Research Article

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Separation and evaluation of potential antioxidant, analgesic, and anti-inflammatory activities of limonene-rich essential oils from *Citrus sinensis* (L.)

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Abstract: The peel of *Citrus sinensis* (L.) Osbeck is a source of essential oils, particularly limonene, which is this plant's characteristic molecule. The main goal of this study was to test the potential analgesic and anti-inflammatory properties of limonene-rich essential oils derived from the peel of *C. sinensis* L. (orange) *in vivo*, as well as their antioxidant activity *in vitro*. Carrageenan-induced paw edema in Wistar rats and the formalin test in Swiss albino mice were used to examine anti-inflammatory activity. The analgesic activity was assessed using hot plate and acetic acid writhing tests, while the antioxidant activity was assessed using the 1,1-diphenyl-2-picrylhydrazyl and ferric reducing antioxidant power methods. The essential oil (EO) safety was determined using an acute toxicity experiment on mice. The phytochemical analysis confirmed the existence of limonene as the primary molecule (88.94%), and *in vivo* experiments revealed that the EO had a significant pain and inflammation-relieving effect, especially at the dose of 50 mg/kg, when compared to the used control drugs. The acute toxicity evaluation reported this EO's safety. This study contributes to the pharmacological valorization of the peel of *C. sinensis* L., confirming that, in addition to its numerous cosmetic and industrial uses, it may be effective in the treatment of inflammatory and pain-related illnesses.

Keywords: limonene, essential oils, acute toxicity, analgesia, gas chromatography

1 Introduction

Traditional medicine continues to be the primary source of therapy for the great majority of the population since it is an important component of their cultural heritage. According to the World Health Organization [1], almost 80% population in the world depends on traditional medicine for basic health care. Natural plants are still thought to be useful in the treatment of the most prevalent illnesses, including acute pain and inflammation [2]. Several studies have supported their traditional use throughout the years, and they are now accessible on the market as phytomedicine [3,4]. Many scientific investigations have been carried out to uncover the functional qualities of plant components that may be useful in the prevention and treatment of diseases [5]. The hunt for novel natural compounds, including essential oils, that are softer, more accessible, and have numerous activities (antimicrobial, antioxidant, anti-inflammatory, and so on) represents an indisputable solution in resolving these issues [6]. Volatile oils have been used for a long time in several industries, including beverages and food [7]. The aromatic plants are mainly composed of terpenes as a major component, besides other constituents such as esters, nitrogen, aldehydes, ketones, phenols, alcohols, and fatty acids [8]. Citrus species have been shown to have antioxidant, hypoglycemic, antibacterial, and anticancer effects. [9]. The sweet orange (*Citrus sinensis* (L.) Osbeck) *is a Rutaceae* fruit shrub species. This ancient hybrid is possibly a cross between grapefruit and tangerine, and it is a sweet

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and edible fruit. It is grown in warm places, such as Mediterranean countries [10]. Citrus peel is very beneficial medicinally and demonstrates a wide spectrum of biological effects due to its high amount of flavonoids (flavone, flavonol, and flavanone), terpenes, carotenes, and coumarines [11]. Citrus essential oils are now frequently used in pharmaceutics to treat bacterial infections, diabetes, reactive oxygen species, cancer, and a variety of other ailments [12]. Many bioactive compounds derived from citrus, such as narirutin, have shown considerable promise in the treatment of a variety of ailments, such as diabetes [13], hesperetin in the treatment of cancer [14], and naringenin to treat kidney injury [15]. *C. sinensis* peel essential oil is well known for its antioxidant, antibacterial, and insecticidal properties. Velázquez-Nuñez et al. reported that essential oil (EO) was effective against the proliferation of *Aspergillus flavus* whether administered via vapor exposure or direct addition [16]. The EO's antibacterial action was validated against a variety of bacteria species, including *Escherichia coli*, *Bacillus subtilis*, *Penicillium chrysogenum*, *Staphylococcus aureus* and *Saccharomyces cerevisiae* [17], *Staphylococcus aureus*, *Listeria monocytogens* and *Pseudomonas aeruginosa* [18], Gram-negative *Pseudomonas aeruginosa*, Gram-positive *Listeria monocytogenes*, *Escherichia coli* and *Staphylococcus aureus* [19–21]. The EO also presented a potential effect on *Streptococcus iniae* infection [22]; it is also effective in controlling *Zabrotes subfasciatus* [23]. The cytotoxicity of the EO was determined in CCD-1059Sk cells, are the fibroblast cells from the skin as well as its antioxidant activity (using the 1,1-diphenyl-2-picrylhydrazyl [DPPH], 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), and β-carotene assays) [24]. The antioxidant activity was further confirmed by Youcef-Ettoumi et al. study using the DPPH assay [25]. Other studies confirmed the insecticidal potential of this EO against *Sitophilus oryzae*, *Callosobruchus maculatus*, and *Tribolium confusum* [26], *Bemisia tabaci* [27], and *Musca domestica* [28]. The EO exhibited other activities such as anti-acetylcholinesterase, Na^+/K^+-ATPase, and anthelmintic [26,29–31]. Despite all those studied activities there is a lack of *in vivo* investigations of the potential biological activities of this EO.

The essential oil of plant species from the same genera was evaluated, such as the EO of *Citrus limon* (antioxidant and antinociceptive in mice [32,33]) and *Citrus aurantium* (anxiolytic [34]) promising results were achieved. In addition, When methanolic and ethanolic extracts of *C. sinensis* were tested in rodents, they showed anti-inflammatory properties [35]. Because there have been few investigations on *C. sinensis* essential oil's anti-inflammatory and analgesic properties, this study was conducted using a variety of *in vivo* assays as well as a comprehensive screening of its phytochemical composition.

2 Experiment

2.1 Extraction of the essential oil (hydrodistillation)

The essential oil of *C. sinensis* (L.) Osbeck (peels collected on September 2020 identified by a specialist botanist [Voucher: BPRN142]) was extracted by hydrodistillation in a Clevenger-type device. Distillation was achieved by boiling 200 g of plant peels in 1 L of water for 3 h. The obtained essential oil was reserved at 4°C in a dark container.

2.2 Animals

Wistar rats (male) weighing 180–210 g and Swiss albino mice (male) weighing 20–30 g were used in the study. These mice were maintained in the animal facility for at least 1 week prior to the experiment under regular lighting conditions (12 h light/dark cycle) and at room temperature (25 ± 1°) in typical plastic cages. They were provided standard rodent food and had unlimited access to water. In this experiment, all treatments were delivered orally through intragastric gavage with gastric syringes (essential oil weighted using capillaries and dose prepared in water + tween 20).

The study was conducted according to the guidelines of the Declaration of Helsinki, conformed to the ARRIVE guidelines, and approved by the Institutional Review board (16/2021/LBBEH-2 and 01/04/2021).

2.3 Identification of phytochemical compounds (Gas chromatography–mass spectrometry (GC-MS) analysis)

The following is how the EO aliquot was created to proceed with the GC-MS analysis: 1 mL of ethyl acetate was added to 1 mg of EO weighted sample. 0.1 µL of the sample was then injected for analysis in split mode using a mass spectrophotometer (Agilent 5973) combined with a gas chromatograph (Agilent 6890 series). In a positive

mode, the HP-5MS Agilent column (Model number 19091S-433) was used with inner diameter 0.25 mm, 30 m long, and 0.25 m film thickness. Helium was employed as a carrier gas. For 10 min, the oven temperature was adjusted from 60 to 300°C, then to 300°C for another 20 min. By contrasting the resulting molecule fragments (mass spectra) with those of the Wiley 7n.L Mass Spectral Library, the chemicals were identified.

2.4 Determination of the antioxidant activity

2.4.1 DPPH method

The ability of an antioxidant to scavenge the free radical DPPH is measured in this test. When a hydrogen donor combines with the DPPH radical, it is converted to its corresponding hydrazine.

The color of the solution changes from violet to yellow as DPPH is reduced. At 517 nm, a spectrophotometer measures the absorption. The lower the absorbance, the better the antiradical activity [36]. Jeong et al. describe a procedure for determining the effect of *C. sinensis* essential oil on DPPH [37]. A 100 µL volume of different concentrations of *C. sinensis* essential oil is added to 1 mL of methanolic solution of DPPH (0.025 g/L) freshly prepared. The negative control is made by combining 100 mL methanol with 1 mL methanolic DPPH solution at the same concentration as the positive control. After 30 min of incubation in the dark and at room temperature, the absorbance at 517 nm was measured using a spectrophotometer. The percentage of DPPH radical scavenging activity was calculated using the following equation:

$$I(\%) = ((A_1 - A_0)/A_0) \times 100,$$

where $I\%$ denotes the percent DPPH inhibition, A_0 represents the absorbance of control, and $A1$ represents the absorbance of sample (essential oil of the peel of *C. sinensis* L.).

2.4.2 Ferric reducing antioxidant power (FRAP) method

Karagözler et al.'s approach was used to calculate reducing power [38]. One milliliter of *C. sinensis* essential oil diluted in distilled water was combined with 2.5 mL of 0.2 M phosphate buffer solution at pH 6.6 and 2.5 mL of 1% potassium ferricyanide ($K_3Fe(CN)_6$). The mixtures are refrigerated after 20 min at 50°C before adding 2.5 mL of 10% trichloroacetic acid. About 2.5 mL supernatant from each concentration is combined with 2.5 mL distilled water and 0.5 mL 0.1% iron chloride in a mixture of 2.5 mL distilled water and 0.5 mL 0.1% iron chloride ($FeCl_3$).

This experiment uses butylated hydroxyanisole (BHA) as a reference antioxidant.

The findings are expressed as an effective concentration (EC_{50}), which represents the antioxidant concentration needed to reach an absorbance of 0.5 nm.

$$EC_{50} = (0.5 - b)/a,$$

where a is the slope and b is the y-intercept.

2.5 Determination of the antinociceptive activity study

2.5.1 Peripheral antinociceptive activity assay

The acetic acid-induced pain assay or torsion test, as developed by Bhowmick et al., was used to assess the pain relief potential of the EO [39].

It entails eliciting an allogenic response in Swiss albino mice by administering acetic acid (1%) intraperitoneally. Five homogenous groups of five mice were formed and fasted for 16 h prior to the start of the test.

- Negative control group: Animals in this group received the vehicle solution (physiological water) 30 min before the injection of acetic acid.
- Positive control group: The animals in this group were given an analgesic (Diclofenac) at a dose of 10 mg/kg orally.
- Treated groups: The animals were given oral doses of 12.5, 25, and 50 mg/kg *C. sinensis* essential oil. The analgesic effect is measured by counting the number of abdominal cramps that occur 30 min after the allogenic agent is injected [40].

Using the following formula, the percentage of cramp inhibition (PI) was calculated:

$$PI = [(NCTe - NCTr)/NCTe] \times 100,$$

where NCTe is the average number of contortions in the negative control group and NCTr is the average number of contortions in the positive/treated group.

2.5.2 Central antinociceptive activity assay: Hot plate

This experiment was carried out on a hot plate calibrated to 55 ± 1°C. The time it takes mice to leap is measured by their nociceptive reaction latency [41]. The mice were

placed on the hot plate for 120 min, with measurements taken every 30 min.

The mice in Group 1 were given physiological water and acted as a control group. Mice in Group 2 (positive control) were given 100 mg/kg sodium salicylate, whereas doses of 12.5, 25, and 50 mg/kg *C. sinensis* essential oil were given to Groups 3, 4, and 5, respectively.

The maximum reaction time was set at 30 s to avoid injury to paw tissue. If the reading exceeds 30 s, it would be considered maximum analgesia.

The following equation was used for calculating the maximum possible analgesia (MPA):

MPA = (reaction time for positive/treated group – reaction time for negative control group)/30 s – reaction for saline × 100.

2.6 Anti-inflammatory activity study

2.6.1 Carrageenan-induced paw edema assay

The carrageenan-induced rat paw edema method was used to assess anti-inflammatory activity *in vivo* [42].

The rats were divided into five groups of five and fasted for 16 h before the experiment. The initial volume ($V0$) of each rat's left hind paw was measured before administration of the treatments.

The different treatments were administered by oral gavage to the following:

- Group 1: Negative control group receiving physiological water.
- Group 2: Positive control group receiving a dose of 20 mg/kg of indomethacin.
- Group 3: Group receiving a dose of 12.5 mg/kg. *of C. sinensis* essential oil
- Group 4: Group receiving a dose of 25 mg/kg *of C. sinensis* essential oil.
- Group 5: Group receiving a dose of 50 mg/kg *of C. sinensis* essential oil.

Each rat got a 100 µL injection of 1% carrageenan solution under the plantar pad of the left hind paw 1 h after the oral administration of the various treatments.

At the first, third, fourth, and fifth hours after carrageenan injection, the volume of the paws was measured.

The increase in paw volume (PV) and edema inhibition (EI) was detected by computing the mean percentage from the following equations:

$$\mathrm{EI} = [(\mathrm{PVTe} - \mathrm{PVTr})/\mathrm{PVTe}] \times 100 \mathrm{PA} = [(Vt - V0)/V0] \times 100,$$

where $V0$ is the initial volume of the paw before induction of edema, Vt is the paw volume after administration of carrageenan and treatment, PVTr is the percent increase in the paw of the positive control/treated group, and PVTe is the percent increase in paw volume in the paw of the negative control group.

2.6.2 Formalin test

Pain was induced in mice by injecting 2% formalin subcutaneously into the right paw 13 min after the following treatments were administered [43]: Group 1: negative control (physiological water); Group 2: positive control (10 mg/kg of indomethacin); Group 3: dose of 12.5 mg/kg of *C. sinensis* essential oil; Group 4: *C. sinensis* essential oil at a dose of 25 mg/kg; Group 5: dose of 50 mg/kg *of C. sinensis* essential oil. The animals' response to pain (lifting of the painful paw) was counted in two phases for 30 min: early phase (0–5 min) and late phase (5–15 min).

The percentages of pain inhibition were computed as follows:

$$\%I = 100 - (\mathrm{MRT}/\mathrm{MRC}) \times 100,$$

where $\%I$ is the percent inhibition, MRT is an abbreviation for mean reaction time in the test group, and MRC is the mean reaction in the control group.

2.7 Acute toxicity

The acute toxicity test was performed by a single administrated dose of the EO to mice with 2 weeks of monitoring [44].

The following treatments were given to four groups of five mice each: Group 1 was given physiological water as a control, whereas dosages of 12.5, 25, and 50 mg/kg of *C. sinensis* essential oil were given to Groups 2, 3, and 4, respectively.

The animals were carefully monitored for the next 2 h after the treatments were administered. They were subsequently monitored for the next 14 days, recording intoxication symptoms (vomiting, ptosis mobility, aggressiveness alertness, stool status, piloerection, etc.) in addition to fatality rates.

Biochemical parameters such as aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), urea, and creatinine were assessed at the end of the experiment by slaughtering the treated mice and drawing the blood.

Table 1: Phytochemical compounds identified in *C. sinensis* EO

	Retention time	Scan	Type	Height (picoamps)	Area (mV/sec)	Total area (%)	Start time	End time	Compound
1	4.370	71	BB	411869.846	7379.984	0.70	4.342	4.394	Sabinene
2	4.510	106	BB	1827329.000	32468.879	3.07	4.478	4.542	Beta-myrcene
3	4.984	224	BB	23444571.286	939768.486	88.94	4.916	5.028	Limonene
4	5.643	388	BB	359867.625	7690.718	0.73	5.619	5.683	Alpha-terpinolene
5	10.614	1626	BB	504252.429	8872.285	0.84	10.594	10.650	Isopropyl myristate
6	11.212	1775	BB	204742.321	9746.720	0.92	11.168	11.280	Palmitoyl chloride
7	11.698	1896	BB	209601.923	8935.057	0.85	11.658	11.762	Nonadecane
8	12.031	1979	BB	172947.400	6564.447	0.62	12.007	12.087	Heptadecane
9	12.159	2011	BB	217469.444	11172.981	1.06	12.119	12.228	Eicosane
10	12.609	2123	BB	239189.667	10693.380	1.01	12.573	12.681	*n*-Ecosane
11	12.922	2201	BB	224430.333	6322.730	0.60	12.906	12.979	Docosane
12	13.990	2467	BB	116221.333	6983.559	0.66	13.926	14.059	Heineicosane
Total						100%			

2.8 Statistical analysis

The data are presented as a mean ± standard error of the mean. The analysis was carried out using GraphPad's Prism 6 software. Repeated measurements ANOVA was used to perform the statistics, followed by a Tukey *post hoc* test. Statistical significance was determined at $P < 0.05$.

3 Results

3.1 Phytochemical analysis

Table 1 presents the phytochemicals identified in the citrus essential oil (Chromatogram in Figure 1) and Table 2 shows its 2D, 3D, and chemical structures to

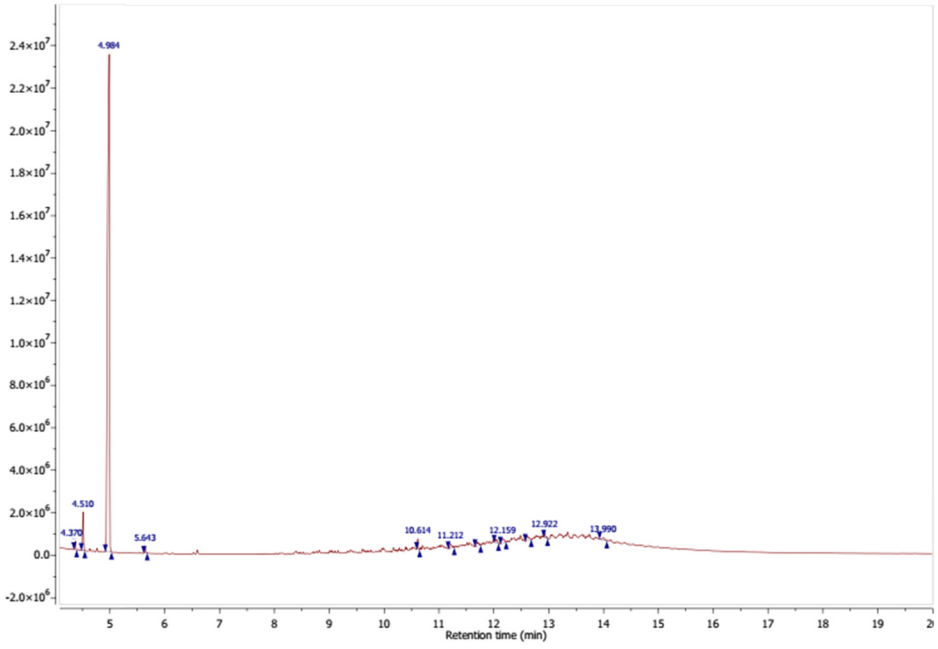
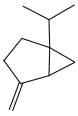
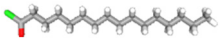
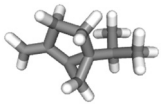
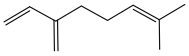
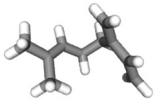
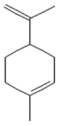
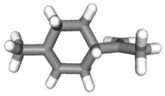
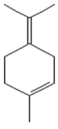
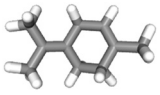
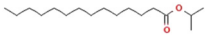
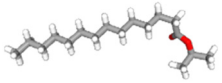
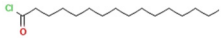


Figure 1: Chromatogram of the GC/MS identified compounds in *C. sinensis* EO.

Table 2: Structural properties of identified compounds from *C. sinensis*

Name	2D structure	IUPAC name	3D structure
Sabinene		4-Methylidene-1-propan-2-ylbicyclo[3.1.0]hexane	
Beta-myrcene		7-Methyl-3-methylideneocta-1,6-diene	
Limonene		1-Methyl-4-prop-1-en-2-ylcyclohexene	
Alpha-terpinolene		1-Methyl-4-propan-2-ylidenecyclohexene	
Isopropyl myristate		Propan-2-yl tetradecanoate	
Palmitoyl chloride		Hexadecanoyl chloride	
Nonadecane		Nonadecane	—
Heptadecane		Heptadecane	
Eicosane		Eicosane	—
Docosane		Docosane	—
Henicosane		Henicosane	—

better visualize the chemical properties of each compound. Among the 12 identified molecules, limonene was the major compound contained in this EO with 88.94%. Similar results were found in different studies, Dias et al. (98.54%) [45], Ferhat et al. (86–95%) [46], Martin et al. (70%) [47], Braddock et al. (90%) [48], and Magalhães et al. (95.12%) [24].

3.2 Antioxidant activity

3.2.1 DPPH and FRAP methods

C. sinensis essential oil demonstrated significant antioxidant activity with an IC_{50} of 0.012 ± 0.0009 mg/mL when compared to butylated hydroxytoluene (BHT), which

Table 3: Antioxidant activity of the EO by DPPH and FRAP methods (IC_{50} [mg/mL])

	Positive control (BHA)	Positive control (BHT)	*C. sinensis* essential oil
DPPH	—	0.0089 ± 0.0005	0.012 ± 0.0009
FRAP	1.61 ± 0.09	—	0.41 ± 0.03

Values are expressed as means ± SD (n = 3).

was used as a positive control and had an IC_{50} of 0.0089 ± 0.0005 mg/mL (Table 3).

Table 2 shows that *C. sinensis* essential oil has a substantial reduction capacity and is more potent than the reference antioxidant (BHA), with an IC_{50} of 1.61 ± 0.09 mg/mL. The FRAP results presented show that the reduction capacity of *C. sinensis* essential oil is significant and more active than the reference antioxidant (BHA) with a IC_{50} of 1.61 ± 0.09 mg/mL. Both tests confirmed the potential antioxidant effect of *C. sinensis* essential oil.

3.3 Antinociceptive activity study

3.3.1 Peripheral antinociceptive activity assay

After 30 min of acetic acid injection, the mice develop abdominal cramps. In the presence of 10 mg/kg of diclofenac and 12.5, 25, and 50 mg/kg of *C. sinensis* essential oil, the number of stomach cramps decreases over time. It results in percentages of contraction inhibition of 61.24, 83.13, and 91.65%, respectively, compared to 75.4% obtained with the positive control, demonstrating a powerful and dose-dependent efficacy (Figure 2).

3.3.2 Central antinociceptive activity assay: Hot plate

Figure 3 illustrates the percentage of analgesia of *C. sinensis* essential oil as a function of dose and time as determined by the hot plate method.

In Swiss Albino mice, all doses tested increased analgesia and pain resistance, as indicated in the results. The 25 and 50 mg/kg doses of *C. sinensis* essential oil presented higher efficacy when compared to the lowest dose (12.5 mg/kg).

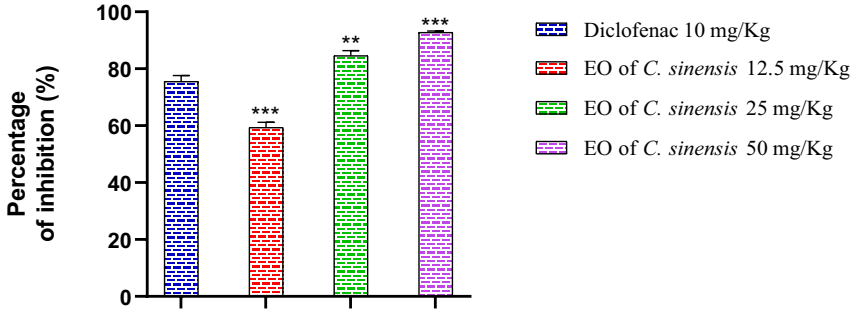


Figure 2: Analgesic activity of the *C. sinensis*'s essential oil of by the writhing method. The values are expressed as mean ± SD (n = 5). $^{**}P < 0.01$, $^{***}P < 0.001$ compared with positive control (one way ANOVA with Tukey test).

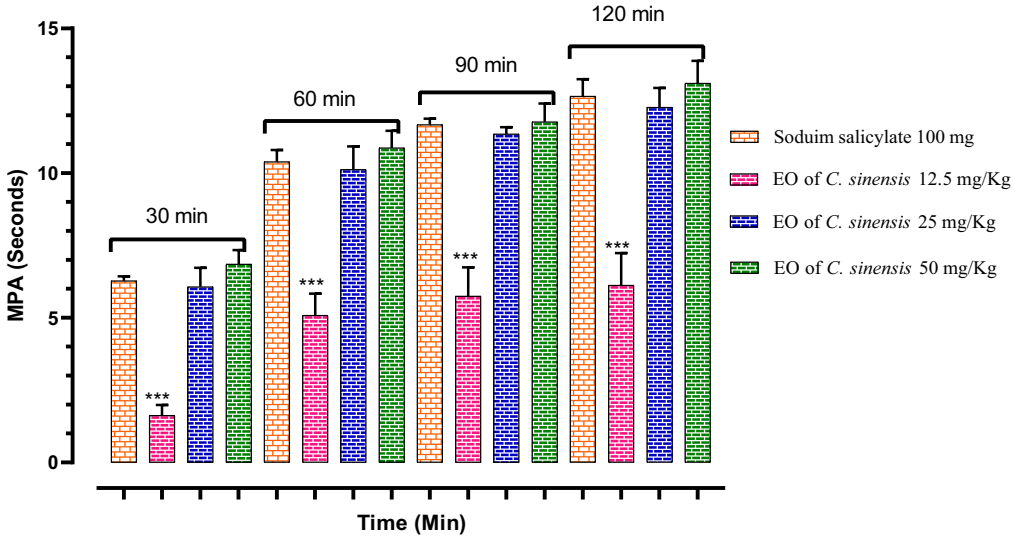


Figure 3: Effect of *C. sinensis* essential oil on the antinociceptive effect of the hot plate. The values compared with positive control are expressed as mean ± SD (n = 5). $^{***}P < 0.001$.

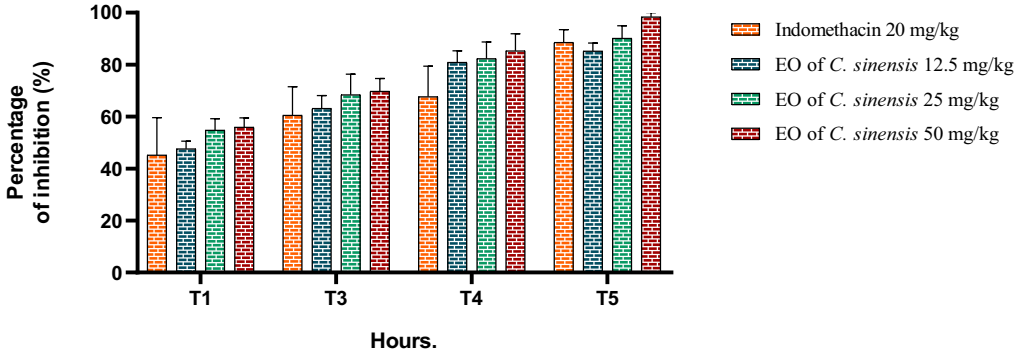


Figure 4: Percentage of edema inhibition. The values are expressed as mean ± SD (n = 5).

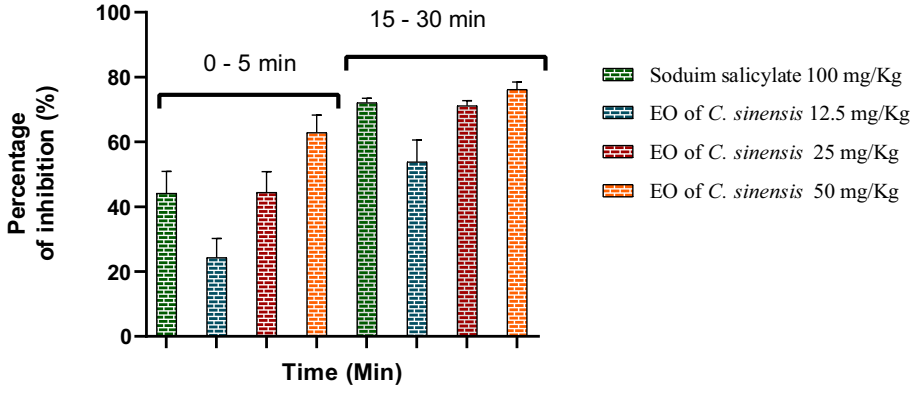


Figure 5: Percentage of pain inhibition. The values are expressed as mean ± SD (n = 5).

As expected, sodium salicylate (100 mg/kg) had a strong analgesic effect.

3.4 Anti-inflammatory activity study

3.4.1 Carrageenan-induced paw edema assay

Figure 4 represents the percentages of inflammatory edema suppression by *C. sinensis* essential oil. From the third hour of the test, *C. sinensis* essential oil inhibits pain at all doses, and this inhibition is slightly better than that of indomethacin.

3.4.2 Formalin test

Figure 5 shows the percentages of inhibition in the formalin test. The findings demonstrate that sodium salicylate and *C. sinensis* essential oil at various doses (12.5, 25, and 50 mg/kg) have a nonsignificant increase in the percentage of pain inhibition during the 0–5 min period (first phase: neurogenic phase). Also, there is no significant increase in the percentage of pain inhibition by both the reference drug and the *C. sinensis* essential oil at the different doses between the 15th and 30th minute (second phase: inflammatory phase), indicating a moderate anti-inflammatory activity but still compared to the reference drug used.

3.5 Acute toxicity

No signs of toxicity appeared in the mice when treated under acute conditions compared to control animals, and this was evident by focusing on the general observations on eyes, fur and skin, motor activity and behavior, feces consistency, respiration, urination (color), mucous membranes, salivation, convulsions and tremors, sleep, itching, and coma mortality.

3.5.1 Corporal weight

Figure 6 shows body weights of control and treated mice. Body weight increased in both treated and control mice, but there was no significant difference between the groups.

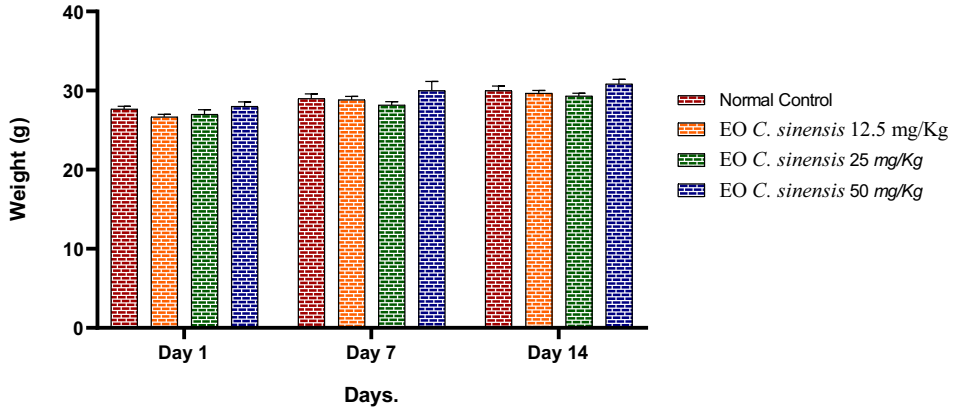


Figure 6: Bodyweight development during acute toxicity assessment in mice. The values are expressed as mean ± SD (n = 5).

Table 4: Relative organ weight of the treated mice in the acute toxicity assessment

	Kidneys	Liver	Spleen
Normal control	1.457 ± 0.13	6.455 ± 0.062	0.71 ± 0.021
EO *C. sinensis* 12.5*	1.48 ± 0.063^{ns}	5.97 ± 0.01^{ns}	0.68 ± 0.02**
EO *C. sinensis* 25*	1.54 ± 0.04^{ns}	6.18 ± 0.03^{ns}	0.703 ± 0.03**
EO *C. sinensis* 50*	1.63 ± 0.01^{ns}	6.45 ± 0.08^{ns}	0.704 ± 0.011**

* Significant at $p < 0.05$, ** significant at $p < 0.01$. The values are expressed as mean ± SD (n = 5).

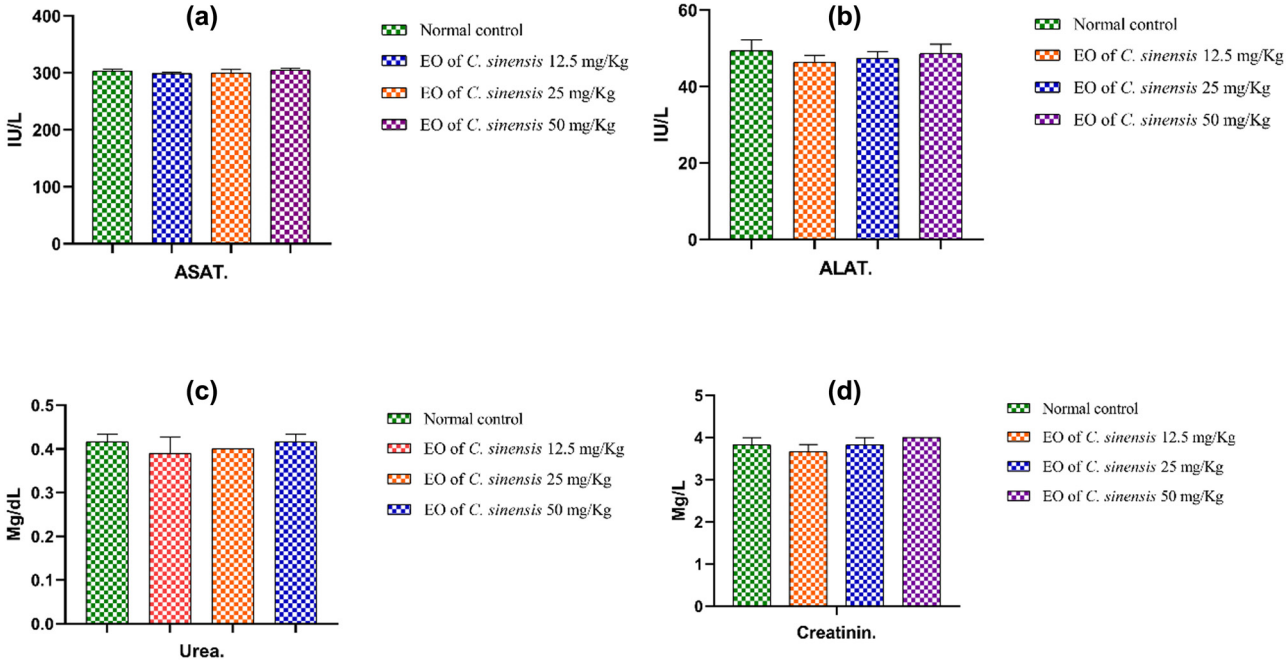


Figure 7: (a) The serum levels of "ASAT" of control and treated mice; (b) serum levels of "ALAT" of control and treated mice; (c) level of serum "urea" of control and treated mice; and (d) serum "creatinine" level of control and treated mice. The values are expressed as mean ± SD (n = 5).

3.5.2 Organ's weight

After 14 days of testing, the weights of the organs of the mice treated with 12.5, 25, and 50 mg/kg of *C. sinensis* essential oil and the control are measured. The treatment at various doses had no effect on the normal weight of the internal organs, as indicated in Table 4.

3.5.3 Biochemical parameters (liver and kidney function)

Biochemical parameters for liver function assessment are shown in Figure 7. Comparing ASAT and ALAT after treatment with *C. sinensis* at doses of 12.5, 25 and 50 mg/kg shows no significant difference (Figure 7a and b). Figure 7c and d shows the biochemical measurements used to determine kidney function (serum creatinine and urea levels), which indicate no significant difference from the control group.

4 Discussion

Essential oils are used in alternative medicine and have recently been used to treat various ailments, including stress, sleeplessness, pain, inflammation, and more. Aromatherapy is becoming increasingly popular in both traditional and modern medicine to improve patient quality of life [49]. The cyclic monoterpene limonene (88%) turned out to be the most common compound in *C. sinensis* essential oil. This molecule's biological activities have been demonstrated through extensive research. Limonene can be found in a wide variety of items, including household cleaners, disinfectants, industrial hand cleaners, fragrances, cosmetics, fire extinguishers, rubber, wax and paint strippers, adhesives, lubricating oils, essential oils, and wetting and dispersing products [50]. It has been utilized in fragrances, soaps, food, and beverages as a scent and flavoring component [51]. Chemopreventive and chemotherapeutic characteristics are among its medical uses [52]. The DPPH method is used to test the antioxidant activity of essential oils, and it is a good predictor of a compound's potential antioxidant activity [53]. The antioxidant activity of *C. sinensis* essential oil was established by measuring the decrease of the DPPH radical. Similarly, the antioxidant capacity of the EO to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) was confirmed in the ferric reducing power test. The presence of molecules with a reducing capacity slows the chain reactions triggered by free radicals, resulting in these activities. In this case, the antioxidant activity reported is most likely due to limonene, the main component of this essential oil. In their investigation, Piccialli et al. discovered that limonene inhibited the elevation of KV3.4 activity caused by A1-42 oligomers by reducing reactive oxygen species generation. Limonene activity caused a decrease in myeloperoxidase (MPO) activity, a biomarker of neutrophil infiltration, and an increase in GPx activity [54], as demonstrated in a study by Hamuel Doughari and Jamila Bazza, indicating an antioxidant effect [55], while in a study by de Souza et al. [56], limonene activity resulted in a reduction in MPO activity, a biomarker of neutrophil.

The analgesic effect *of C. sinensis* essential oil was also tested in a model of abdominal contortions caused by an intraperitoneal injection of acetic acid. Acetic acid induction of contortions is an experimental methodology used in the biological evaluation of analgesic properties. Chemical mediators including serotonin, histamine, bradykinin, substance P, and prostaglandins (PGE2 and PGF2) are released in response to acetic acid treatment, causing pain [39]. Abdominal contractions could be caused by local peritoneal receptors. The essential oil of *C. sinensis* at 25 and 50 mg/kg was found to have better analgesic power to the reference molecule employed, diclofenac, at a dose of 10 mg/kg, in this investigation. Nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit cyclooxygenase and/or lipo-oxygenase or reduce the pain response produced by peripheral nociceptors are frequently used to counteract the peripheral analgesic effect [57]. Heat is often used as a noxious stimulus in models of acute pain (hot plate test). The animal's response time to stimulation is usually the dependent variable. To see if a test drug has analgesic properties, the latency reactions of the treated animals and the control animals are compared. Compared to a control treatment, an antinociceptive or analgesic response is characterized by a significant increase in the latency to respond to a thermal stimulus after therapeutic therapy [58]. According to the findings, the *C. sinensis* essential oil considerably increased the latency period in the hot plate test, implying that this analgesic action was mediated mostly through the central nervous system. Early research also revealed that limonene has powerful antinociceptive properties. The writhing test revealed that limonene has antinociceptive activity, according to Adriana Estrella's research. In a model of oxazolone-induced colitis, it also significantly reduced hyperalgesia, pathological biomarkers, and inflammatory cytokines in the colon and prevented gastric damage [59]. Arajo-Filho et al. found that limonene improves regeneration and reduces neuropathic pain after peripheral nerve injury in [60].

This standardized testing paradigm for acute inflammation uses carrageenan, a chemical that stimulates the production of inflammatory mediators. Edema develops and quinine, histamine and serotonin are released in the first hour after injection, followed by the release of prostaglandins 2–3 h later. The most important mediators of acute inflammation are prostaglandins [61]. The results of this study show that EO has anti-inflammatory properties in both the first and second stages of the carrageenan-induced inflammatory response by inhibiting paw edema volume in rats induced by 1% carrageenan, which is higher than the positive control (diclofenac 10 mg/kg). The formalin test has a biphasic response, with the first phase (the first 10 min) and the second phase (the 15–20 min after the formalin injection). The first reaction is thought to be caused by a burst of activity of the pain fibers (particularly the C fibers), while the second is caused by peripheral inflammation and can be reduced by NSAIDs. Furthermore, central sensitization is believed to play a role in the behavioral response in the second phase [58]. As can be observed from the data, EO treatment effectively ameliorates these phases even while having an analgesic and anti-inflammatory effect. A study carreid out by Matuka et al. [62] confirmed the anti-inflammatory activity of *C. sinensis* from South Africa, as well as a study by de Souza et al. [56] in which limonene exhibited anti-inflammatory effects by lowering TNF-α, interlrukin (IL)-6, and IL-1 levels while increasing IL-10 levels. Limonene has been demonstrated to suppress Nf-b, Il-1, and Mpo expressions while enhancing Gpx expression.

Tumor necrosis factor (TNF) levels in rat peritoneal exudate have been shown to be significantly reduced by limonene [63]. Limonene, the major ingredient of *C. sinensis* peel essential oil, has been proven to have anti-inflammatory activities, inhibiting the production of TNF and prostaglandin E2 [64]. It has also been suggested that it has anti-inflammatory qualities through lowering heat-induced albumin denaturation [65]. During the testing period, *C. sinensis* essential oil exhibited no toxicity by oral route in mice, despite all of the identified pharmacological activity (14 days). The EO had no influence on the behavior of the animals who were given it. The levels of ALAT, ASAT, urea, and creatinine did not change significantly, and all of the internal organs examined appeared normal, with no significant differences in organ weights.

5 Conclusions

This research looked at the antioxidant, nociceptive, and anti-inflammatory activities of *C. sinensis* essential oil. The EO demonstrated pharmacological activities that were similar to or even better than those obtained with the reference drugs. In this study, limonene was reported as the major compound of this EO with 88%. This compound is well known, and its activities are already established and can be behind all the activities reported in this study. The acute toxicity assessment proved that the EO is completely safe, confirming the conventional usage of this EO while also opening up new potential for its application in other sectors, notably for pharmaceutical industry.

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Conflict of interest: The authors declare that there is no conflict of interest.

Ethical approval: The study was conducted according to the guidelines of the Declaration of Helsinki, conformed to the ARRIVE guidelines, and approved by the Institutional Review board (16/2021/LBBEH-2 and 01/04/2021).

Data availability statement: All data are available within the manuscript.

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