ₂	270.5	1.044
10	Benzenepropanoic acid	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate Methylox	15.410	C ₁₈ H ₂₈ O ₃	292.41	1.487
11	Benzenedicarboxylic acid	1,2-Benzenedicarboxylic acid, butyl octyl ester	Plasticizer BOP or Phthalic acid 1-butyl 2-octyl ester	15.680	C ₂₀ H ₃₀ O ₄	334.44	17.493
12	Saturated fatty acid	n-Hexadecanoic acid	Palmitic acid	16.017	C ₁₆ H ₃₂ O ₂	256.42	2.366
13	Saturated fatty acid	Octadecanoic acid	Stearic acid	16.062	C ₁₈ H ₃₆ O ₂	284.47	3.263
14	Fatty acid esters	Hexadecanoic acid ethyl ester	Ethyl palmitate	16.677	C ₁₈ H ₃₆ O ₂	284.47	4.412
15	Fatty alcohols	2-Methyl-Z,Z-3,13-octadecadienol	(z,z)-2-methyl-3,13-octadecadienol	19.223	C ₁₉ H ₃₆ O	280.5	1.169
16	Phthalate ester and a diester	Diisooctyl phthalate	Isooctyl phthalate	19.656	C ₂₄ H ₃₈ O ₄	390.6	1.183
17	Phytosterol	Stigmasterol	Stigmasterin	23.515	C ₂₉ H ₄₈ O	412.70	0.389
18	Phytosterol	β-Sitosterol	β-Sitosterin	25.835	C ₂₉ H ₅₀ O	414.718	4.257
19	Sapogenin	Spirost-8-en-11-one, 3-hydroxy-, (3β,5α,14β,20β,22β,25R)	3-Hydroxyspirost-8-én-11-one	26.065	C ₂₇ H ₄₀ O ₄	428.6	12.304
20	Organosilicon	Eicosamethylcyclodecasiloxane	Icosamethyl-cyclodecasiloxane	28.760	C ₂₀ H ₆₀ O ₁₀ Si ₁₀	741.5	13.035
21	Organosilicon	Cyclodecasiloxane, eicosamethyl	-	29.859	C ₂₀ H ₆₀ O ₁₀ Si ₁₀	740	0.976
22	Phenyl ester	4-Chlorobutyric acid, 4-methoxyphenyl ester	4-methoxyphenyl 4-chlorobutanoate	33.243	C ₁₁ H ₁₃ ClO ₃	228.67	1.031

the EEPLL at a retention time of 26.065 minutes and an area percentage of 12.304%. At the same time, the other two major phytosterols were also recorded from EEPLL *viz.*, Stigmasterol and β-Sitosterol at a retention time of 23.515 and 25.835 minutes, with an area percentage of 0.389 per

cent and 4.257 per cent. Accordingly, some minor constituents from the EEPLL were also identified, consisting mainly of two acyclic olefins, three saturated hydrocarbons, saturated fatty acids and fatty acid esters, fatty alcohols, phenyl ester and phthalate diester derivative compounds *viz.*, 1-Tetradecene

Table 3 Evaluation of analgesic activity of ethanol extract of *P. loureiroi* leaves by tail immersion method in mice

Group	Treatment	Dose (mg/kg, p.o)	Average tail withdrawing time (Sec)				
			0 min	15 min	30 min	45 min	60 min
I	Control	3 ml/kg	2.17±0.11	2.21±0.21	2.78±0.13	3.73±0.19	2.76±0.33
II	Pentazocine	30	2.39±0.23	6.2±0.33**	8.15±0.16**	8.83±0.33**	10.45±0.39**
III	EEPLL	200	3.22±0.22	3.48±0.13	4.35±0.24*	5.75±0.17*	5.83±0.18**
IV	EEPLL	400	2.11±0.11	4.35±0.22*	5.84±0.15**	6.34±0.16**	6.85±0.28**
V	EEPLL	600	2.36±0.11	5.55±0.3**	7.72±0.22**	7.98±0.26**	8.33±0.27**

Values are expressed as mean ± SEM (n=6). All columns are significant using ANOVA *P<0.05, **P<0.01 when compared to control; Dunnet's t-test.

Table 4 Effect of ethanol extract of *P. loureiroi* leaves on yeast induced pyrexia in rats

Treatment	Dose (mg/kg, p.o.)	Initial rectal temperature (°C)	Rectal temperature (°C) after drug administration			
			0 h	1 h	2 h	6 h
Control	3 ml/kg, p.o.	37.42±0.03	38.87±0.03	39.33±0.21	39.69±0.06	39.13±0.08
Paracetamol	150	37.32±0.02	38.83±0.02	38.11±0.03**	38.02±0.02**	37.36±0.01**
EEPLL	200	37.52±0.05	38.88±0.03	38.91±0.21	38.42±0.04**	37.83±0.02**
EEPLL	400	37.47±0.03	38.93±0.01	38.42±0.11**	38.21±0.03**	37.51±0.03**
EEPLL	600	37.51±0.01	38.88±0.02	38.23±0.09**	38.12±0.02**	37.61±0.01**

Values are expressed as mean ± SEM (n=6). All columns are significant using ANOVA *P<0.05, **P<0.01 when compared to control; Dunnet's t-test.

(7.435%), 1-Nonadecene (1.30%), Tetradecane (2.793%), Tetradecane, 2,6,10-trimethyl (1.179%), Pentatriacont-9-ene (3.046%), n-Hexadecanoic acid (2.366), Octadecanoic acid (3.263%), Hexadecanoic acid methyl ester (1.044%), Hexadecanoic acid ethyl ester (4.412%), 1-Hexadecanol (9.925%), 2-Methyl-Z,Z-3,13-ocetadecadienol (1.169%), 4-Chlorobutyric acid, 4-methoxyphenyl ester (1.031%) and Diisooctyl phthalate (1.183%). Correspondingly, the derivative of benzenepropanoic acid, benzenedicarboxylic acid, ethanoanthracene and oxaspiro compound, a lactone, an enone with a cyclic ketone derivatives compounds was also measured from the EEPLL at a retention time of 15.410, 15.680, 12.109 and 12.859 minutes viz., Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester (1.487%), 1,2-Benzenedicarboxylic acid, butyl octyl ester (17.4937%), 9,10-Ethanoanthracene, 9,10-dihydro-11,12-diacetyl (1.630%) and 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione (5.899%). Whereas three unknown compounds (0.317, 0.344 and 2.381 per cent) were found in the ethanol extract at 33.275, 33.297 and 33.720 minutes of retention time.

Acute Toxicity Studies

No mortality or morbidity was observed in animals (Wistar albino rats) through the 14 day period following single oral administration. Morphological characteristics (fur, skin, eyes and nose) appeared normal. The extract induced sedation, mild

diuresis, and purgation at all tested doses. No tremors, convulsion, salivation, lethargy or unusual behaviors such as self-mutilation, walking backward etc. were observed. Gait and posture, reactivity to handling or sensory stimuli, grip strength was all normal. There was no significant difference in body weights between control and treatment groups. Food and water intake showed daily fluctuations within the range of control animals. This indicates that the ethanol extract from *Phoenix loureiroi* leaves was safe to a single dose of 2000 mg/kg body weight. Hence, the 1/5th of the preceding dose i.e 400 mg/kg body weight, p.o., was taken as the testing dose for pharmacological evaluation and lower upper dose of 200 and 600 mg/kg body weight, p.o., also tested to find out whether there is any dose dependent pharmacological effect or not.

Effect of EEPLL on analgesic activity

Assessment of peripheral analgesic effect through acetic acid induced writhing analysis was evaluated on the basis of the average number of abdominal constrictions indicated by the extension of hind paw of animals (mice) during the writhing test. The analgesic effects induced by different doses of EEPLL and aspirin on the acetic acid writhing in mice are portrayed in Figure 4. The EEPLL significantly (P <0.05) reduced the numbers of writhings induced by intraperitoneal injection of acetic acid in mice in dose dependent manner, simultaneously the reference drug aspirin (100 mg/kg) produced significant analgesic activity against chemically

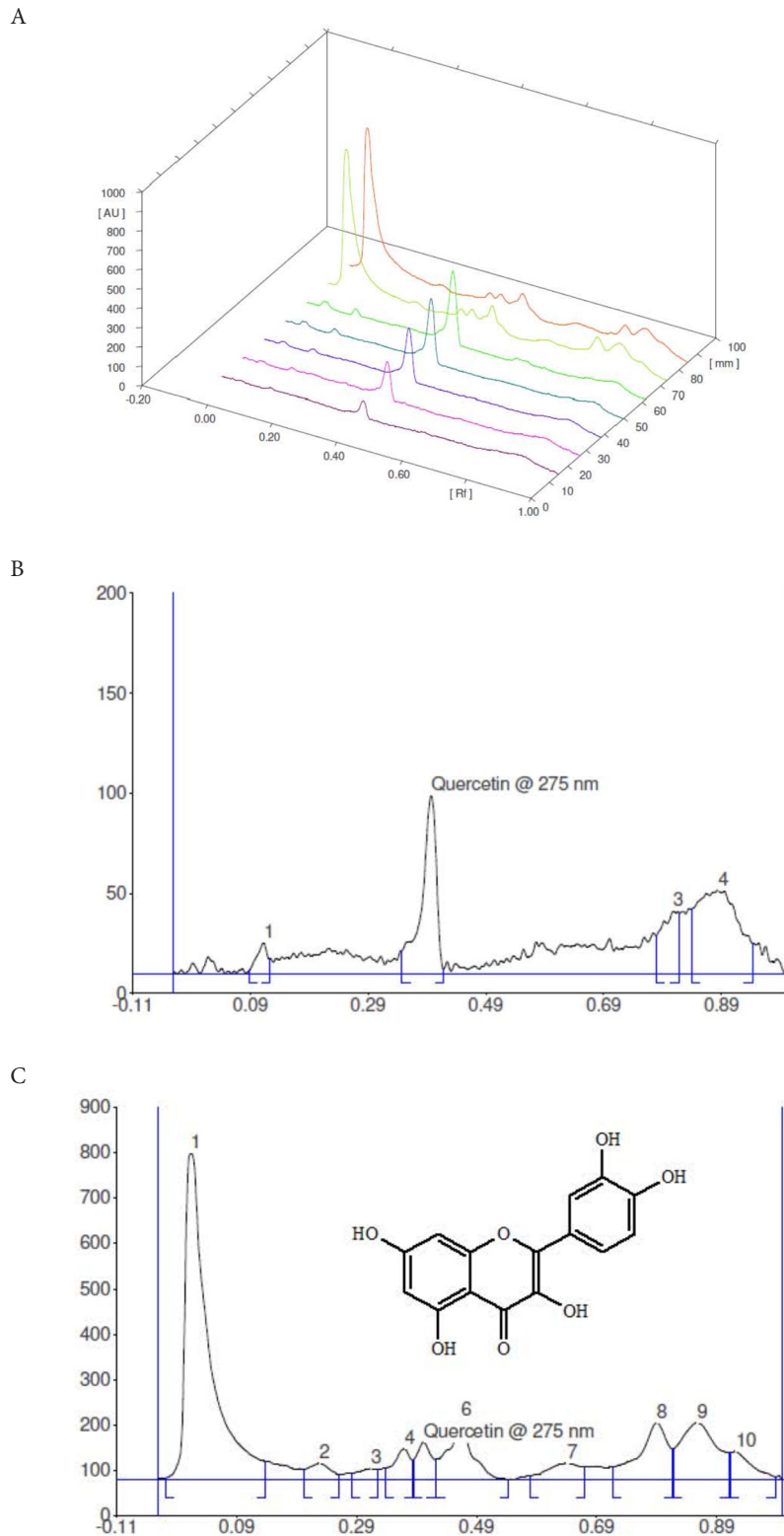


Figure 1 (A) 3D display of HPTLC chromatogram of *Phoenix loureiro* ethanol extract for detection of polyphenolic compound quercetin
 (B) Peak densitogram of reference standard
 (C) Peak densitogram of *Phoenix loureiro* ethanol extract showing the presence of quercetin

induced pain. The percentage inhibition (Figure 5) of writhes are 33.34%, 57.04% and 66.62% were obtained at a dose of 200, 400 and 600 mg/kg, p.o., of EEPLL, whereas the percentage inhibition of writhes for standard drug aspirin (69.02%) was obtained at a dose of 100 mg/kg, p.o. Similarly, the mean latency of nociceptive responses to thermal stimuli in the tail immersion test is summarized in Table 3. The EEPLL at dose of 200, 400 and 600 mg/kg, p.o., produced a significant increase in mean latency time throughout the observation period, i.e. at 15, 30, 45 and 60 min when compared to the control group animals. The reference drug Pentazocine (30 mg/kg, p.o) also showed a significant increase in mean latency time.

Effect of carrageenan-induced anti-inflammatory activity of *P. loureiro* leaves

The circumference on carrageenan-induced rat hind paw edema signifies the extreme anti-inflammatory impact of EEPLL (Figure 6). Pretreatment with EEPLL at 200, 400 and 600 mg/kg, prevented a substantial reduction in paw oedema volume relative to control group animals. The extract showed maximum inhibition of 32.24%, 38.16% and 45.4% at the dose of 200, 400 and 600 mg/kg, p.o., after 3 h of drug treatment, whereas the usual drug ibuprofen (10 mg/kg, p.o.) showed 46.72% inhibition. The percent inhibition of the test extract in acute inflammation within the paw of rats was determined to be comparable however little lower when as compared to the standard drug ibuprofen (Figure 7).

Antipyretic activity evaluation yeast mediated hyperpyrexia

Antipyretic effects of ethanol extract from *Phoenix loureiro* leaves (EEPLL) on rectal temperature are presented in Table 4 and Figure 8. The subcutaneous injection of yeast markedly increased the rectal temperature and the mean increment recorded was $1.45 \pm 0.02^\circ\text{C}$ after 17 hrs of administration. The EEPLL showed a significant dose dependent decrease in pyrexia when compared to control. EEPLL at doses of 200, 400, and 600 mg/kg, p.o., showed a progressive decline in mean temperature pattern with the increase in the dose. Paracetamol demonstrated a substantial reduction in the rectal temperature from 1 to 6 h. Paracetamol's onset of action was 60 mins, and after 6 h the body temperature was normal. In comparison, EEPLL demonstrated its onset of action at doses of 200 mg/kg in 2 h and EEPLL 400 and 600 mg/kg, in 60 min. The line graph shows that paracetamol and EEPLL registered a remarkable drop in mean temperature between 60 min and 6 h, and retained a steady mean temperature thereafter.

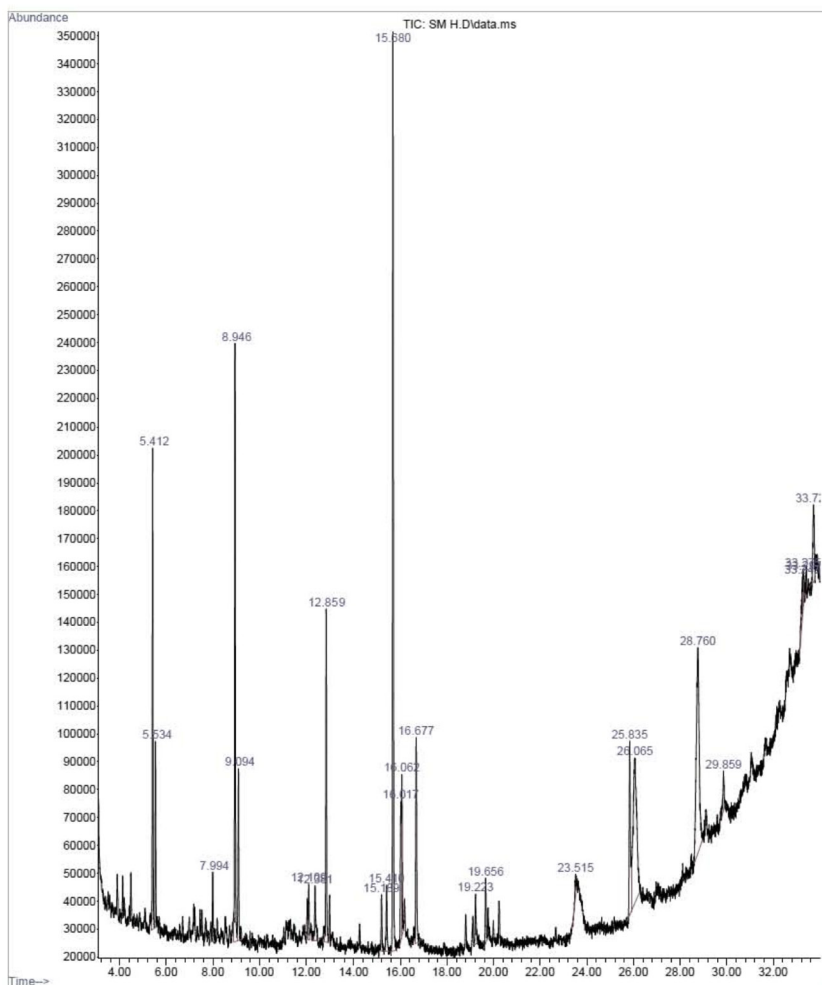


Figure 2 A typical chromatogram of the bioactive compounds present in the ethanol extracts from *Phoenix loureiroi* leaves

DISCUSSION

There was a wonderful development within the discipline of artificial drugs for the duration of current era. But they're observed to have a few or different side effects, while flora nonetheless preserve their personal specific vicinity, *via* the way of having no aspect outcomes. Therefore diverse pharmaceutical firms round the sector are inquisitive about growing safer and more powerful drugs to treat ache, fever and infection.²⁹

Phoenix loureiroi leaves preliminary phytochemical test of ethanol extract was performed and the result revealed the presence of carbohydrates, tannins and phenolic compounds, steroids and sterols, triterpenoids, saponins and flavonoids. Therefore, the preliminary phytochemical test helps in determining the class of chemical compound present within the extracts which might also result in their quantitative estimation and also identifying the source of pharmacologically active phytoconstituents.³³ Similarly, HPTLC is an important

quality assurance method for evaluating botanical materials and allows the accurate and cost-effective study of a wide variety of compounds. It also helps to test the pureness of crude products, to quantify and classify marker compounds in crude extracts.³⁴ Analysis of the HPTLC was carried out to identify potential polyphenolic compounds in EEPLL. Analysis of the extract for flavonoids confirmed the presence of ten peaks out of which one peak is similar with to standard quercetin, while EEPLL confirmed the presence of quercetin, which has been formerly said to own analgesic and anti-inflammatory properties.³⁵ The name quercetin (3,3', 4',5,7-pentahydroxyflavone) derives from the Latin word "Quercetum," meaning oak forest, belongs to the class called flavonols which can't be produced inside the human frame.³⁶ One of the center most brilliant properties of quercetin is its potential to modulate infection. Quercetin inhibits cyclooxygenase (COX) and lipooxygenase inflammatory enzymes and thus decreases inflammatory mediators such as prostaglandins and leukotrienes.^{37,38} While this HPTLC approach has been effective in the detection of polyphenolic compound quercetin and this is the first research to report quercetin presence in this plant. Consequently we should assume that these known compounds may be responsible for their analgesic and anti-inflammatory effects in rats, either individually or synergistically.

At the same time, the necessity of unambiguous recognition of different components in complex mixtures was the impetus for the development of various instrumental coupling techniques. One of the most effective and commonly used techniques of coupling was gas chromatography, combined with mass spectrometry (GC-MS). Through analysing GC-MS more precise and reliable knowledge can be gained in qualitative analysis. GC-MS analysis has been increasingly used in medicinal plant analyses in recent years as this analytical method has proven to be very useful in the study of volatile essential oil, fatty acids, lipids, sterols, non-polar components and alkaloids.³⁹ Our GC-MS analysis of ethanol extract from *P. loureiroi* leaves revealed the presence of 22 different phytoconstituents, consisting mainly of phytosterols, sapogenin, acyclic olefins, saturated hydrocarbons, fatty alcohol, saturated fatty acids, fatty acid esters, phthalate esters, benzenedicarboxylic acid and benzenepropanoic acid mimic compounds, along with organosilicon, ethanoanthracene and oxaspiro-lactone-enone cyclic ketone derivative compounds.

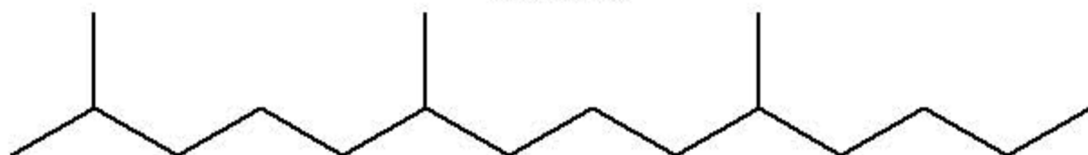
Despite the extensive use of herbal medicine, there were only a few clinical research performed on herbals to provide knowledge approximately their efficacy and safety.⁴⁰ Plant products also can produce toxic outcomes and as a consequence to



1-Tetradecene



Tetradecane



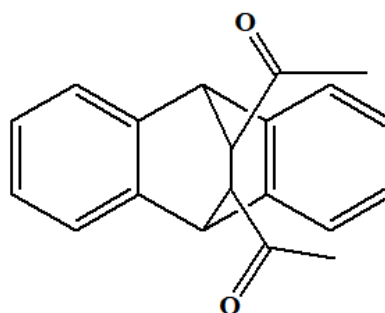
Tetradecane, 2,6,10-trimethyl



1-Hexadecanol (palmityl alcohol)



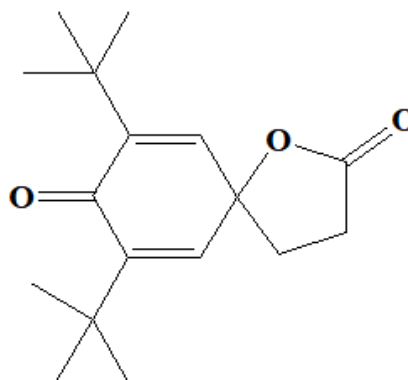
Pentatriacont-9-ene



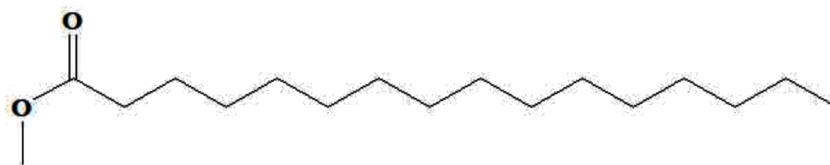
9,10-Ethanoanthracene, 9,10-dihydro-11,12-diacetyl



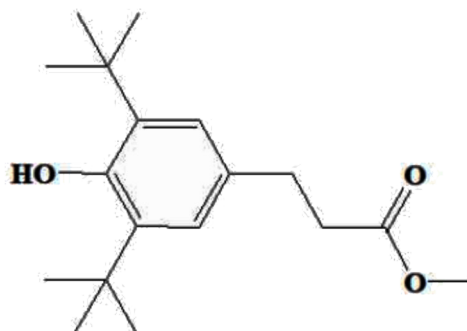
1-Nonadecene



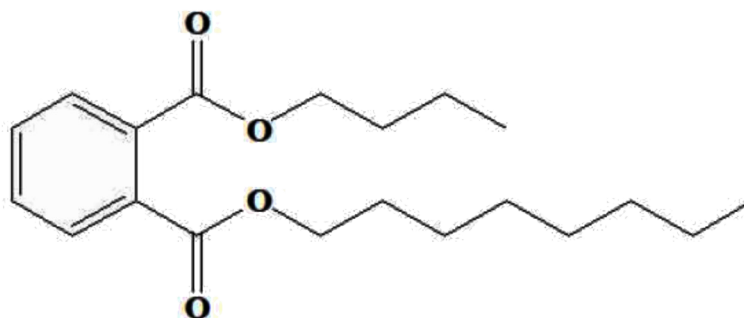
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione



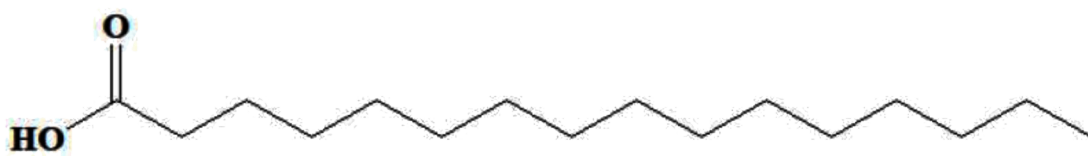
Hexadecanoic acid methyl ester (Palmitic acid methyl ester)



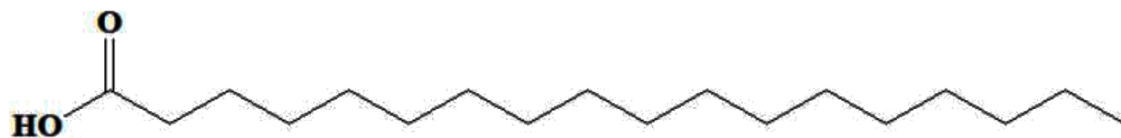
Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-,methyl ester
or
methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propanoate



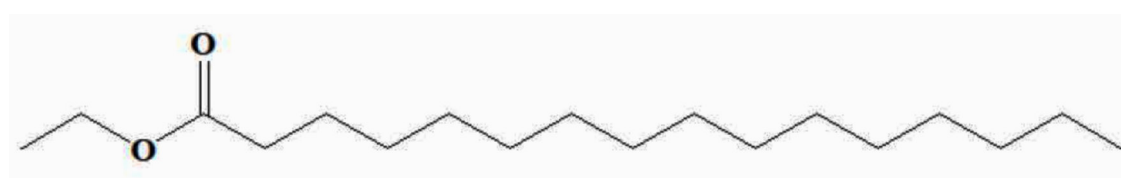
1,2-Benzenedicarboxylic acid, butyl octyl ester



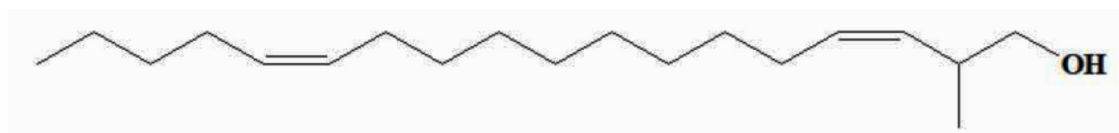
n-Hexadecanoic acid (Palmitic acid)



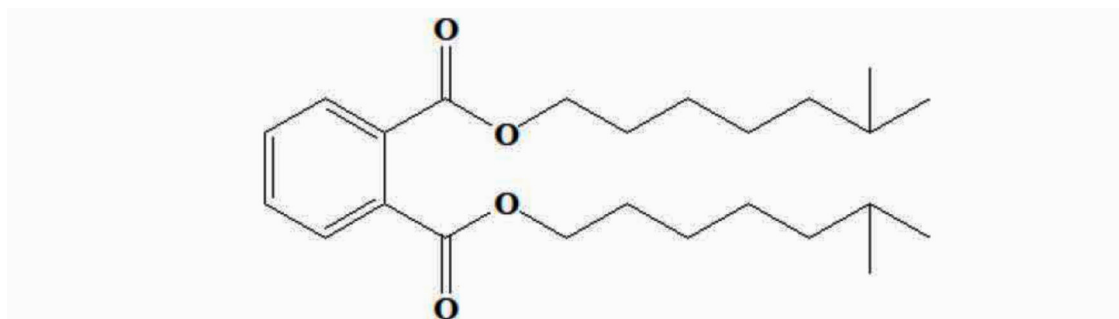
Octadecanoic acid (Stearic acid)



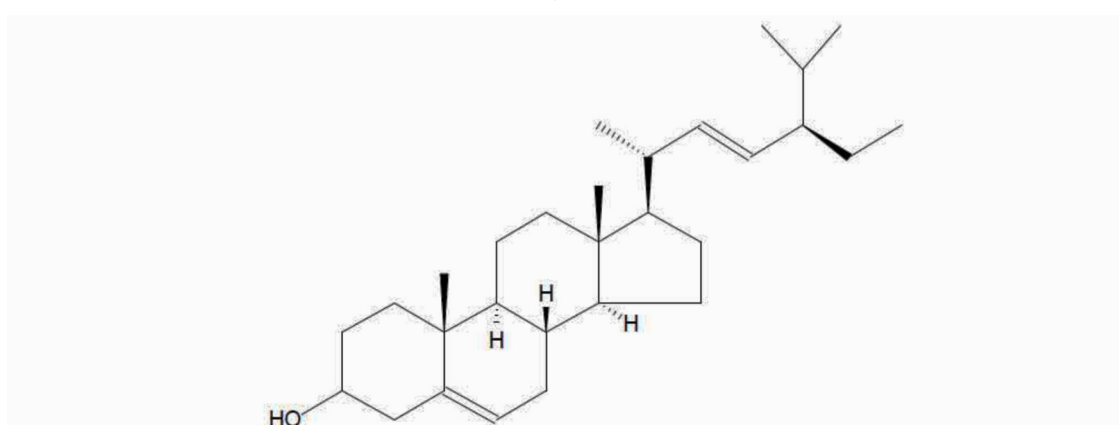
Hexadecanoic acid ethyl ester



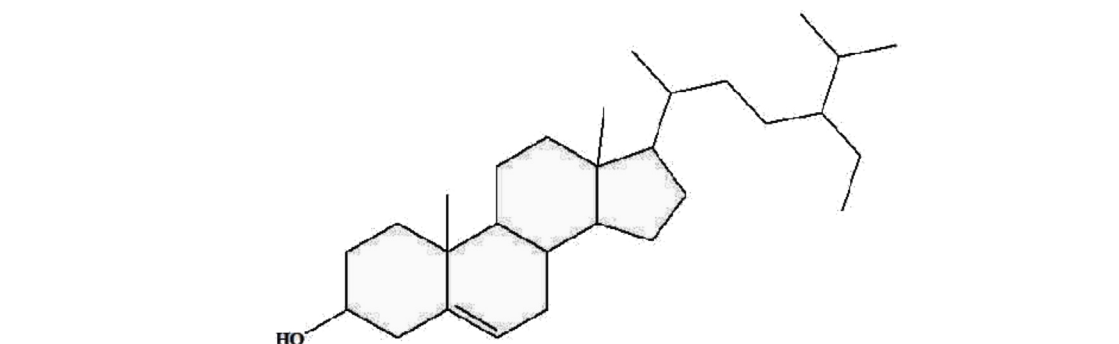
2-Methyl-Z,Z-3,13-octadecadienol



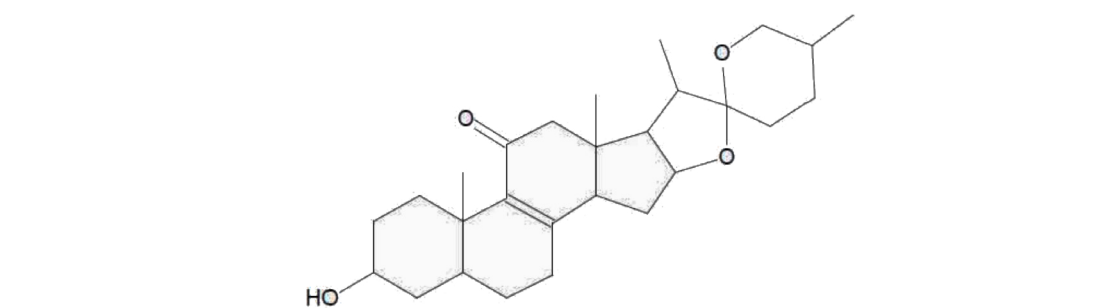
Diisooctyl phthalate



Stigmasterol



β -Sitosterol



Spirost-8-en-11-one, 3-hydroxy-, (3 β ,5 α ,14 β ,20 β ,22 β ,25R)

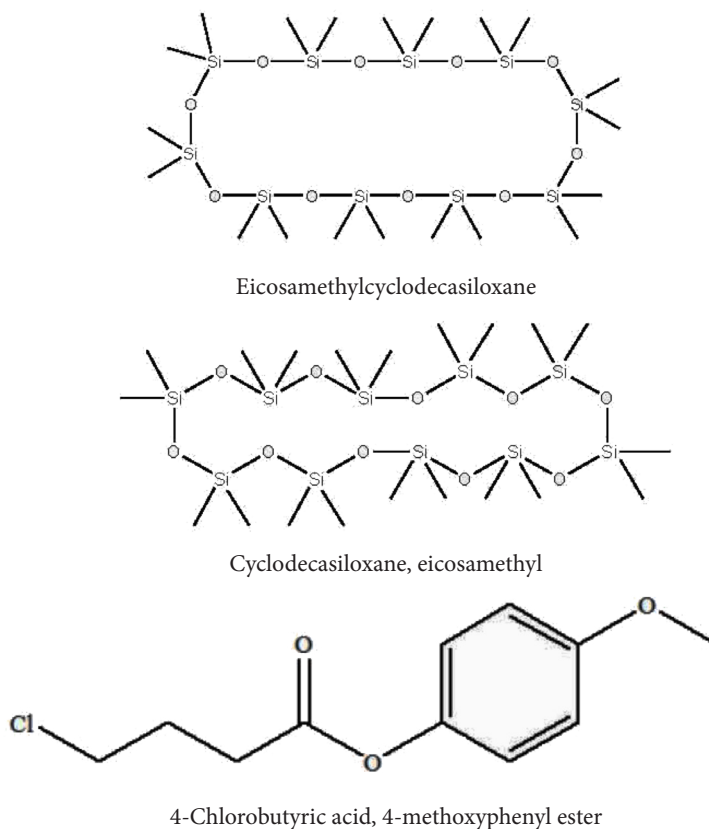
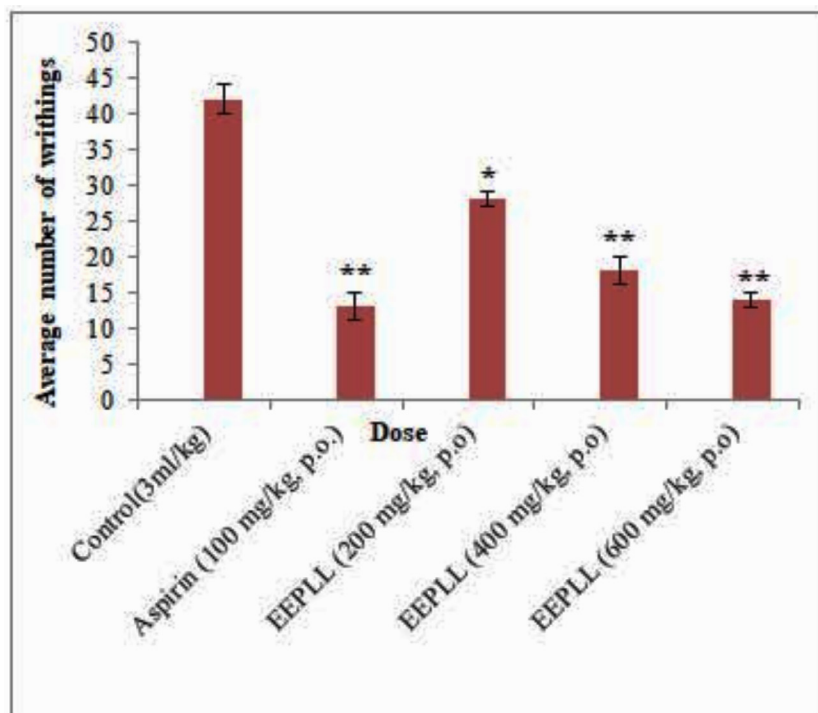


Figure 3 Chemical structures of the compounds identified in GC-MS analysis



Values are expressed as mean \pm S.E. (n=6). All columns are significant using ANOVA; *P<0.05, **P<0.01 when compared to control; Dunnet's t-test.

Figure 4 Analgesic activity of EEPLL on acetic acid induced writhing in mice

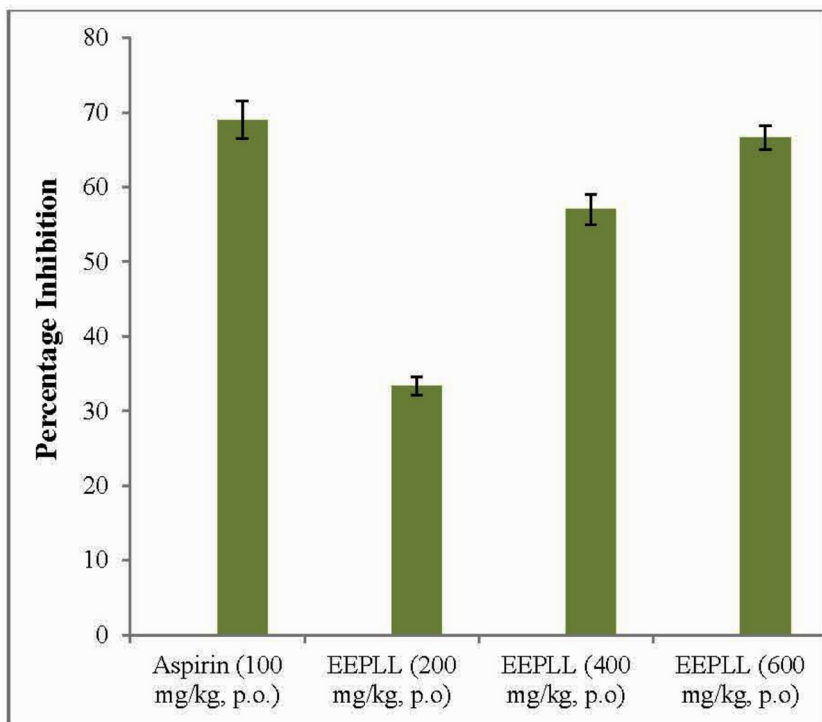


Figure 5 Percentage Inhibition of EEPLL and aspirin on acetic acid induced writhing in mice

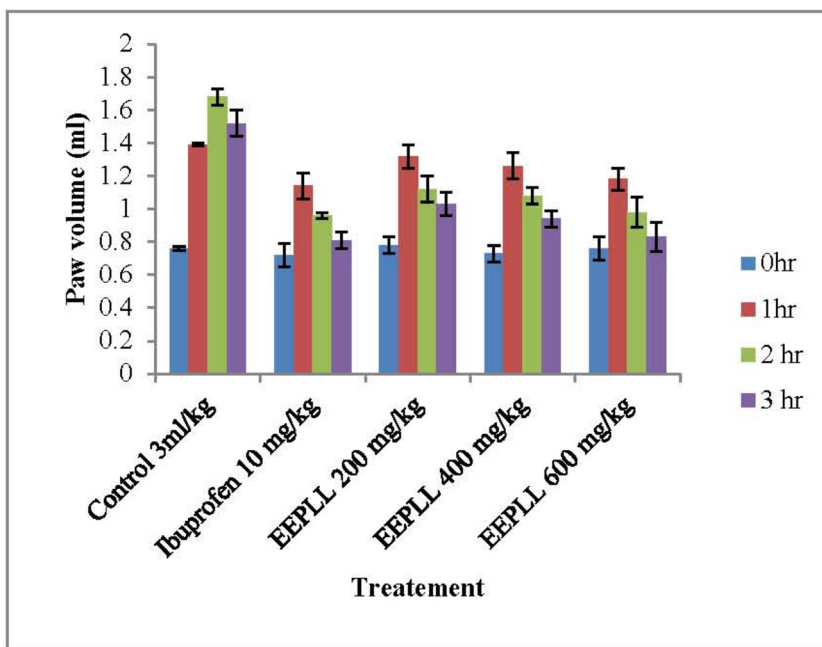


Figure 6 Evaluation of anti-inflammatory activity of ethanol extract of *P. loureiroi* leaves on carrageenan induced rats paw edema

make certain its safety, systematic research regarding its poisonous outcomes desires to be evaluated as an end result supplying scientific statistics for deciding on safe doses for animals together with humans.⁴¹ Consistent with the modern literature the extreme toxicity effects of *P. loureiroi* leaves has not been studied at all. In fact, there are very few clinical reviews of toxicity have a look at

conducted on mountain dates palm which might be extensively used by nearby healers for treatment of diverse diseases and such folkloric makes use of these dates palm without understanding their toxic consequences scientifically might be dangerous in long term. For this reason, the existing studies will provide the primary information document of acute toxicity effect which is traditionally utilized by the nearby healers for curing pain and inflammation. Acute toxicity is considered an initial study which presents us the premise for category and labelling. This also offers initial knowledge about a substance's mode of toxic action, from which we can correct a dose of a new compound and assist in animal experiments in assessing the dose.⁴² In our observation a single administration of EEPLL with increasing doses did not produce any mortality or any critical abnormalities at all doses in acute toxicity, however, the moderate sedative impact changed into noticed in both male and female rats at a single dose of 2000mg/ kg, b.wt. The limit test dose for the study was 2000 mg/kg, b.wt., which showed no significant toxic effects and mortality. Thus, the 1/5th of the preceding dose, i.e. 400 mg/kg body weight, p.o., was used as the test dose for pharmacological evaluation and the lower upper dose of 200 and 600 mg/kg body weight, p.o., was also evaluated to assess whether or not there is any dose-dependent pharmacological impact.

Ethanol extract from *Phoenix loureiroi* leaves (EEPLL) protected against both thermal and chemical induced stimuli, which were evidence from tail immersion and acetic acid induced writhing test. In acetic acid-induced writhing and tail immersion studies, variability in order of action for ethanol extract suggested that the different constituents present in the extract might be responsible for central and peripheral analgesia. Acetic acid, which is used as an inducer for writhing syndromes, causes algisia by means of liberating of endogenous substances, which then excite the pain nerve endings; the abdominal constriction is associated with the sensitization of nociceptive receptors to prostaglandins.^{43,44} It is feasible that EEPLL exerts an analgesic effect probable with the aid of inhibiting the synthesis of prostaglandins. The general analgesic impact of EEPLL (200, 400, and 600 mg/kg body weight, p.o.) was little lower than the standard drugs (aspirin, and pentazocine). The presence of flavonoids, sterols, and sapogenin compounds in *Phoenix loureiroi* leaves ethanol extract may be responsible for the analgesic effect by inhibiting the effects of prostaglandin mediated synthesis, release or receptor response.

In addition, the EEPLL showed a substantial dose-dependent decrease in fever induced by the yeast. The extract was seen in yeast mediated

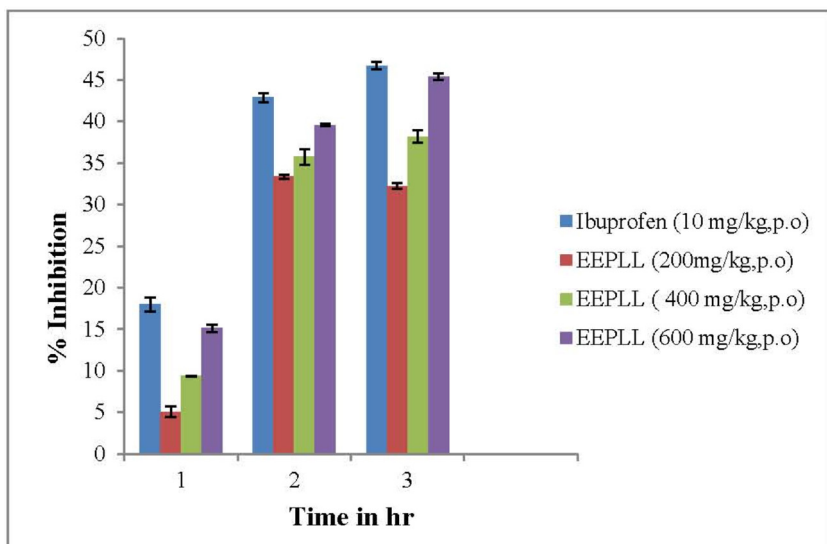


Figure 7 Percentage Inhibition of EEPLL and ibuprofen on carrageenan induced rats paw edema

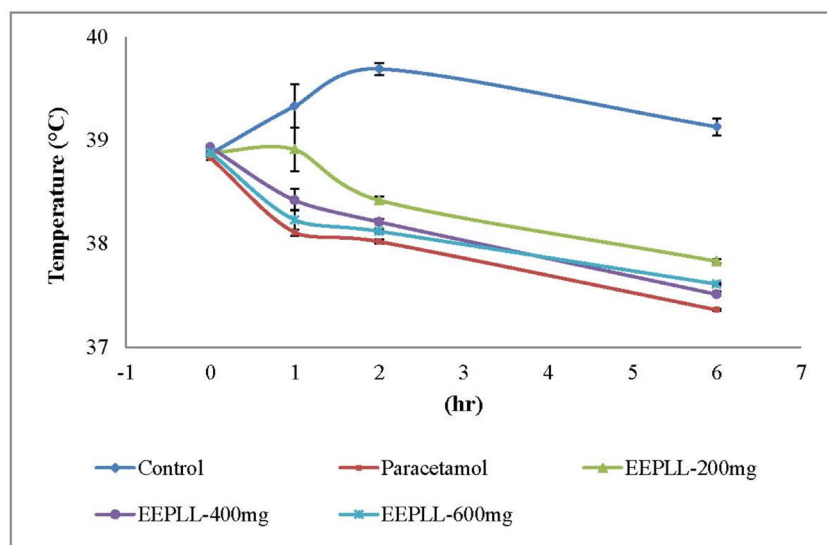


Figure 8 Effect of control, standard: Paracetamol, ethanol extracts of *Phoenix loureiroi* leaves (EEPLL 200 mg/kg, EEPLL 400mg/kg, and EEPLL 600mg/kg)

hyperthermia in rats during an antipyretic study. Pyrexia caused by yeast is called pathogenic fever. Subcutaneous Brewer’s injection causes pyrexia by increasing prostaglandin synthesis.⁴⁵ Prostaglandin synthesis inhibition may be the potential mechanism of antipyretic action as that of paracetamol and prostaglandin inhibition can be accomplished by blocking the enzyme activity of cyclooxygenase. Here our test extract drastically reduced the pyrexia induced by means of yeast in rats. The reference drug paracetamol (150 mg/kg, p.o.) additionally suppressed the fever by inhibiting prostaglandin E₂ synthesis.^{46,47} This finding seems to support the view that leaves extract from *P. loureiroi* has some effect on prostaglandin biosynthesis because

prostaglandin is believed to be a temperature regulator for the body.

Subsequently, anti-inflammatory effects of the ethanol extract from *P. loureiroi* leaves were demonstrated in carrageenan induced model. Carrageenan triggered on rat paw is an appropriate test for evaluating acute anti-inflammatory model. Paw edema caused by carrageenan is a biphasic phenomenon. Kinins, serotonin and histamine are released in the first phase, while in the second phase edema is caused by the release of prostaglandins, lysosome and protease, this process is very prone to most clinically effective anti-inflammatory drugs.⁴⁸ The outcome of carrageenan-induced rat paw edema showed the EEPLL’s role in this model of acute inflammation was significant. The extract at dose of 200, 400, and 600 mg/kg body weight, p.o., showed considerable and significant dose dependent reduction in paw size and elicited comparable anti-inflammatory response reaction similar with widespread drug ibuprofen (10 mg/kg, p.o). This may be due to the carrageenan-induced inhibition of biphasic response. *P. loureiroi* leaves can be involve multiple mechanisms like inhibition of both cyclooxygenase and/or lipooxygenase enzyme or inhibition of synthesis, release and action of above inflammatory mediators. Anti-inflammatory effect of ethanol extract from *P. loureiroi* leaves can be attributed due to the presence of flavonoid, sterols or sapogenin because as we know that flavonoids, sterols and sapogenin have various biochemical outcomes, which inhibit a number of enzymes like aldose reductase, xanthine oxidase, phosphodiesterase, lipooxygenase, cyclooxygenase and many others.^{28,49}

Previous data on preclinical *in-vitro* and *in-vivo* studies have shown that β -sitosterol,⁵⁰ stigmasterol,⁵¹ and quercetin³⁸ generally promote pain relief by inhibiting inflammatory cascades. Pronounced effects on the reduction of inflammatory cytokines (IL, TNF- α) and PGE₂ levels, in addition to the reduction of COX-2, iNOS and 5-LOX expressions, were the most relevant targets. On the other hand, these compounds may also inhibit the conductive pathways of nociceptive stimulus by blocking the Na⁺ channels, NMDA, and TRPV₁ receptors. In general, these compounds may inhibit the sensitization of primary afferent pain fibers by their anti-inflammatory effect or block the nerve conduction of painful stimuli with peripheral and central action drugs.^{52,53} The distinct scientific opinions of EEPLL in terms of its analgesic, antipyretic and anti-inflammatory actions indicates therapeutic efficacy, which has been found to be comparable to standard drugs. The EEPLL was found to be considerably effective against pain, fever and inflammation and there was no substantial acute toxicity observed in

the course of the preclinical study. Analgesic, anti-pyretic and anti-inflammatory properties have been known to be inter related and inter dependent and could be attributed due to the presence of the above said phytoconstituents in the ethanolic extract of *Phoenix loureiroi* leaves.

CONCLUSION

The prevailing study demonstrated that the ethanolic extract of *Phoenix loureiroi* leaves (family: Arecaceae) possesses widespread analgesic, anti-pyretic and anti-inflammatory effects. Acute toxicity tests in Swiss albino rats have shown that EEPLL has a fair safety profile. The HPTLC fingerprint analysis of EEPLL confirmed the presence of quercetin. That is the first file of the presence of quercetin in this mountain dates palm and, through the GC-MS evaluation of the *P. loureiroi* leaf ethanol extract; it recognizes 22 unique phyto-constituents and three unknown compounds. Consequently, this study shows that there is a prospective destiny in the use of this plant as a source of natural medicine for the treatment of various diseases due to the existence of medicinally essential phyto-constituents and it may presage further studies to better understand the mechanism of such action scientifically.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENT

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