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Original Research Article

Chemical Composition and Pharmacological Activities of *Calamintha nepeta* Essential Oil

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ABSTRACT

Calamintha nepeta (Lamiaceae), widely distributed throughout the Mediterranean area, is a small and fragrant perennial herb traditionally used for both culinary and medicinal purposes. It serves as an antiseptic, spasmolytic, diuretic, and cough suppressant. The objective of this research is to characterise the chemical constituents of *Calamintha nepeta* essential oil (EO) through GC-MS analysis and evaluate its antioxidant and antimicrobial properties against nine multidrug resistant bacterial strains and one yeast. The study also conducted an *in vitro* anti-inflammatory 5-lipoxygenase inhibition test and determined its lethal dose 50 (LD_{50}) as well as the effects against carrageenan-induced paw edema *in vivo*. Analysis revealed that the primary components of *C. nepeta* included oxygenated monoterpenes, notably pulegone (58.36%), isoborneol (10.40%), menthone (8.91%), and piperitenone (3.86%). Antimicrobial tests demonstrated significant inhibition zones ranging from 10.2 to 37.12 mm. Essential oil exhibited minimum inhibitory concentrations and bactericidal/fungicidal concentrations ranging from 0.937 to 3.75 µL/mL and 0.937 to 15 µL/mL, respectively. Furthermore, it showed potent antioxidant activity against DPPH and a high total reducing power. Acute oral toxicity tests in Wistar rats indicated an LD_{50} of 2500 mg/kg. *C. nepeta* EO also exhibited strong anti-inflammatory properties against carrageenan-induced paw edema, with an IC_{50} value of 17.23 ± 0.32 µg/mL compared to diclofinac. Interestingly, this research highlights the importance of the phytochemical and antimicrobial properties of the essential oil of the *Calamintha nepeta* and accentuats its importance in pharmacological applications for treating inflammatory conditions.

***Keywords*:** *Calamintha nepeta*; Essential oil; GC-MS; Antimicrobial activity; Antioxidant activity; Anti-inflammatory activity; phytochemical; pharmological activites.

Introduction

Inflammation is a physiological reaction that occurs within the tissues of mammals in response to injury. The body exhibits a defensive response aimed at eradicating or restricting the dissemination of harmful agents.[1,2] The inflammatory response involves various elements that can lead to the appearance of symptoms and tissue injury. Swelling, the influx of white blood cells, and the formation of granulomas are among the significant components seen in inflammation. However, this reaction can be assumed to act as a protective mechanism. The complex mechanisms and substances involved in inflammation have the ability to trigger or worsen numerous reactions.[3,4]

The efficacy of conventional drug therapies for the management of various inflammatory diseases is often limited. Furthermore, patients may experience many side effects.[5]

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Based on the reports of the World Health Organisation (WHO), it has been observed that a significant proportion, approximately 70 to 80%, of the global population predominantly depends on alternative forms of medicine for their primary healthcare, particularly those derived from herbal sources.[6,7]

Over the past decade, medicinal and aromatic plants (MAPs) have been increasingly used for their therapeutic and pharmaceutical properties in the prevention and treatment of many diseases.MAPs still representing an important role and are a valuable resource for improving human health. The essential oils (EOs) distilled from these plants are extensively investigated and used for industrial purposes(cosmetic, chemical, food, pharmaceutical, and fragrance) owing to the substantial quantities of bioactive and aromatic compounds [8,9]. Furthermore, EOs exhibit a diverse array of biological actions, including antibacterial, anti-inflammatory, antioxidant, antiproliferative and genoprotective properties.[10,11]

Calamintha nepeta (Meuta or Nebta in the Algerian language) is a small aromatic perennial plant with ovals and small leaves, reaching 60 cm in height and classified as members of the Lamiaceae family, which is largely widespread in the Mediterranean region .[12,15]

Traditionally, it was employed for both culinary and medicinal purposes as an antiseptic, spasmolytic, diuretic, and cough suppressant. Furthermore, *C. nepeta* has been widely used as a therapeutic agent for the treatment of depression, cramps, convulsions, insomnia, and gastroenterologic and respiratory diseases.[16,19]EOs are also known for their biological activities due to the existence of bioactive natural compounds. There was an interesting variation in the chemical

composition depending on the source of the samples; others highlighted the major impact of environmental influences on the chemical contents of plants.[20]

EOs derived from these native aromatic *Calamintha nepeta* plants exhibit little toxicity,[21,22]possess significant antioxidant, antiproliferative and antifungal properties and have a wide range of antibacterial activity properties ,[20,13,17,23]which has the potential to be used in many sectors, such as biotechnology, food, and pharmaceuticals. This study presents an initial investigation of the anti-inflammatory characteristics of *C. nepeta* essential oil *in vitro* and its effects on carrageenan-induced paw edema *in vivo*. The evaluation included an evaluation of the antibacterial and antifungal capabilities, along with an examination of acute and subacute toxicity.

Materials and Methods

Plant Material

The small leaves of *C. nepeta* were collected in May 2021 from the Beni-Saf region in the northwest of Algeria (latitude 35°18'8''N, altitude 25 m, and longitude 1°23'1''O). The plant was authenticated, and a specimen was deposited at the Plant Biodiversity Laboratory of Sidi Bel-Abbes University with the voucher specimen number LBV/032021. The small leaves were washed and dried for 15 days in the dark at room temperature.

C. nepeta essential oil extraction

Essential oil was extracted from small leaves of *C. nepeta* (100 g) by hydrodistillation using a Clevenger-type apparatus for 3 h. The resulting EO was stored in the dark at 4°C until further use. The following equation was used to evaluate the EO yield:[24]

$$EO_{yield}(\%) = \frac{volumeofEOobtained(g)}{massofdrymatter(g)} \times 100 \qquad (1)$$

Gas chromatograph mass spectrometer analysis of essential oil of C. nepeta

The composition of volatile EO compounds was analyzed using a gas chromatograph coupled to a mass spectrometer (Shimadzu GC-MS QP 2020, Shimadzu, Kyoto, Japan). The separation of the compounds was performed using a Zebron ZB-5 MSi capillary column (dimensions: 30 m, 0.25 mm, film thickness: 0.25 µm; Phenomenex, Torrance, CA, USA). GC-MS analysis was performed under the following conditions: scan range from 35 to 320 m/z in mode 3 scans·s-1. Helium was used as a carrier gas at a flow rate of 1.01 mL/min and a split ratio of 1:20. Furthermore, annealing was carried out under air in an oven as follows: the temperature was raised from 45 ° C to 150 °C at a rate of 2 ° C/min, then heated to 270 ° C at a rate of 15 °C and kept for 5 min. The compounds were identified through their mass spectra and their retention indices (RI) compared to the NIST 20 (National Institute of Standards and Technology) library and FFNSC (Mass Spectra of Flavours and Fragrances of Natural and Synthetic Compounds), comparing their unknown retention times with authentic standards. Quantification of the identified EO constituents was performed by normalizing the peaks against the added internal standard. GCMS Post-run Analysis software (Shimadzu, Kyoto, Japan) and AMDIS (v. 2.73) were used for the analysis of the experimental data. The identification of compounds where based on comparison of obtained MS spectra with NIST20 database as well as with comparison of obtained retention indices with this database by GCMS solution v. 4.45 version .[25,26]

Antimicrobial activity

Microorganisms

The antimicrobial efficacy of essential oil (EO) was assessed against a panel of microorganisms obtained from the American Type Culture Collection (ATCC). (i) Five Gram-positive strains, including *Staphylococcus aureus ATCC25923, Enterococcus fecalisATTC29212, Bacillus subtilis ATCC6633, Bacillus cereus ATCC10876*, and *Bacillus cereus ATCC11778*; (ii) Four Gram-negative strains, including *Pseudomonas aeruginosa ATCC27853, Klebsiella pneumoniae ATCC700603, Escherichia coli ATCC25922*, and *Proteus mirabilis ATCC35659*; (iii) one yeast, which is *Candida albicans ATCC10231*.

The antibacterial effect of *C.nepeta* EO was tested by the disc diffusion method according to the Committee on Clinical Laboratory Standard Guidelines. [27]A sterile physiological saline solution (0.9% w/v) was used to prepare a suspension of each examined microorganism. Then, 100 µL of each bacteria inoculum (10^8 CFU/mL) were streaked in Petri dishes containing 15 mL of Muller-Hinton agar. A quantity of 15 µL of EO, which was impregnated in a sterile filter paper disc (Ø = 6 mm), was placed on the agar surface using a sterile swab. The plates were kept at room temperature for 30 min before incubation at 37°C for 24 h. Doxycycline (30 µg/disc) and ampicillin (10 µg/disc) were used as positive controls.

The antifungal activity of *C. nepeta* EO was performed on 48 h cultures of *Candida albicans* in Sabouraud medium at 37°C. 100 µL of *C. albicans* suspension (10^6 CFU/mL) was equally dispersed over new Sabouraud medium plates. To test antifungal activity, a quantity of 15 µL of EO, which was impregnated on a sterile filter paper disc (Ø = 6 mm), was placed on the agar surface using a sterile swab and incubated for 48 h at 37 ° C. Econazole (50 µg/disc) was used as a positive control. All antimicrobial experiments were carried out in triplicate, which was determined by measuring the diameter of the inhibition zone formed around each disc.

The determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of EO against the tested microbial strains was carried out by the microdilution method .[28,29]The stock solution of EO was dissolved in dimethylsulfoxide (DMSO) in the ratio of 1:1 (v/v) to obtain the highest concentration. The EO was then diluted two-fold serially (30 to 0.029 µL/mL) by using Mueller Hinton broth (MHB) for bacteria and Sabouraud dextrose broth (SDB) for yeast. A final volume of 200 µL in each microplate well contained 100 µL of each EO dilution and 90 µL of MHB or SDB supplemented with 10 µL of 10^6CFU / mL of bacterial suspension and 10^7CFU / mL of *C. albicans*. The last well of each strip containing the broth and inoculum served as a positive control, while the negative control contained only broth. The microplates were then incubated at 37 ° C for 24 h for bacteria and 48 h for *C. albicans*. After incubation, 40 µL of 0.4 mg/mL Triphenyltetrazolium chloride (TTC) solution was added to each well and further incubated at 37 °C for 30 min. The microplates were then examined for colour change. The microbial growth was detected by the development of a red colour (caused by the reduction of the dye). The lowest concentration of EO at which no microbial growth was observed was recorded as MIC. 10 µL of each well with no visible growth were streaked onto MHA and SBA, followed by incubation for 24 h at 37 ° C to determine the MBC values that correspond to the lowest concentration at which 99.9% of the inoculated microorganisms were killed.[30]

Antioxidant activity in vitro

DPPH Assay

The assessment of the capacity to scavenge free radicals was conducted using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method as outlined in the study conducted by Boussena et *al.*[31] The stock solution was prepared by dissolving 24 mg of DPPH in 100 mL of ethanol, followed by storage at a temperature of -20 °C. The preparation of the working solution involved the utilization of a spectrophotometer. Specifically, a volume of 10 mL from of stock solution was combined with 45 mL of ethanol to achieve an absorbance reading of 1.1 ± 0.02 at a wavelength of 515 nm. A volume of 150 µL of EO was subjected to different dilutions and subsequently mixed with 2850 µL of DPPH solution. The reaction was carried out for a duration of 30 minutes under conditions of darkness. Subsequently, the measurement of absorbance (OD) was conducted at a wavelength of 515 nm. The standard substance employed in the experiment was ascorbic acid, which was subjected to identical experimental conditions. All measurements were made in triplicate to ensure accuracy and reliability. The percentage of DPPH radical scavenging was determined using the provided equation,[32] followed by the application of linear regression analysis to determine the inhibition concentration (IC_{50})values:

$$DPPH\ scavenging\ (\%) = \left[\frac{A_{contol} - A_{sample}}{A_{contol}}\right] \times 100 \qquad (2)$$

FRAP assay

The reducing power of EO was determined according to Bentaiba et *al*.[33]In this experiment, 250 µL of EO ethanolic solutions at different concentrations were mixed with 625 µL of phosphate buffer (0.2M, pH 6.6) and 625 µL of 1% potassium ferrocyanide $K_3Fe\ (CN)_6$. The mixture was incubated at 50°C in a water bath for 20 min, cooled to room temperature, and then 625 µL of trichloroacetic acid (10%) was added to stop the reaction. The mixture was centrifuged for 10 min at 3000 rpm, and then 625 µL of supernatant was mixed with 625 µL of distilled water and 125 µL of 0.1% $FeCl_3$ solution. After 10 minutes of incubation, the absorbance (OD) was measured at 700 nm using a spectrophotometer against a blank with ethanol. Ascorbic acid was used as a standard, and then a linear regression analysis was performed to determine the effectiveness concentration values (EC_{50}).

Anti-inflammatory activity in vivo

Animals

Wistar female rats, weighing 150 ± 15g, were purchased from the Pasteur Institute of Algeria (Algiers). Animals were kept in polypropylenecages for 14 days prior to the experiments to allow acclimatization to the laboratory conditions. Standard housing conditions are a temperature of 22 ± 3 ° C with a 12-hour light/dark cycle system. Rats were fed dry pellets with water access ad libitum.

Acute oral toxicity

The acute oral toxicity test was carried out in accordance with Organization for Economic Cooperation and Development (OECD) recommendations No. 423 .[34]The rats fasted for 18 hours with free access to water before the experiment. *Calamintha nepeta* EO was solubilized in 1% Tween 80 and administered to each group (n = 3) by intragastric gavage at doses of 50, 300, and 2000 mg/kg, respectively. The control group received the vehicle (10 mL/kg of 1% Tween 80). During 2 hours, all rats were monitored for any signs of toxicity, such as changes in the skin, eyes, and fur, as well as changes in the respiratory and central nervous systems, autonomic diarrhea, lethargy, and salivation. The observations were continued for any mortality for 48 hours and then for 7 days. When no deaths occurred in any group, this test was repeated with higher doses of up to 5000 mg/kg.

Carrageenan-induced paw edema

A carrageenan-induced inflammatory paw edema assay was used to determine the anti-inflammatory activity of *C. nepeta* EO *in vivo***.**[35]All animals in each group (n=6) received a subplantar injection of 100 µL carrageenan (1%, w/v) in the right hind paw. Rats were treated with 1% of Tween 80 at a dose of 10 ml / kg (vehicle) as a positive control, Diclofenac at a dose of 10 mg/kg as a reference drug (standard) and *Calamintha nepeta* EO at doses of 100, 200, and 400 mg/kg (experimental). All the treatments were performed by i.g.g. The thickness (mm) was measured using a digital vernier caliper 1 h before the injection of carrageenan and every 1 for 6 h. The percentage of edema inhibition (INH %) was calculated according to the following formula:

$$INH(\%) = \frac{[(V_t - V_0)_{contol} - (V_t - V_0)_{tested}]}{(V_t - V_0)} \times 100 \qquad (3)$$

Where:

V_t: represents the volume of the paw at T time after carrageenan injection.

V_0: represents the paw volume at T_0 before carrageenan injection.

In Vitro Anti-Inflammatory Activity

The *in vitro* evaluation of the lipoxygenase inhibition assay was carried out. The inhibitory activity of *C. nepeta* EO on 5-lipoxygenase (5-LOX) was evaluated by monitoring the oxidation of linoleic acid at a wavelength of 234 nm, as described in the study conducted by Andrade et al ,[36]albeit with minor modifications. A mixture was prepared by combining 20 µL of *C. nepeta* EO and 20 µL of Glycine max 5-lipoxygenase (100 U/mL). This mixture was then subjected to pre-incubation with 200 µL of phosphate buffer (0.1 M, pH 9) at room temperature for a duration of 5 minutes. The experimental procedure involves the addition of 20 µL of linolenic acid, which is present at a concentration of 4.18 mM in ethanol. The subsequent observation was carried out for a duration of 3 minutes, with measurements taken at a wavelength of 234 nm. The results presented here represent the average value ± standard error value (SEM) obtained from three independent and replicated experiments. The positive control utilised in the study was quercetin.[37]

Statistical analysis

Data are presented asmean ± standard deviation. The statistical analysis of the collected data was conducted using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Significant differences were determined based on the following thresholds: ***P< 0.001, **P< 0.01, *P< 0.05.

Ethical note

All experiments adhered to the regulations of Algerian legislation (Law Number 95–322/1995) regarding the protection of animals intended for experimental and scientific purposes. Furthermore, the procedures followed the guidelines outlined by the Algerian Association of Experimental Animal Sciences (AASEA authorisation number 45/DGLPAG/DVA/SDA/14) concerning the welfare and protection of animals used in experimental and scientific endeavours.

Results and Discussion

Chemical characterization of essential oil from Calamintha nepeta

Hydro-distillation of *Calamintha nepeta* EO had a yield of 2.32±0.06 % (W/W).Compared to previous studies; the yield of EO obtained in this study was higher than obtained from samples of *Calamintha from* different regions of Algeria, with values of 1.3% and 1.4%.[14,38]

16 compounds were identified in *C. nepeta* EO with a content greater than or equal to 0.1% by GC-MS analysis; the results are presented in Table 1 and Figure 1. These compounds represent ~98.56% of the total chemical composition. This oil is characterized by the majority presence of pulegone (58.36%), isoborneol (10.40%), menthone (8.91%), piperitenone (3.86%), bicyclogermacren (3.29%), and caryophyllene (2.88%). GC-MS analysis showed that the chemical compounds of *C. nepeta* EO are strongly dominated by oxygenated monoterpenes (73.39%), sesquiterpene hydrocarbons (8.21%), monoterpene hydrocarbons (4.72%), oxygenated sesquiterpenes (2.44%) and non-terpene derivatives (1.15%). Previous studies carried out in several Algerian regions discovered that the chemical composition varies depending on the origin of the samples. The results indicated the existence of three chemotypes of EO from *C. nepeta*. The first chemotype from the Bouhanifia region (West Algeria) is characterized by the predominance of pulegone (73.54%), isomenthone (7.89%), and cis-peperitone oxide (2.28%).[14] The second Jijel chemotype (Northeastern Algeria) is characterised by the predominance of menthone (26.46%), piperitone oxide (22.26%) and pulegone (14.04%).[39] The third chemotype from Blida (50 km south of Algiers) is characterized by the predominance of pulegone (39.5%), neo-menthol (33%), and isomenthone (19.6%)[38]**.** Several studies in Italy also indicated the presence of many chemotypes with pulegone (44.7%), menthone (16.4%), piperitenone (13.3%) and piperitone (6.01%) as major components .[40] However, pulegone was identified as the main compound (64.4%) followed by piperitenone (6.4%) and piperitenone oxide (2.5%).[19] Furthermore, it was discovered that EO derived from *C. nepeta* plants harvested in various years in the Alentejo region in Portugal exhibits a high concentration of oxygenated monoterpenes. The major constituents of this EO were identified as isopulegol, isopulegone, and 1,8-cineole.[23,41]A separate investigation conducted on the EO derived from *C. nepeta* originating from the Alentejo region revealed that the predominant constituent was 1, 8-cineole, accounting for 28% of the composition. This was followed by menthone at 22%, menthol at 16%, and pulegone at 5% .[21]The results indicate that the chemical composition of the examined EO differs from that of other sources due to the existence of qualitative and quantitative disparities in the individual constituents. The observed diversity in

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chemical composition can be attributed to several factors, including the selection of specific genera, species, and plant parts for extraction, as well as variations in climatic, geographical, and seasonal conditions.

Antimicrobial Activity

The antimicrobial activity findings of EO were examined *in vitro* using the disc diffusion method and manifested a variable effect against all strains tested strains (Table 2). The diameter of the growth inhibition zone varied considerably, from 10.2 to 37.12 mm. The most sensitive strains were found to be *C. albicans ATCC10231, K. pneumoniae ATCC700603, B. cereus ATCC11778, and B. subtilis* ATCC6633, with diameters in the inhibition zone of 37.12, 20.17, 18.62, and 18.37 mm, respectively. An important effect is observed against *E. fecalis ATCC29212, P. aeruginosa ATCC27853, K. pneumoniaeATCC700603, P. mirabilis ATCC35659*, and *C. albicans ATCC10231* compared with the positive control (disc antibiotic or antifungal). The *C. nepeta* EO shows a similar or greater effect on Gram-positive and Gram-negative bacteria. Whereas the antimicrobial activity of the *C. nepeta* EO extracted from Turkey reflects no significant difference in susceptibility between Gram-positive and Gram-negative bacteria[42].Compared to the previous study,[38]EO of *C. nepeta* exhibited an inhibition zone greater than the results obtained in this study against *S. aureus ATCC 6538* (40.66 mm) and *B. subtilis ATCC 9372* (20 mm), was nearly comparable against *K. pneumoniae ATCC 4352* (19.66 mm), *P. aeruginosa ATCC 9027* (10.33 mm), and E. coli *ATCC 4157* (16.33 mm), and had a low effect against *C. albicans ATCC 24433 (11.3* mm). The results of the MICs and MBCs of *C. nepeta* EO are summarized in Table 3.

Table 1: Chemical composition of essential oil extracted from *C. nepeta*

Number	Compound	RT (min)	Content (%)
1	*α*-Pinene	6.704	1.080
2	Camphene	7.114	1.227
3	*β*-Pinene	7.921	0.637
4	3-Octanol	8.448	1.158
5	Limonene+Eucalyptol	9.530	1.749
6	Unknown	10.789	0.108
7	Linalool	11.878	0.502
8	menthone	13.705	8.915
9	Isoborneol	14.105	10.406
10	Pulegone	16.698	58.361
11	Piperitenone	19.984	3.865
12	Caryophyllene	20.791	2.884
13	Unknown	22.543	1.329
14	Germacrene D	24.448	2.042
15	Bicyclogermacren	24.917	3.291
16	Spathulenol	27.305	2.446
		Total:	98.56

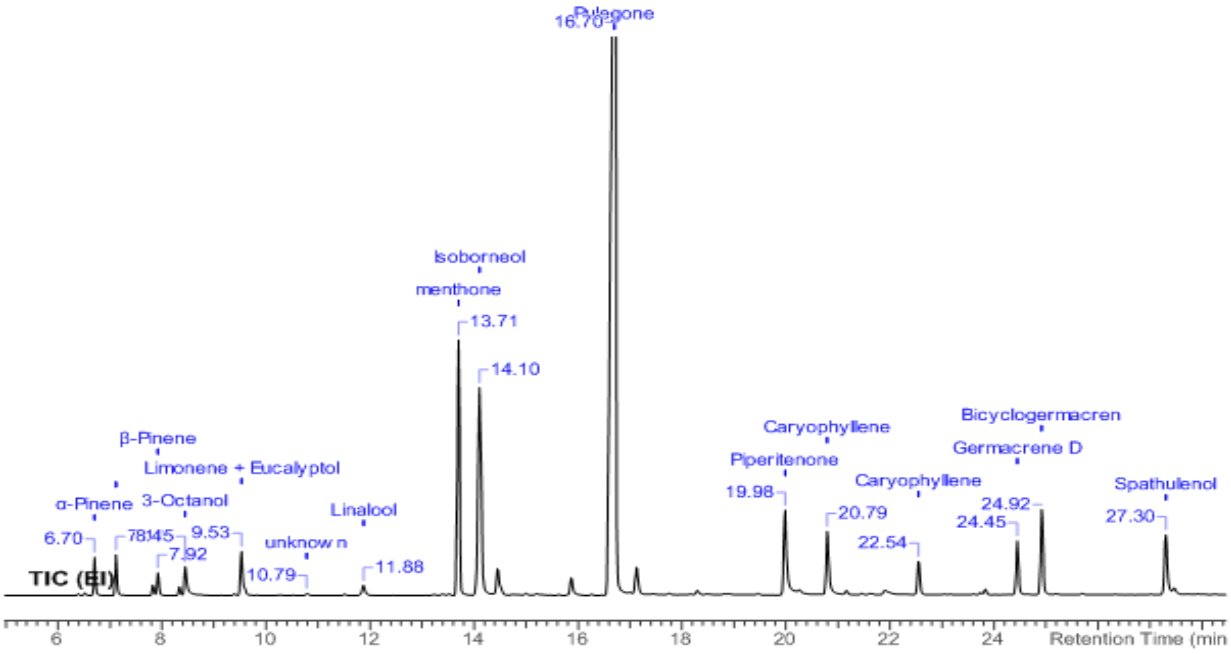

Figure 1: GC-MS spectral chromatogram of essential oil extracted from *C. nepeta*.

Data indicate that *C. nepeta* possesses antimicrobicidal properties against all microorganisms tested, with MIC ranging from 0.937 to 3.75 µL/mL and MBC ranging from 0.937 to 15 µL/mL. Interestingly, *C. albicans ATCC10231* was found to be the most sensitive strain, with MIC and MBC values of 0.937 µL/mL.When the MBC/MIC ratio of an antimicrobial agent is less than or equal to 4, it is considered to be bactericidal or fungicidal; however, if the ratio is greater than 4, it is considered to be bacteriostatic or fungistatic.[43] In this research, it was shown that *C. nepeta* exhibited antimicrobial properties against both Gram-negative and Gram-positive bacteria that were subjected to testing. Interestingly, *C. albicans*ATCC10231 is found to be the most sensitive strain with MIC and MBC values of 0.937 µL/mL. These findings are comparable to those of researchers who found that *C. albicans* was a particularly sensitive strain to *C. nepeta* EO isolated at various periods from Tarquinia city (Italy), with MIC ranging from 0.78 to 1.56 mg/mL.[16] The presence of oxygenated monoterpenes in EO accounts for its antibacterial effect. GC-MS research showed that the predominant monoterpene component of *C. nepeta* EO is pulegone (58.36%). Previous studies have demonstrated that pulegone and menthone are responsible for a considerable degree of antimicrobial activity.[19,20,40]However, other oil components can have a synergistic impact with the main component, which may not be the only one responsible for the antimicrobial effect .[44]

Antioxidant activity in vitro

The antioxidant activity of *C. nepeta* EO, assessed by the DPPH and FRAP assays, are presented in Table 4. The EO of C. nepeta was observed to have a free-radical scavenging capacity (3.77±0.71 mg/mL) and a ferric reduction power (544.6±12 µg/mL) lower than ascorbic acid (3.01±0.08 µg/mL for the DPPH assay and 4±0.18 µg/mL for the FRAP assay).In this study, the antioxidant activity results of *C. nepeta* EO by DPPH and FRAP assay are better than those estimated for the antioxidant activity of *C. nepeta* EO collected from the Bejaia region that showed 8.33±0.37 mg/mL for DPPH scavenging activity and 10.1 mg / ml for FRAP assay, In their investigation, pulegone was recognized as one of the main constituents with a percentage of 33.46%. When compared with ,the value obtained in this study, pulegone represents a ratio of 58.36%.[45]This difference may explain the influence of this compound on the neutralization and/or inhibition of reactive oxygen space generation in the studied sample. Several studies carried out with *C. nepeta* EO and extract confirmed the effectiveness of pulegone in its antioxidant capacity. [20,41]Antioxidants play an essential role in the prevention and protection of human health against dangerous free radicals, which are the primary cause of many age-related disorders. It is suggested that essential oils high in sesquiterpenes and/or oxygenated monoterpenes exhibit significant antioxidative effects. [46]

Table 4: Antioxidant Activity of *C. nepeta* EO determined by the DPPH and FRAP methods

Samples	DPPH(IC_{50})	FRAP (EC_{50})
C. nepeta EO	3.77 ± 0.7 mg/mL	544.6 ±12 µg/mL
Ascorbic acid	3.01 ± 0.08 µg/mL	4 ± 0.18 µg/mL

Data are represented as mean ± standard deviation.

Table 2: Antimicrobial activity of *C. nepeta* EO and standard drugs

Microorganisms	Inhibition diameter (mm)			
	Essential oil	Doxycycline	Ampicillin	Econazole
B. cereus ATCC10876	17.18 ± 0.2	22.56 ± 0.54 ***	0	_
B. cereus ATCC11778	18.62 ± 0.83	19.21 ± 0.63	0	_
B. subtilis ATCC6633	18 .37 ± 0.22	19.34 ± 0.12 **	14.57 ± 0.39∞∞∞	_
S. aureus ATCC25923	17.21 ± 0.63	29.30 ± 0.01 ***	10.83 ± 0.53 ∞∞∞	_
E. fecalis ATCC29212	12.29 ± 0.54	10.72 ± 0.39	0	_
E. coli ATCC25922	16,45 ± 0.92	20.23 ± 0.05 **	0	_
P. aeruginosa ATCC27853	10.2 ± 0.54	0	0	_
K. pneumoniae ATCC700603	20.17 ± 0.42	15.44 ± 0.48 ***	0	_
P. mirabilis ATCC35659	17.28 ± 0.54	8.68 ± 0.38 ***	13.62 ± 0.46∞∞∞	_
C. albicans ATCC10231	37.12 ± 0.65	_	_	23.46 ± 0.35ØØØ

*, ∞, Ø compared to Essential oil; ***P< 0.001, ** P< 0.01, * P< 0.05; Data are represented as mean ± standard deviation of three experiments.

Table 3: Minimum Inhibitory and Bactericidal Concentration values (µL/mL) of *C. nepeta* EO

Microorganisms	MICs (µL/mL)	MBCs (µL/mL)	MICs/ MBCs	Activity
B. cereus ATCC10876	1.875	3.75	2	Bactericidal
B. cereus ATCC11778	1.875	3.75	2	Bactericidal
B. subtilis ATCC6633	0.937	1.875	2	Bactericidal
S. aureus ATCC25923	0.937	1.875	2	Bactericidal
E. fecalis ATTC29212	0.937	3.75	4	Bactericidal
E. coli ATCC25922	0.937	1.875	2	Bactericidal
P. aeruginosa ATCC27853	3.75	15	4	Bactericidal
K. pneumoniae ATCC700603	1.875	3.75	2	Bactericidal
P. mirabilis ATCC35659	1.875	3.75	2	Bactericidal
C. albicans ATCC10231	0.937	0.937	1	Fungicidal

Anti-inflammatory activity

Acute toxicity

The acute oral toxicity test did not show an observed adverse effect level for EO of *C. nepeta* in Wistar rats. The results indicated that the EO of *C. nepeta* is classified in the category 5 range (>2000–5000 mg/kg) with an LD_{50} of 2500 mg/kg. The dose of 5000 mg/kg is found to be a lethal dose after 24 hours of oral administration, with the appearance of some clinical symptoms that include convulsions,dyspnea, and salivation. The acute oral toxicity test for *C. nepeta* revealed no detectable deleterious effects, according to the Global Harmonized Classification System (GHS).[34]A recent investigation carried out with EO of *C. nepeta* EO in Swiss mice observed an oral lethal toxicity dose of 1500 mg/kg.[21]Compared to other species, a toxicological screening of *Calamintha officinal* EO recorded low intraperitoneal toxicity with an LD_{50} of 100 mg/kg.[47] Indeed, acute oral toxicity performed in Balb/c mice with a crude extract of *Calamintha vulgaris* was found to be safe up to a dose of 5000 mg/kg.[48]

Carrageenan-induced paw size edema

The findings of rats' paw thickness, diameter, and inhibition of inflammation induced by carrageenan in experiment groups are shown in Table 5 and Figure 2. The obtained results showed that *C. nepeta* EO pre-treated rats at various concentrations reduced progressively the size of paw edema after the second hour of inflammation induced with a high significant difference ($P<0.001$) when compared to the control rats. After 6 h, EO indicates an inhibition percentage of 75.17, 79.44, and 88.96% at doses of 100, 200, and 400 mg/kg body weight, respectively. While diclofenac-treated rats revealed an effective reduction in paw size edema only after 2 h, with a maximum inhibition percentage of 82.30 % in 6 h,The antiedemic effect of *C. nepeta* EO at a dose of 400 mg/kg Bw was comparable to that of the diclofenac drug. The paw edema, thermal hyperalgesia, and mechanical allodynia generated by carrageenan injection were considerably decreased after the administration of *C. nepeta* EO at doses of 100, 200, and 400 mg/kg.The paradigm of carrageenan-induced paw edema in rats is well recognized as a valuable tool for evaluating the efficacy of prospective anti-inflammatory and analgesic medications.[33,52,54]Furthermore, carrageenan is a frequently used phlogistic agent that serves as a model to investigate the effects of non-steroidal anti-inflammatory drugs due to its ability to induce acute inflammation.[52]The development of carrageenan-induced paw edema is a biphasic inflammatory response.[51,53] Histamine, serotonin, and bradykinin are released in the first phase, which lasts from 0 to 150 minutes after carrageenan administration. During the late phase, which occurs 2-30 min to 6 h after Carrageenan injection, macrophages and neutrophils produce free radicals, secrete cytokines (including IL-1, IL-6, TNF- and IL-10), infiltrate the area, activate cyclooxygenase (COX-2), synthesise nitric oxide, and eventually produce prostaglandins.[56,57]The findings showed that *C. nepeta* EO has a strong anti-inflammatory effect against Carrageenan-induced paw edema at a dose of 400 mg/kg when compared to Diclofenac and other doses (100 and 200 mg/kg). This herb was previously used as an anti-inflammatory and analgesic treatment against febrile colds and respiratory disorders.[18]Compared to another study, it was observed that the methanolic extract of *C. nepeta* is less effective in inhibiting carrageenan-induced inflammation ,with an inhibition percentage of 49% after 3 h at a dose of 200 mg/kg.[58]The significance of the anti-inflammatory activity in this study can be attributed to the presence of pulegone as a predominant constituent.

Table 5: Inhibition percentage (%) on carrageenan induced paw edema in rats

Hours	Diclofenac	*C. nepeta* EO		
	10mg/kg	100mg/Kg	200mg/Kg	400mg/Kg
H1	51.69 ± 17.26	39.25 ± 16.87	42.9 ± 18.13	50.26 ± 13.67
H2	54.05 ± 8.33	56.37 ± 8.79	57.23 ± 14.14	58.78 ± 6.90
H3	62.44 ± 13.53	52.04 ± 8.19	50.57 ± 16.42	56.17 ± 15.59
H4	64.12 ± 16.49	50.4 ± 18.49	55.13 ± 13.10	58.58 ± 12.20
H5	69.59 ± 13.87	61.56 ± 11.08	64.28 ± 14.84	72.52 ± 11.19
H6	82.3 ± 11.70	75.17 ± 19.19	79.44 ± 11.42	88.96 ± 8.45

Values are presented as means ±standard deviation.

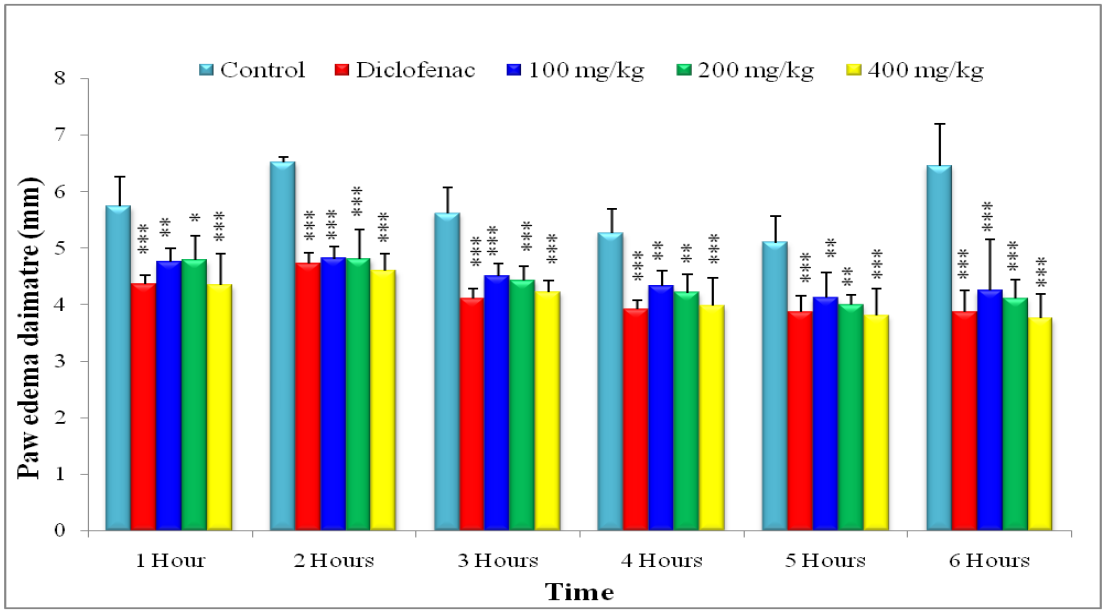

Figure 2: Effect of *C. nepeta* EO in paws edema diameter after 6 h of carrageenan injection (mm), Values are presented as means ±standard deviation (n = 6); *p< 0.05; **p< 0.01; ***p< 0.001 compared to the control group.

Previous research found that pulegone (PUL) had an anti-inflammatory impact by reducing the production of inflammatory mediators.[59]This terpene has been demonstrated to decrease the formation of prostaglandins and other inflammatory mediators in diarrhea, which might account for some of its anti-secretory effect. According to previous studies, pulegone has been found to demonstrate anti-inflammatory properties through inhibition of prostaglandin synthesis and the release of pro-inflammatory mediators.[20,60]Pulegone was reported to reduce the expression of inducible nitric oxide synthase (iNOS), COX-2, and nuclear factor kappa B (NF-κB) generation.[61]

In vitro anti-inflammatory test 5-Lipoxygenase (5-LOX) inhibition assay

The anti-inflammatory activity of *C. nepeta* EO was assessed and compared to the standard quercetin *in vitro*; the findings are presented in Table 6. The 5-LOX enzyme was inhibited by *C. nepeta*, with an IC_{50} value of 17.23 ± 0.32 µg/mL.On the contrary, quercetin exhibited an IC_{50} value of 1.13 ± 0.12 µg/mL under identical experimental conditions; *C. nepeta* demonstrated significant anti-inflammatory properties compared to the control group. The data related to the inhibition activity of the LOX enzyme are consistent with the findings of the *in vivo* assessment of anti-inflammatory activity, demonstrating a similar pattern in terms of structure-activity relationship. In various disorders, inflammation assumes a significant role, necessitating the recruitment of various cell types such as monocytes/macrophages, neutrophils, dendritic cells, and lymphocytes to affected tissue [36,39].A previous study indicated that effective inhibition of 5-lipoxygenase (5-LOX) plays a significant role in promoting anti-inflammatory effects.[66]C. nepeta's EO was evaluated for its *in vitro* anti-inflammatory activities and contrasted with quercetin. EO of *C. nepeta* inhibited the 5-LOX enzyme with an IC_{50} value of 17.23 ± 0.32 µg/mL. This investigation closely resembled the research conducted to evaluate the *in vitro* anti-inflammatory properties of thyme honey and different varieties of EOs derived from *Origanum vulgare* L., *Eucalyptus globules* L., and *Mentha spicata* L.[56] The experimental findings demonstrated that thyme honey displayed notable inhibitory properties on the enzyme, as indicated by an IC_{50} value of 29.53 ± 0.17 µg/mL.The EO of *O. vulgare*, *E. globulus* and *M. spicata* exhibited IC_{50} values of 13.23 ± 0.02, 15.53 ± 0.17, and 27.14 ± 0.07 µg/mL, respectively.[56]
This study effectively achieved the extraction of EOs from *C. nepeta* and observed that excavatolide *C. nepeta* EOs had a considerable down regulation effect on Lipoxygenase (5-LOX) *in vitro* as well as on carrageenan-induced paw edema in rats.In this reasearch, the fact that pulegone was the major compound in the EO of *C. nepeta*, makesit difficult to determine whether the anti-inflammatory activity could be attributed to this compound. The findings presented in this study demonstrate that the chemicals produced from *C. nepeta* have anti-inflammatory and analgesic properties in both *in vitro* and *in vivo* settings.

Conclusion

The essential oil derived from *C. nepeta*, sourced from the Beni-Saf region, exhibited a high concentration of oxygenated monoterpenes, with pulegone being the predominant constituent. Essential oil manifested a broad spectrum of antimicrobial activity against the specific microorganism. Additionally, it exhibited notable antioxidant capacity, effectively functioning as a scavenger of radicals and a reducer of ferric ions .Furthermore, an *in vivo* study of essential oil from*C. nepeta* revealed high anti-inflammatory activity and very low toxicity in Wistar rats. The findings indicate that *C. nepeta* essential oil can be considered a potential source of bioactive compounds to be used in pharmaceutical products.

Conflict of Interest

The authors declare no conflict of interest.

Table 6: Anti-inflammatory activity of EO of *C.nepeta* using 5-Lipoxygenase (5-LOX) Inhibition Assay

Samples	Quercetin	*C. nepeta* EO
IC_{50} (µg/mL)	1.13 ± 0.12	17.23 ± 0.32

Values are presented as means ±standard deviation.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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