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Research Article

Spectral characterization, analgesic, and anti-inflammatory effects of ethanolic extract of *Calotropis procera* leaf and dry latex from Jazan, Kingdom of Saudi Arabia

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Abstract

Traditional healers have used the shrub *Calotropis procera* (CP) for many years for various therapies. The present study investigated the bioactive constituents of ethanolic extract of CP leaf and dried latex using gas chromatography-mass spectrometry and Fourier transforms infrared spectroscopy. The identification and characterization of the compounds were confirmed by examining the constituents' mass spectrum fragmentations and FT-IR spectra and comparing the results with those in the literature. The tail-flick method was used to investigate the analgesic properties of the extract and its anti-inflammatory activities using a rat model of formalin-induced oedema. Acute oral toxicity in rats was studied per OECD recommendations. Twenty male rats were divided into four groups and received an ethanolic extract of the leaves and dried milky sap of CP (200 mg/Kg) in groups 1, 2, and 3. Group 4 rats were administered aspirin 50 mg/kg as a positive control. The CP dried latex extract has the highest content of lupeol and its acetate derivative compared to its leaf extract. The CP dried latex extract inhibited inflammation more significantly than the ethanolic leaf extract and the drug indomethacin at a higher dosage (200 mg/kg). The ethanolic extracts showed analgesia comparable to aspirin. It suggests that fatty acids and their esters, particularly ethyl linoleate (8.96%), ethyl palmitate (7.99%), ethyl linoleate (6.98%), and palmitic acid (5.18%), may be valuable biomarkers for characterizing leaf and latex samples and describing the medicinal potential of CP.

Keywords: analgesic activity; acute toxicity; anti-inflammatory activity; bioactive principles; *Calotropis procera*

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Introduction

Calotropis procera (CP) R. Br. (*Asclepiadaceae*), common name Ushar or Madar, is a well-known medicinal plant in Greeco-Arab medicine extensively dispersed in tropical and subtropical Asia and Africa. (Miller & Morrison, 1987). It is recommended to cure various disorders, such as skin ailments, antiparasitic, antifungal, anti-inflammatory, anti-rheumatic etc. CP has captured substantial interest from scientists worldwide. Isolated compounds and crude extracts of CP have distinct biological activities (antioxidant, antimicrobial, anti-inflammatory, antimalarial, anticancer) (Kaur *et al*., 2021). Fruits, leaves, roots and latex were found effective in treatment of jaundice, diphtheria, gonorrhoea, ringworm and eye infections. Earlier research on a different part of CP (leaves, fruit, latex, roots) disclosed the existence of acyclic ditepenes (Mittal *et al*., 2013; Mittal *et al*., 2015), pentacyclic triterpenes (Alam *et al*., 2009; Shaker *et al*., 2010; Chundattu *et al*., 2016), flavonoids (Salunke *et al*., 2005; Mittal *et al*., 2012; Nenaah *et al*., 2013), alkaloids, and numerous cardenolides (Ramos *et al*., 2006; Shetty *et al*., 2015) which makes this plant scientifically important.

Researchers have always been interested in the phytochemistry of CP because, amidst its toxicity, it has a variety of uses in traditional medicine. The first chemical from this plant, calotropin, was isolated in 1936. Since ancient times, tribes have utilized the root powder of this plant as a uterotonic and to induce abortion in females. Finally, it was found that the compound calotropin was responsible for this activity. Other cardiac glycosides were also identified, including uscharidin, calactin, calotropin, calotoxin, uscharin, and voruscharin (Wadhwani *et al*., 2021).

CP is proven to be pharmacologically active as a potent antioxidant, antibacterial, anthelmintic, anticonvulsant, and anti-inflammatory (Yogi *et al*., 2016). One study found that the extract (100 mg/kg b.w.) effectively inhibited the development of paw oedema compared to Indomethacin. In the initial stage of the formalin response, the itching was significantly reduced and was comparable to Indomethacin (10 mg/kg b.w.). At 100 mg/kg body weight, the extract similarly suppressed the writhing movement to aspirin (15 mg/kg b.w.). The tail flick model in mice showed the same pattern as well. The study demonstrated that the leaf extract's analgesic or anti-inflammatory activity is mediated centrally and peripherally (Saba *et al*., 2011). Plant latex has shown significant anti-inflammatory activity against formalin and carrageenan-induced antipyretic and paw oedema effects (Kumar *et al*., 1994; Dewan *et al*., 2000), which is rich in secondary metabolites, tannins, proteins, flavonoids and glucosides. Anti-inflammatory and antioxidant effects in a rat model of colorectal cancer showed that the levels of MPO, nitrite, PGE2 and TNF-α markers were regulated when the rats were treated with methanolic extract of dried latex of *Calotropis procera* (Kumar *et al*., 2022).

Aqueous and methanolic extracts of CPDL had an anti-inflammatory action against carrageenin that was more evident than phenylbutazone (PBZ). It was equivalent to phenylbutazone (PBZ) and chlorpheniramine (CHP) against histamine and PGE2, respectively. Around 80%, 40%, and 30% of the inhibition of inflammation were reported for both the extracts against BK, compound 48/80, and serotonin, respectively (Arya and Kumar, 2005). The extract's ability to scavenge DPPH radicals was found to be dependent on its concentration in a recent study. The study showed that the DPPH radical scavenging ability of the extract was lower than that of Ascorbic acid, as evidenced by the extract's higher IC_{50} values (Namadina *et al*., 2023).

Conventional anti-inflammatory drugs can have multiple side effects with prolonged use, so researchers are searching for alternative treatments with minimal side effects (Wahid *et al*., 2021). In Saudi Arabia, the extract of the aerial part of CP has been traditionally used to cure multiple disorders, including muscular pain, joint pain, constipation and fever. Researching this plant thoroughly is vital to provide alternatives to traditional medicine and improve people's health and well-being. Continuing our earlier works, we explored the analgesic, anti-inflammatory and acute toxicity activity of the CP's ethanolic leaf and latex extract. Furthermore, we have studied the biologically active components in the plant extract by using gas chromatography-mass spectrometry (GC-MS) and Fourier-transform infrared (FTIR) spectroscopy.

Materials and Methods

Chemicals and reagents

All chemicals, solvents, and reagents utilized in this study were of chromatographic grade and were sourced from Sigma Aldrich (USA).

Study area, collection, and identification

The aerial part of the plant was procured from Jazan, which is located in the southwest of Saudi Arabia along the Red Sea coast (Figure 1A-C), and immediately transported to the laboratory in bio-hazard plastic bags. To remove all adhesive impurities, the leaves were thoroughly washed with tap water. Dr. Remesh Moochikkal, curator of the Jazan University Herbarium (JAZUH), verified the plant's authenticity after receiving a voucher specimen with the identification reference number JAZUH 1317. CP leaves were detached from the obtained branches, properly cleaned with Millipore water, and then dried outside for 30 minutes. Ten days were spent in a ventilated space drying the aerial section. For further analysis, the dried aerial parts were gathered, crushed, and stored at room temperature. The latex was collected in summer from the aerial parts of the plant growing in the wild, which was then dried in the shade (Arya and Kumar, 2005).

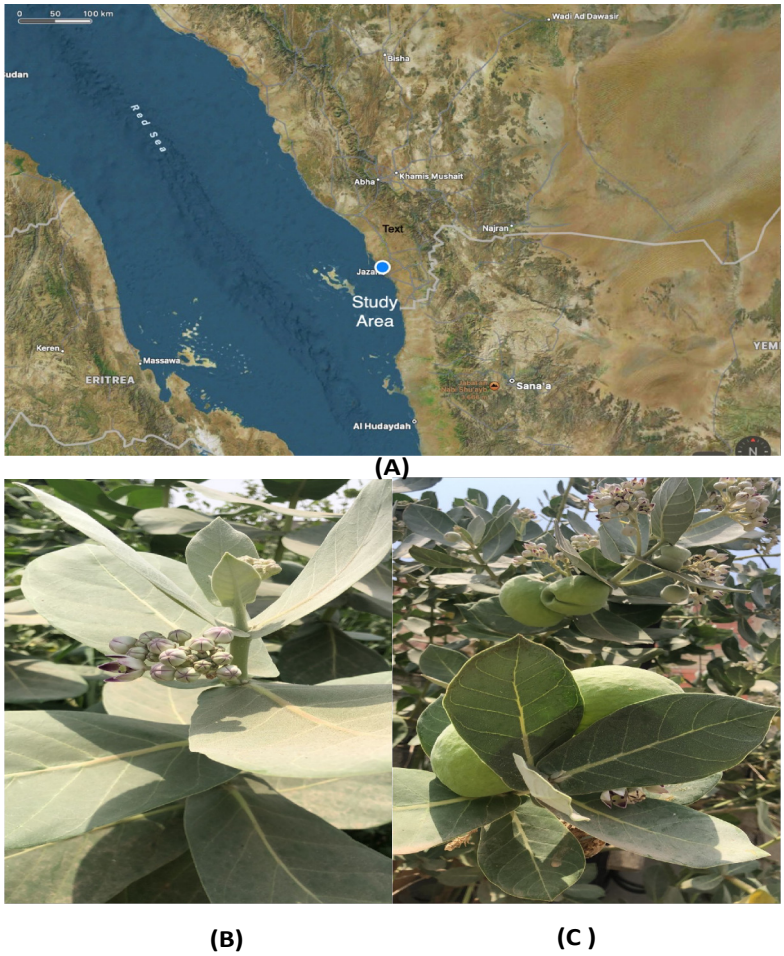

Figure 1. (A) The study area, Jazan, Saudi Arabia (B) Aerial image of Calotropis shrub with branches and cluster of leaves (C) Stem of Calotropis shrub with fleshy & inflated fruits and greenish-white, purple flowers

Organic solvent extraction

Through a cold ethanol maceration process, the CP's bioactive components were extracted. A hotplate magnetic stirrer was used to stir 50 g of dried CP leaf powder at room temperature for one hour after being dissolved in 100 mL of ethanol. The mixture was refrigerated at 4 °C overnight. Then, during the following week, the mixture was stirred using a magnetic stirrer. Using a Sigma tabletop centrifuge, the macerated mixture was centrifuged at 2000 g for 10 min. The filtrate was stored at 4 °C for subsequent use after the supernatant solution was filtered through Whatman filter paper, no. 1. The dried latex sample was subjected to the same procedure to extract the bioactive constituents.

GC-MS analysis of ethanolic extract of CP leaves and latex

Thermo Scientific GC-MS fitted with AS 3000 autosampler, trace ultra-GC and ISQ detector (Serial No: ISQ 110330) was used for identification of components present in the ethanolic leaf extract of CP. Approximately 10 mg of ethanolic leaf extract was diluted with 10 mL of methanol and filtered through 0.45 µm nylon filter. The injection port was set at 290 °C and filtered 2 µL of diluted sample injected in split-less mode into a TR-5MS capillary column (30 m × 0.25 mm ID × 0.25 µm). Helium was used as a carrier gas with a flow rate of 1.2 mL/min. The oven was set with a ramping program having initial temperature set at 70 °C for 5 min and subsequently ramped to 100 °C, 150 °C, 200 °C, 250 °C and 290 °C at a rate 5 °C/min with holding time of 10 min at each ramped stage (total run time of 130 minutes). The MS-detector ISQ was set to identify the molecular masses ranging from 40-650 amu at 70 eV in positive ion mode and spectra was recorded with delay time of 5 min to avoid initial solvent peaks. Ion source temperature and MS transfer line temperature were set at 300 °C and 310 °C respectively.

ATR-FTIR analysis

The ATR-FTIR spectra of ethanolic CPL and CPDL extracts were recorded using an FT-IR spectrophotometer (Nicolet iS10, Thermo Fischer Scientific, USA). Approximately 0.1 mL samples were placed on zinc selenide crystal and the spectra were gathered at 4 cm^{-1} resolution and 100 scans were made over the transmittance range of 4000-400 cm^{-1} (Patel *et al*., 2016).

Pharmacological activity

Study design: Twenty Wistar rats (male) (200-220 gm) were used for this study, obtained from the Medical Research Centre animal house at Jazan University, Saudi Arabia. The rats were housed in a controlled setting with constant ventilation, temperature (22±2 °C), humidity (45-55%), and light/dark cycles (12 hours light/12 hours dark). Rats were acclimatised before starting the experiments. Water and normal diets (pellets) were freely provided during this study.

Further, rats were randomly classified into four groups, each group consisting of five animals as below:

Group-1: Normal Control (vehicle)

Group-2: Single oral dose (gavage) of ethanolic CP leaf extract-200 mg/kg body weight was given, represented as CPL-200

Group-3: Single oral dose (gavage) of ethanolic CP dried latex extract-200 mg/kg body weight was given, represented as CPDL-200

Group-4: Single oral dose (gavage) of Asprin-50 mg/kg body weight as positive control for analgesic, represented as ASP-50 or Indomethacin 10 mg/kg body weight as positive control of anti-inflammatory represented as INDM-10

Acute oral toxicity study

Using OECD Guidelines No. 423 (OECD 2002), toxicological research was carried out. All the animals were divided into six groups, each with five rats. The five groups (Group-2, Group-3, Group-4, Group-5, and Group-6) received 50, 200, 400, 600, and 1000 mg/kg, respectively, of a single dose of CPL extract, whereas the control group (Group-1) received just vehicle. Another five groups (Group-2, Group-3, Group-4, Group-5, and Group-6) received the same single dose of CPDL extract 50, 200, 400, 600, and 1000 mg/kg, respectively. The rats' mortality and other visual changes were observed. Rats were observed in this way every day for 14 days. Rats were given access to clean water, and we kept daily records of what they ate. At the end of the experiment, the rats were anaesthetized and sacrificed; the major organs (liver, heart, kidney and lungs) were then isolated. An additional organ/body ratio was calculated (Porwal *et al*., 2017).

Analgesic activity

Tail-flick Methods: By using the tail-flick techniques mentioned in Sewell and Spencer *et al*. (1976), analgesic activity was evaluated at a single dose of 200 mg/kg body weight. Each rat tail was specifically submerged in warm water at 50 °C + 1 °C for 2-3 cm at the distal end. Rats flicked their tails in discomfort to signal the second response time. After the treatments (oral gavage 200 mg/kg body weight), we measured the latency period (response time) at intervals of 30, 60, and 90 minutes. The maximum reaction time was established at 15 seconds to avoid any harm to the tail tissue. Maximum analgesia was defined as a response lasting more than 15 seconds, and it was computed as follows:

$$Max\ Possible\ Effect\ (\%) = \frac{\text{Treatment Reaction Time} - \text{Control Reaction Time}}{15\ \text{sec} - \text{Control Reaction Time}} \times 100$$

Anti-inflammatory activity

Formalin-induced Oedema test: Four groups of five rats each were made. To the first group, only distilled water as vehicle was given. CPL-200 and CPDL-200 extracts were administered to the second and third groups, respectively. The dosage of indomethacin for the fourth group (the positive control) was 10 mg/kg. According to Roy *et al*. (1982), formalin was injected into the limb of the rats 30 minutes after the drug was administered (0.05 ml of 1% formalin was given to animals that had been fasting overnight). A percentage increase in paw volume was calculated and compared with both the normal control and the indomethacin positive control groups. At 180 minutes following the formalin treatment, the paw oedema was measured. An inflammatory response was seen as oedema on the paws, which was detected as an increase in linear circumference at 180-minute intervals. The calculation of the inhibitory activity used the formula: The percentage of inhibition of oedema is calculated as (Vc-Vt)/Vc *100.

Where, Control group volume is Vc and treatment group volume is Vt.

Statistical analysis

Utilizing the Graph Pad Instat software system, version 3.10, statistical analyses were carried out. Data was represented as mean ± sd (n=5) and the $p < 0.05$ values were considered as significant. Dunnett's post hoc analysis was used to compare the test results to those obtained using the standard drugs.

Results and Discussion

GC-MS analysis

The components of *Calotropis procera* leaf (CPL) and *Calotropis procera* dried latex (CPDL) extract were identified by using software generated match factor (SI) and reverse match factor (RSI) having thresholds of 900 and above. The fragmentation pattern of the EI mass spectrum is compared via spectrum matching with the reference mass spectra in the library, and a list of most probable identities is produced based on the best

(forward or reverse-search) mass spectrum matching score. The relative content (%) of each component was calculated by dividing the peak area of each component to the total peaks area using Xcalibur software. The peaks area was calculated without any internal standard and are uncorrected. The details of all identified compounds with their retention time, molecular weight, molecular formula, and relative content (%) and chemical class are represented in Table 1 and Figures 2-3.

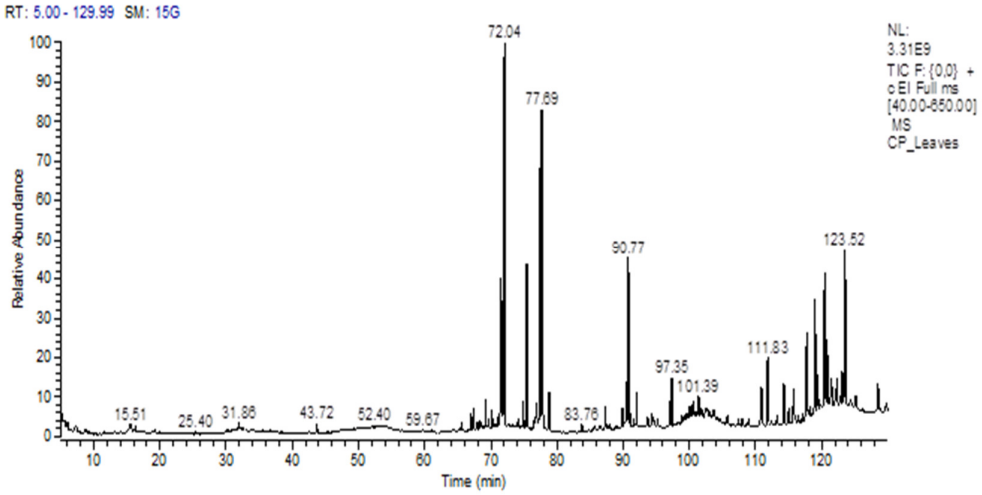

Figure 2. GC-MS Total ion chromatogram of ethanolic CPL extract

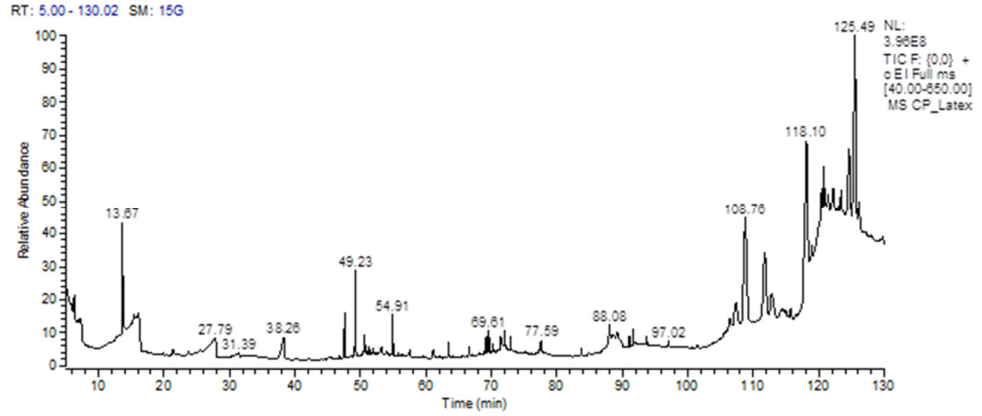

Figure 3. GC-MS Total ion chromatogram of ethanolic CPDL extract

The ethanolic CPL extract appeared as a light green liquid having very little aromatic odor. The GC-MS analysis revealed the presence of fatty acids and its esters as the major components (36.46%). The long chain fatty acids esters such ethyl linolenate (8.96%), ethyl palmitate (7.99%), ethyl linoleate (6.98%), and palmitic acid (5.18%) are animals and plant metabolites and have anti-inflammatory properties. The pentacyclic triterpenoids (25.70%) were the next major class with lupeol and its acetate (13.82%). Recent studies have suggested its role as a therapeutic and chemo preventive agent for the treatment of inflammation and cancer. Lesser quantities of monoterpenes such as limonene (0.14%) and terpineol acetate (0.25%) were also found responsible for slight aromatic odour. Amongst the plant phytosterols (8.25%), stigmasterol, clionasterol and camp sterol together constituted 5.38% of the total content suggesting significant cholesterol lowering effect of the extract. The monoglycerides such as palmitoyl glycerol and glyceryl monostearate were also present in small quantities (1.58%). Phthalate esters and diesters were also found to be present (7.96%).

Overall, a total of 58 compounds were identified with a mass balance of 91.89%. The details of these compounds with relative percent area of 0.30% and above are given in Table 1 and Figure 2.

The ethanolic CPDL extract appeared as a buff white suspension with no odour. The GC-MS analysis revealed the presence of pentacyclic triterpenoids as the only major constituents. The lupeol and its acetate derivative was present in maximum quantity (36.39%) when compared with the CP leaf extract (13.82%). Similarly, cycloartenol acetate was also found in good quantities (12.35%) when compared with CPL extract (1.07%). The other class of chemical constituents such as fatty acids and its esters, phytosterols, triglycerides, phthalate ester and diesters etc. were found in minor quantities. Overall, a total of 28 compounds were identified with a mass balance of 89.98%. The details of these compounds with relative percent area of 0.30% and above are given in Table 1 and Figure 3.

Table 1. Chemical compounds of CPL & CPDL extract by gas chromatography mass spectrometry (GCMS)

S. No.	RT	Extract	Name of Compound	Molecular Formula	Molecular Weight	Relative content %	Chemical Class
1	13.67	CPL	--	--	--	--	Aromatic ester
		CPDL	Cyclopentyl 4-ethylbenzoate	$C_{14}H_{18}O_2$	218	3.24	
2	31.86	CPL	Diethyl succinate	$C_8H_{14}O_4$	174	0.41	Fatty acid ester
		CPDL	--	--	--	--	
3	47.59	CPL	--	--	--	--	Bicyclic sesquiterpene
		CPDL	Beta Caryophyllene	$C_{15}H_{24}$	204	1.32	
4	50.61	CPL	--	--	--	--	Naphthelene derivative
		CPDL	Alpha Selinene	$C_{15}H_{24}$	204	1.14	
5	51.32	CPL	--	--	--	--	Fatty acid silyl ester
		CPDL	2,3-bis(trimethylsilyloxy)propyl octadecenoate	$C_{27}H_{58}O_4Si_2$	502	0.30	
6	52.23	CPL	--	--	--	--	Farnesane Sesquiterpenoid
		CPDL	Nerolidol	$C_{15}H_{26}O$	222	0.42	
7	61.11	CPL	--	--	--	--	Fatty acid aldehyde.
		CPDL	Myristaldehyde	$C_{14}H_{28}O$	212	0.73	
8	65.56	CPL	--	--	--	--	Fatty acid ester
		CPDL	Ethyl palmitate	$C_{18}H_{36}O_2$	284	0.61	
9	67.40	CPL	Phytone (6,10,14-Trimethylpentadecan-2-one 502-69-2)	$C_{18}H_{36}O$	268	0.48	Aliphatic ketone
		CPDL	--	--	--	--	
10	67.97	CPL	Ethyl 13-methyl-tetradecanoate	$C_{17}H_{34}O_2$	270	0.34	Fatty acid ester
		CPDL	--	--	--	--	
11	69.22	CPL	Ethyl pentadecanoate	$C_{17}H_{34}O_2$	270	0.62	Fatty acid ester
		CPDL	--	--	--	--	
12	69.61	CPL	--	--	--	--	oxaspiro compound, (lactone, an enone and a cyclic ketone)
		CPDL	7,9-di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	276	0.65	
13	70.19	CPL	Methyl palmitate	$C_{17}H_{34}O_2$	270	0.31	Fatty acid ester
		CPDL				0.30	
14	71.55	CPL	Palmitic acid	$C_{16}H_{32}O_2$	256	5.18	Fatty acid
	71.41	CPDL				0.76	
15	72.77	CPL	Ethyl palmitate (Ethyl hexadecanoate)	$C_{18}H_{36}O_2$	284	7.99	Fatty acid ester
		CPDL	--	--	--	--	
16	74.12	CPL	oleic acid (cis-9-Octadecenoic acid)	$C_{18}H_{34}O_2$	282	0.52	Fatty acid
		CPDL	--	--	--	--	
17	74.92	CPL	Ethyl heptadecanoate (Ethyl margarate)	$C_{19}H_{38}O_2$	298	0.55	Fatty acid ester
		CPDL	--	--	--	--	
18	75.42	CPL	Phytol	$C_{20}H_{40}O$	296	4.59	Acyclic diterpenoid
		CPDL	--	--	--	--	
19	76.60	CPL	Linoleic acid	$C_{18}H_{32}O_2$	280	0.34	Fatty acid

		CPDL	--	--	--	--	
20	76.86	CPL	Linolenic acid	$C_{18}H_{30}O_2$	278	0.85	Fatty acid
		CPDL	--	--	--	--	
21	77.41	CPL	Ethyl linoleate	$C_{20}H_{36}O_2$	308	6.98	Fatty acid ester
		CPDL	--	--	--	--	
22	77.59	CPL	--	--	--	--	Fatty acid ester
		CPDL	Ethyl oleate	$C_{20}H_{38}O_2$	310	0.37	
23	77.69	CPL	Ethyl linolenate	$C_{20}H_{34}O_2$	306	8.96	Fatty acid ester
		CPDL	--	--	--	--	
24	78.80	CPL	Ethyl stearate	$C_{20}H_{40}O_2$	312	1.04	Fatty acid ester
		CPDL	--	--	--	--	
25	87.27	CPL	Ethyl arachidate	$C_{22}H_{44}O_2$	340	1.35	Fatty acid ester
		CPDL	--	--	--	--	
26	89.89	CPL	Tetratriacontane	$C_{34}H_{70}$	478	0.37	Long chain alkane
		CPDL	--	--	--	--	
27	90.48	CPL	2-Palmitoylglycerol (2-Monopalmitin)	$C_{19}H_{38}O_4$	330	1.19	2-Monoglyceride
		CPDL	--	--	--	--	
28	90.77	CPL	Mono(2-ethylhexyl) phthalate	$C_{16}H_{22}O_4$	278	3.33	Dicarboxylic acid ester
		CPDL	--	--	--	--	
29	92.05	CPL	Ethyl docosanoate	$C_{24}H_{48}O_2$	368	1.21	Fatty acid ester
		CPDL	--	--	--	--	
30	97.35	CPL	Squalene	$C_{30}H_{50}$	410	1.22	Triterpene
	91.63	CPDL					
31	97.76	CPL	Diisodecyl phthalate	$C_{28}H_{46}O_4$	446	4.63	Phthalate ester and a diester.
		CPDL	--	--	--	--	
32	106.39	CPL	--	--	--	--	
		CPDL	Beta-amyrone	$C_{30}H_{48}O$	424	0.70	Pentacyclic triterpenoid
33	110.89	CPL	Campesterol	$C_{27}H_{48}O$	400	1.54	Plant phytosterol
		CPDL	--	--	--	--	
34	111.83	CPL	Stigmasterol	$C_{27}H_{48}O$	400	2.28	Plant phytosterol
		CPDL	--	--	--	--	
35	114.28	CPL	Clionasterol (Gamma Sitosterol)	$C_{29}H_{50}O$	414	1.56	Plant phytosterol
		CPDL	--	--	--	--	
36	115.01	CPL	Isofucosterol	$C_{29}H_{48}O$	412	0.60	Plant phytosterol
		CPDL	--	--	--	--	
37	115.74	CPL	Beta-amyrin	$C_{30}H_{50}O$	426	2.32	Pentacyclic triterpenoid
		CPDL	--	--	--	--	
38	117.74	CPL	Alpha-amyrin	$C_{30}H_{50}O$	426	3.88	Pentacyclic triterpenoid
	111.69	CPDL				5.46	
39	118.98	CPL	Methyl 3-oxours-12-en-23-oate	$C_{31}H_{48}O_3$	468	3.39	Triterpenoid ester
		CPDL	--	--	--	--	
40	119.40	CPL	Cycloartenol acetate	$C_{32}H_{52}O_2$	468	1.07	Pentacyclic triterpenoid
		CPDL	--	--	--	--	
41	120.47	CPL	Lupeol acetate	$C_{32}H_{52}O_2$	468	5.62	Pentacyclic triterpenoid
	118.09	CPDL				18.46	
42	120.83	CPL	Lupeol	$C_{30}H_{50}O$	426	8.2	Pentacyclic triterpenoid
	108.76	CPDL				17.93	
43	121.47	CPL	(5beta,22E)-3',6alpha-Dihydrocyclopropa[5,6]stigmast-22-en-3-one	$C_{30}H_{48}O$	424	0.39	Plant phytosterol
		CPDL	--	--	--	--	
44	122.29	CPL	(22E)-3beta-Methoxystigmasta-5,22-diene	$C_{30}H_{50}O$	426	0.85	Plant phytosterol
		CPDL	--	--	--	--	
45	124.60	CPL	--	--	--	--	1-monoglyceride
		CPDL	Glyceryl monolinoleate	$C_{21}H_{38}O_4$	354	7.06	
46	125.49	CPL	--	--	--	--	Pentacyclic triterpenoid
		CPDL	Cycloartenol acetate	$C_{32}H_{52}O_2$	468	12.35	

The phytochemical screening of the crude ethanolic extract of leaves of CP showed that the extract contains terpenoids and steroids. This is coherent with the previous reports (Hassan *et al.*, 2006; Oladimeji *et al.*, 2006). In addition, fatty acids, fatty acid esters and aldehydes are also found in the ethanolic extract that matches the previous studies (Verma *et al.*, 2013). In the present study, free and ester forms of n-hexadecanoic acid are identified with relative contents of 5.18% and 7.99%, respectively, compared to one of the previous studies where the authors reported only ester derivative of n-hexadecanoic acid (10.24%) (Verma *et al.*, 2013). Similarly, the 9-octadecenoic acid content (21.36%) was reported by some researchers from *Calotropis procera* leaf extract, which is very high compared to the present study, 0.52% only. Interestingly, phytoconstituents phytol (4.59%), linoleic acid (0.34%), linolenic acid (0.85%) and diisodecyl phthalate (4.63%) are identified in the present study but were absent in the previous study (Verma *et al.*, 2013). Phytol attenuates the inflammatory response by inhibiting neutrophil migration, partly caused by reduced IL-1β and TNF-α levels and oxidative stress (Silva *et al.*, 2014).

FT-IR spectroscopy analysis

The analysis of ethanolic extracts of CPL and CPDL using FT-IR revealed the presence of unique peak patterns. (Figure 4). The presence of bioactive substances, particularly fatty acids, fatty acid esters, acyclic diterpenes, phytosterol, pentacyclic triterpenoids, sesquiterpenoid, and sesquiterpene, was consistent with the major functional groups that corresponded to them (Tables 2 and 3).

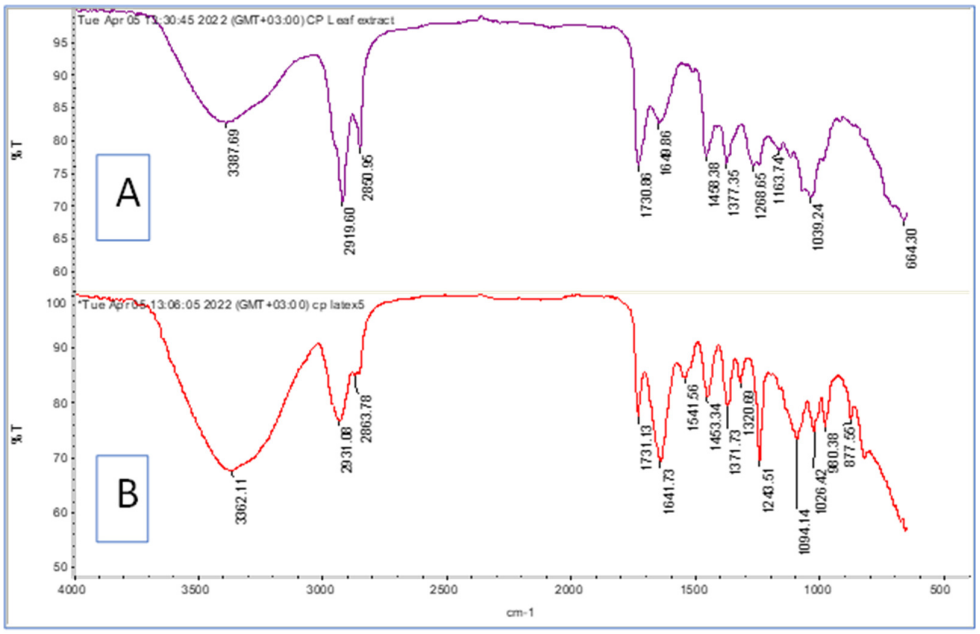

Figure 4. FT-IR spectral pattern of ethanolic CPL extract (A) and ethanolic CPDL extract (B)

Table 2. Chemical composition of CPL extract by FTIR spectroscopy

Wave number (cm^{-1})	Intensity Estimation	Functional group/Class	Type of vibration	Possible Compounds	References
3387	Strong &Braod	O-H (Alcohol)	Stretching	Lupeol Phytol, 2-Palmitoylglycerol, Campesterol, Stigmasterol, Clionasterol, Isofucosterol, Alpha-amyrin	Ranade *et al*., 2017
2919	Strong	C-H str. (Asymmetrical)CH_2(Csp3)/=C-H (Csp2)	Stretching	Lupeol and Lupeol acetate	Souza *et al*., 2022
2850	Strong	CH str. (Symmetrical CH_2, Alkane)	Stretching	Lupeol and Lupeol acetate	Kinda *et al*., 2020 Souza *et al*., 2022
1730	Strong	C=O , (Carboxylic, Ketones & Fatty acid ester groups)	C=O Stretching	Phytone, Ethyl palmitate, oleic acid, Linoleic acid, Ethyl linoleate, Ethyl stearate, Ethyl docosanoate, Cycloartenol acetate, (22E)-3beta-Methoxystigmasta-5,22-diene	Kinda *et al*., 2020 Souza *et al*., 2022
1649	Strong	C=C (Alkenes)	Stretching	Alkenes, oleic acid, Phytol, Squalene, Campesterol, Stigmasterol	Ranade *et al*., 2017
1458	Strong	C-H	Asymmetrical bending	Aliphatic compounds, Squalene	Ranade *et al*., 2017
1377	Medium	O-H (Hydroxyl)	bending	Phenolic compounds	Ranade *et al*., 2017
1268	Strong	C-N (Amine)	Stretching	Aromatic amine	Kinda *et al*., 2020
1163	Strong	C-O	Stretching	Esters, Ethyl heptadecanoate,	Kinda *et al*., 2020
1039	Medium	C-O	Stretching	Tetrahydropyran-4-one	Kinda *et al*., 2020
664	Strong	C-S (Thio)	Stretching	Thiodiglycol	

Table 3. Chemical composition of CPDL extract as obtained from FTIR spectroscopy

Wave number (cm^{-1})	Intensity Estimation	Functional group/Class	Type of vibration	Possible Compounds	References
3362	Strong &Braod	O-H (Alcohol)	Stretching	Lupeol, Alpha amyrin, Phenolic compounds	Ranade *et al*., 2017
2931	Strong	C-H (Alkane)	Asymmetrical Stretching	Aliphatic compounds, squalene, Alpha amyrin, Lupeol and Lupeol acetate	Ranade *et al*., 2017 Kinda *et al*., 2020 Souza *et al*., 2022

2863	Strong	CH_2 str. (Symmetrical, (Alkane)	Stretching	Alkanes, Carboxylic acids and Derivatives, Lupeol and Lupeol acetate	Ranade *et al*., 2017 Kinda *et al*., 2020
1731	Strong	C=O (Carboxylic, Ketones & Ester groups)	C=O Stretching	Cyclopentyl 4-ethylbenzoate, Ethyl palmitate, Palmitic acid, Beta-amyrone, Glyceryl monolinoleate, Cycloartenol acetate	Ranade *et al*., 2017 Kinda *et al*., 2020
1641	Strong	C=C (Alkene)	Stretching	Beta Caryophyllene, Nerolidol	Ranade *et al*., 2017
1453	Strong	C-H (Alkyl)	Asymmetrical bending	Methyl group (Aliphatic compounds)	Ranade *et al*., 2017
1371	Medium	O-H (hydroxyl)	Bending	Phenolic compounds	Ranade *et al*., 2017
1320	Strong	S=O (Sulfone)	Stretching	Sulfone	Ranade *et al*., 2017
1243	Medium	C-N (Amine)	Stretching	Amine	Ranade *et al*., 2017
1094	Medium	C-O (Secondary alcohol)	Stretching	Lupeol	Kinda *et al*., 2020
980	Strong	C=C (Alkene)	Bending	Alkene	Ranade *et al*., 2017
877	Medium	C-H	Out of plane bending	Arenes, Amines	Ranade *et al*., 2017

The broad band at 3387 cm^{-1} with stretching vibrations in the spectrum of the ethanolic CPL extract (Figure 4A) indicates the presence of secondary cyclic hydroxyl (-OH-) groups, which are related to sterols (Cholesterol, Campesterol, Stigmasterol, Clionasterol, Alpha-amyrin, and Isofucosterol), and steroid molecules (Digoxigenin). Additionally, it suggests to Gamma-Tocopherol's phenolic hydroxyl (-OH) group. The results of the current analysis revealed two distinct peaks at 2913 and 2850 cm-1 with stretching vibrations indicative of the presence of 10-Octadecenal and 1,3,3-Trimethoxybutane, respectively. The stretching and bending vibrations at 1730, 1649, 1458, 1377, 1268, 1163, 1039 and 1664 cm^{-1} indicate the presence of vitamin E, squalene, sterols, and fatty acids.

The broad band at 3362 cm^{-1} with stretching vibrations in the spectrum of the CPDL (Figure 4B) indicates the presence of secondary cyclic hydroxyl (-OH-) groups, which conform to sterols (Lupeol, Alpha-amyrin), and phenolic hydroxyl (-OH) groups, such as Gamma-Tocopherol. The current analysis also revealed two distinct peaks with C-H/CH2 stretching vibrations at 2931 and 2863 cm^{-1}, which correspond to the existence of 2-Epoxyhexadecane, aliphatic compounds, squalene, alpha amyrin, and carboxylic acids & derivatives. The shift of the band from 2919 cm^{-1} to 2931 cm^{-1} may be due to the increase in the number of CH_3 groups or =C-H (Csp2) terminal (vinyl) in CPDL with a high content of Lupeol and Lupeol acetate. Similarly, the shift of the band from 3387 cm^{-1} to 3362 cm^{-1} may be because of increase in inter and intra-molecular hydrogen bondings due to the large number of hydroxy groups in CPDL with a high content of Lupeol and Lupeol acetate. The stretching and bending vibrations in the fingerprint region at 1731, 1641, 1453, 1371, 1243, 1094, 980 and 877 cm^{-1} also reveal the occurrence of fatty acids, terpenoids, aliphatic compounds, amines, alkenes and arenes. According to a recent study, the CPDL demonstrated distinct stretching frequency peaks for the functional group's alcohols, phenols, amines, fatty acids and derivatives, aldehydes, ketones, and alkenes at 3417, 2929, 2872, 1736, 1643, 1245, 1115, and 1035 cm^{-1} (Ranade *et al*., 2017). Peaks of bending vibration frequencies were also observed at 1643, 1453, 1383, 1322, 983, 900, 878, 779, and 618 cm^{-1}, indicating the presence of carboxylic acid and its derivatives, alkanes, alcohols, phenols,

sulfone, alkenes, esters, arenes, amines, and alkynes (Ranade *et al*., 2017). Hence, in the current study, the FTIR spectra confirm the functional groups and correlate with the compounds identified through GC-MS analysis.

Effects of leaf extract and dry latex on acute Oral Toxicity

When dosages of 50 mg/kg, 200 mg/kg, 400 mg/kg, 600 mg/kg, and 1000 mg/kg were applied, behavioural abnormalities and mortality were not seen during the observation period. The ratio of organ (liver, heart, kidney and lungs) weight to total body weight resembled control and therapy. Neither the treatment group nor the control group displayed any behavioural changes. In the first and second weeks, the body weight of each group rat decreased. While no appreciable variations between the treatment and control groups were found, the weight of the remaining organs was found to be within the normal range (Tables 4 and 5).

Table 4. Acute toxicity of CPL and CPDL on body weight gain, and food and water consumption in rats

Calotropis procera **leaf (CPL) extract**						
Observation	**Control**	**CPL-50**	**CPL-200**	**CPL-400**	**CPL-600**	**CPL-1000**
Initial weight	183.2±3.18	186.2±2.71	185.4±2.01	183.2±3.65	185.8±3.72	182.4±1.86
Weight (After 1 week)	180.1±2.28	184.8±2.94	182.4±2.42	181.4±3.01	183.2±1.94	179.8±2.33
Weight (After 2 weeks)	180.3±3.01	178.8±2.06	172.6±2.34	170.4±1.96	173.8±2.94	171.4±1.78
Food intake (g/day)	16.88±2.48	16.16±1.16	16.5±1.87	16.33±1.96	17.66±2.80	15.66±2.25
Water intake (ml/day)	21.33±1.03	20.83±1.47	19.83±2.92	21.5±1.04	23.83±1.47	22.5±3.01
Calotropis procera **dried latex (CPDL) extract**						
Observation	**Control**	**CPDL-50**	**CPDL-200**	**CPDL-400**	**CPDL-600**	**CPDL-1000**
Initial weight	183.2±3.18	184.1±1.32	183.5±1.01	184.1±2.35	183.6±1.93	184.3±1.72
Weight (After 1 week)	180.1±2.28	185.2±1.52	184.7±2.33	183.1±2.13	185.4±2.53	181.8±2.33
Weight (After 2 weeks)	180.3±3.01	179.3±2.33	178.4±3.22	177.4±1.11	175.6±1.84	174.5±1.63
Food intake (g/day)	16.88±2.48	15.13±1.12	17.5±1.73	16.55±1.34	16.21±1.90	17.52±1.35
Water intake (ml/day)	21.33±1.03	22.24±2.35	21.35±2.90	20.4±1.32	22.67±1.53	23.7±2.85

Values are expressed in mean±sd; There were no significant changes observed in body weight, food intake and water intake as compared to the control group (P>0.05).

Table 5. Acute toxicity effects of CPL and CPDL on the organ and body weight ratio of rats

Observation	**Control**	**Leaf extract**					**Dried Latex extract**				
		CPL-50	**CPL - 200**	**CPL - 400**	**CPL-600**	**CPL - 1000**	**CPDL-50**	**CPDL-200**	**CPDL-400**	**CPDL-600**	**CPDL-1000**
Liver	2.79 ±0.23	2.76 ±0.08	2.80 ±0.03	2.88 ±0.20	2.92 ± 0.13	3.04 ± 0.13	2.75 ±0.31	2.72 ±0.07	2.95 ±0.07	2.78 ±0.20	2.82 ± 0.33
Heart	0.85 ± 0.060	0.875 ±0.049	0.91 ± 0.068	0.92 ± 0.059	0.93 ± 0.077	0.89 ± 0.013	0.95 ± 0.08	0.83 ±0.039	0.89 ± 0.062	0.81 ± 0.065	0.91 ± 0.057
Kidney	0.37 ± 0.047	0.35 ± 0.036	0.40 ± 0.033	0.36 ± 0.026	0.425 ±0.044	0.36 ± 0.01	0.39 ± 0.032	0.38 ± 0.033	0.45 ± 0.013	0.36 ± 0.026	0.38 ±0.034
Lungs	0.41 ± 0.011	0.4 ±0.021	0.4 ± 0.026	0.41 ± 0.032	0.39 ± 0.033	0.41 ± 0.019	0.39 ± 0.021	0.51 ±0.031	0.44 ± 0.016	0.51 ± 0.022	0.43 ± 0.051

Values are expressed in mean ± sd; There were no significant changes observed in organ/body weight ratio in rats as compared to the control group(P>0.05).

Analgesic/ antinociceptive effects

Previously, authors (Kolar *et al*., 2019; Consuelo *et al*., 2023) have found that fatty acid such as oleic acid, linoleic acid and linoleic acid along with their esters have promising anti-inflammatory and analgesic properties showing good correlation between the observed anti-inflammatory and analgesic activities and the compounds reported in the ethanolic leaf extract (34.46% fatty acid and their esters).

Analgesic activity by the tail-flick response to thermal pain was significantly high with Eth nolic CPL extract at 200 mg/kg (4.02± 0.09 sec, 3.86±0.16, and 3.54±0.24) and CPDL at 200 mg/Kg (4.28±0.18 sec, 4.06±0.17 sec and 3.84± 0.14 sec) at different time intervals of 30 min, 60 min and 90 min as compared to normal control (3.66±0.31 sec, 3.06±0.19 sec and 2.77±0.33 sec). Rats administered with aspirin 50 mg/kg showed the 4.61± 0.41 sec, 4.12±0.07 sec and 3.96±0.16 sec, which is a slightly higher thermal reaction time than the response of CPL at 200 mg/kg b.w and CPDL at 200 mg/kg b.w which is represented in Table 6. Analgesic activity measured by tail flick can be obtained via both central acting and peripheral acting methods. In peripheral tissues, this is achieved by interfering with the activation of primary afferent n ciceptors by inhibiting prostaglandin production and cyclooxygenase activity (Ramabardan, 1989; Field, 1987). The analgesic activity of ethanolic extract and dry latex was not as prominent as an aspirin 50 mg/kg, but it was equivalent to aspirin 50 mg/kg. It was also noticed that CPDL was more potent than CPL, but it was equivalent to aspirin at 50 mg/kg. CPDL may exert its effects either in the CNS or the periphery via a method independent to opioid receptors. Moreover, the effects of CPDL on spontaneous motility and motor coordination were minimal. It was comparable to aspirin in this aspect and is unlikely to significantly lower pain threshold. Hence, the research suggests that CPDL functions as an aspirin-like non-narcotic analgesic (Dewan *et al*., 2000).

Table 6. Analgesic effect of CPL and CPDL extracts by tail-flick methods at different time interval

Groups	30 min	60 min	90 min
Gropu-1 (Normal Control)	3.66±0.31	3.06±0.19	2.77±0.33
Group-2 (CPL extract-200)	4.02±0.09	3.86±0.16	3.54±0.24
Group-3 (CPDL extract-200)	4.28±0.18	4.06±0.17	3.84±0.14
Group-4 (ASP-50)	4.61±0.41	4.12±0.07	3.96±0.16

Data represents in Mean ± SD (n=5).

Anti-inflammatory effects

The CPDL inhibited paw oedema formation to significant levels in rats treated with formalin. At a dose of 200 mg/kg, the CPL produced 34% inhibition in the case of formalin-induced oedema ($P < 0.05$), at an amount of 200 mg/kg, the CPDL of around 42% inhibition was o served for formalin-induced oedema ($P < 0.05$). However, a higher dose (200 mg/rat) of CPDL was high in percentage inhibition of inflammation than ethanolic extract and Indomethacin oedema groups (Table 7).

Table 7. Anti-inflammatory effect of CPL and CPDL extracts against formalin-induced rat paw oedema

	Oedema after 180 min	
	Volume (ml)	% Inhibition
Group-1 Negative control	2.35 ± 0.026	---
Group-2 (CPL extract-200 mg)	1.74 ± 0.013	34%
Group-3 (CPDL extract -200 mg)	1.65 ± 0.027	42%
Group-4 (INDM-10mg/kg	1.76 ± 0.031	33%

Conclusions

The GC–MS analysis of ethanolic CPL extract revealed the presence of fatty acids, their esters, and pentacyclic triterpenoids as the major components. The lupeol and its acetate derivative were present in maximum quantity in CPDL extract compared with the CPL extract. Similarly, compared to CPL extract, cycloartenol acetate was also found in reasonable quantities. The results of the FT-IR investigation revealed the existence of distinct patterns of peaks. In the analgesic activity, the ethanolic CPL and CPDL extracts were not as prominent as an aspirin, but it was equivalent to aspirin. The CPDL extract was more analgesic than the CPL. It was observed that at a high dose (200 mg/rat) of CPDL, the percentage inhibition of inflammation was more than the ethanolic leaf extract and the drug Indomethacin. Thus, this investigation indicates that CP has an effective analgesic and anti-inflammatory action. Both CPL and CPDL extracts have no significant effects on acute oral toxicity. The current results reveal that active analgesic and anti-inflammatory principles can be isolated from CP, and it would help develop new drug molecules. The outcomes highlight the significance of CP as a prospective means for industrial and pharmaceutical applications.

Authors' Contributions

Conceptualization: MSA, and MFA; Data curation: MSA, BRR, and MMH; Funding acquisition; KZ; Investigation: MSA, MFA, ZUR and SSM; Project administration: MA, HAA, and AN; Supervision: MSA, and MFA; Validation: ZUR and SSM; Visualization: MSA; Writing - original draft: MSA, MFA; Writing - review and editing: MA, HAA, AN. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

All the experiments were carried out after the ethical approval of the Standing Committee for Scientific Research - Jazan University (Reference No.: REC-44/07/493.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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