Peppermint (*Mentha piperita* L.) essential oil as a potent anti-inflammatory, wound healing and anti-nociceptive drug

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**ABSTRACT:** The present investigation was designed to study the chemical composition of Algerian peppermint essential oil (PEO) as well as the *in vitro* and *in vivo* anti-inflammatory, wound-healing and anti-nociceptive properties. Twenty-three compounds were identified in the PEO with the main chemical component as menthol (53.29%). Also, PEO showed a high content of oxygenated monoterpene compounds (92.75%). Topical application of PEO at doses of 200 and 20 µL/kg significantly reduced the acute ear edema in 38.09% and 36.50, respectively. Histological observation confirmed that PEO inhibited the skin inflammatory response. *In-vivo* wound healing activity of the cream prepared from PEO (0.5% w/w) was assessed by circular excision wound model followed by histological examination. The topical administration of PEO cream showed a significant decrease of unhealed wound area rate between the 6th (1.67±0.14 mm²) and the 9th (0.49±0.22 mm²) days of treatment when compared with the vehicle (2.32±0.77 mm²; p<0.05) and Madecassol® 0.1% creams (2.23±0.35 mm²; p<0.05). The PEO reduced nociceptive behavior at all doses tested in the acetic acid-induced nociception test (p<0.05). These findings support the anti-inflammatory, wound-healing and analgesic properties of PEO. We suggest that PEO is a promising candidate for use in skin care products with anti-inflammatory and wound-healing properties.

**Keywords:** Topical anti-inflammatory; Peppermint essential oil; Menthol; Anti-nociceptive activity; Wound healing.
1. INTRODUCTION

Wound healing is a dynamic and complex process of reestablishing cellular tissue layers and structures in damaged tissue as closely as possible to its normal state. Wound contraction has different steps such as inflammatory and maturation and is dependent upon the extent and type of injury, the common state of the patient’s health, and the aptitude of the tissue and cutaneous structure for healing [1]. The inflammatory phase is categorized by homeostasis and edema, followed by angiogenesis, epithelization and collagen installation. Inflammation is considered as a defensive host reply to external antigenic challenge or tissue injury that, if unrestricted, could lead to loss of function as well as tissue structure [2]. Inflammatory phase is frequently linked with pain and discomfort as a secondary process resulting from the discharge of analgesic mediators. Nonsteroidal anti-inflammatory drugs (NSAIDs), which have been used generally in the relief of inflammatory sicknesses and pain for decades. However, these chemical drugs are often associated with severe adverse side effects, such as gastrointestinal bleeding and peptic ulcers, cardiovascular and kidney toxicities [3]. For these reasons, there is a necessity for anti-inflammatory molecules having fewer side effects to use for pain or inflammatory disease as well.

Recently, several natural and alternative medicines derived from aromatic and medicinal plants were considered as active and safer for the management of different illnesses including inflammation, wound and pain, but there is a lack of appropriate scientific evidence [4-6]. The application of natural therapy or alternative medicine represents attractive and good approach for the cure of several inflammatory skin and dermatological disorders. Various phytochemical extracts, essential oils (EOs), and isolated pure molecules of natural product origin have been explored and studied for possible pharmacological activities in vitro and in several animal models and reported to have significant anti-inflammatory, analgesic or wound healing properties [7-9]. For example, the molecules of EOs are small enough to pass through the skin barrier. EO will be absorbed without trouble into the skin within 20-50 min depending on its chemical and physical nature [10]. In this context, medicinal plants give an enormous reserve for the discovery, development and improvement of novel and original drug leads [4, 6].

Among these long-established natural medications, the EO of the flowering aerial part of peppermint possesses an exceptional place in Algerian traditional medicine. Peppermint (Mentha piperita L.) is a cultivated hybrid of two species (M. aquatica and M. spicata L.). Although peppermint is a native genus of the Mediterranean area, it has been spread all over the world for use in fragrance, flavor, cosmetic medical and pharmaceutical applications [11-12]. Dried peppermint leaves and flowers were found in the Egyptian pyramids, displaying that the usage of this aromatic plant may date back to at least 1000 BC. Peppermint has been described to have several biological properties. Peppermint is extensively used in traditional remedies for treatment of digestive complaints and nervous system actions because of its antimicrobial, anti-allergenic and anticancer activities, chemopreventive potential, its renal effect, and also for decreasing anorexia, cramping, diarrhea and nausea [13, 14]. Peppermint is cultivated mainly for its EO, which is obtained by...
steam or hydrodistillation [15]. Besides, peppermint essential oil (PEO) is used as a raw material in mouth fresheners, analgesic balms, toothpaste, perfumes, chewing gums, candies and the tobacco industry [16]. However, PEO can be toxic and even lethal at excessive concentrations; it has been linked with interstitial nephritis and acute renal failure. Else, PEO is moderately contraindicated in patients with hiatal hernia or important gastroesophageal reflux disease. Possible side effects of PEO include allergic reactions, heartburn, nausea and vomiting. Further, PEO should be avoided during pregnancy. There are not sufficient data to assess its safety during lactation. PEO should also not be administered internally or near the face in young children because of its potential to cause bronchospasm [12-17].

Different publications have demonstrated that PEO has antibacterial and antifungal properties, potent antioxidant and anticancer actions, and anti-allergenic potential. The extensive application of PEO in phytomedicines has motivated us to more evaluate its potential pharmacological properties, knowing that there are limited studies reporting its biological activities [13, 17, 18]. Based on the above considerations, the present study reports the chemical composition of essential oil obtained from peppermint grown in Algeria as well as the \textit{in vitro} and \textit{in vivo} anti-inflammatory, wound-healing and anti-nociceptive activities.

2. MATERIAL AND METHODS

2.1. Material

2.1.1. Extraction of peppermint essential oil

Peppermint (\textit{Mentha piperita} L.) aerial part was collected at the Cherchell region (Tipaza, Algeria). PEO was extracted from the aerial part with alembic steam distillation. This method is used to obtain PEO from \textit{Mentha piperita} by passing steam generated in a pot still through the plant material. A quantity of fresh plant (leaves and stems) was loaded in the still and stacked in layers to allow appropriate delivery of the steam. When the steam passes through the peppermint tiny pockets that hold the oil open release the volatile compounds. This is referred to as the distillate which is a mix of hydrosol (aromatic water) and PEO. The essential oil was stored in sealed glass bottles at +4 °C until tested and analyzed.

2.1.2. Solvents, drugs and chemicals

The following drugs and chemicals were used: dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), sodium diclofenac, Alsever solution, acetic acid, carrageenan, xylene, tween 80, isosaline (0.85%) and phosphate-buffered saline (PBS) solutions were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ketum gel® 2.5% (Ketoprofene 60 g, Laboratoire Menarini, Barcelona, Spain), Spasfon® 80 mg (Phloroglucinol, Teva Sante, Paris, France), Madecassol® 1% cream (Asiaticoside, Roche, France), Ketamile® (Ketamine chloride, El Kendi Pharmaceuticals, Algiers, Algeria) and Votrex® 50 mg (sodium diclofenac, Hikma Pharmaceuticals, Jordan) were also used. All cosmetic ingredients (Sweet Almond Oil, Beeswax, Stearic Acid, Cetyl Alcohol, Ceteareth-20, Trolamine, Glycerin) were purchased from GireneCosmetic Company (Ain Benian, Algiers, Algeria).

2.1.3. Animals

Male Wistar rats (158.37±14.384 g) and Swiss albino mice of both sexes (25.28±1.75 g) were purchased from the animal breeding of “Institut Pasteur d’Algérie” (Algiers, Algeria). Mice and rats were left for one week at room conditions for acclimatization. A minimum of 6 animals were used in each group, and were kept at room temperature with a 12 h light/dark cycle. They were kept on a standard pellet diet and
water *ad libitum* during the experiment. The experimental method was agreed and approved by current institutional guidelines for animal handling and experiments.

2.2. Methods

2.2.1. Determination of chemical composition

Gas chromatography and mass spectrometry were carried out using a GC-MS QP 5050 Shimadzu. A Scientific DB-5 MS (50 m × 0.25 mm × 0.25 µm) capillary non-polar column was used. The oven temperature was set at 50°C with an increase of 2°C/min until 270°C, and preserved for 10 min. Helium was the carrier gas with a constant flow of 1.4 mL/min. The temperature of the ionization source was conserved at 280°C, the ionization energy at 70 eV, and the ionization current at 0.7 kV. Mass spectra were recorded from 30 to 450 m/z. The individual components were identified by matching and comparing their mass spectra with those of the spectrometer data base using the Wiley library and by comparing their retention indices with those of NIST computer MS library.

2.2.2. In vitro and in vivo anti-inflammatory activities

2.2.2.1. In vitro irritation test in red blood cell (RBC) system cellular model

This technique permits the determination of adverse effects of PEO on the membrane of RBCs, and the resulting release of hemoglobin (hemolysis), which allows the quantification of the irritation level of the PEO [19]. The human venous blood samples were freshly collected from healthy volunteer and put into test tubes containing anticoagulant (EDTA-Na₂ 10.0%). The collected blood was mixed with equal volume of Alsever solution (sodium citrate 0.8%, sodium chloride 0.42%, dextrose 2%, citric acid 0.05%, and distilled water 100 mL) and centrifuged at 2500 rpm. The human RBCs were washed with an isosaline solution (NaCl 0.85%) and a 10% (v/v) suspension was made with isosaline. The PEO was dissolved in PBS in order to obtain different concentrations (6 mg/mL to 0.4 mg/mL). To this solution 1 mL of PBS (pH = 7.4), 2 mL of hyposaline solution (0.4%) and 0.5 mL of the RBC suspension (10% v/v) were added. The PEO samples and those containing anticoagulant (EDTA-Na₂ 10.0%) were incubated at 37ºC for 30 min and then centrifuged at 25000 rpm. The hemoglobin concentration in the supernatant solution was calculated by using a spectrophotometric method at 560 nm. The erythrocyte membrane stability (%) was estimated using the following formula:

\[
\text{Erythrocyte membrane stability (\%)} = 100 - \left( \frac{A_{\text{sample}}}{A_{\text{blood}}} \right) \times 100
\]

where: A sample = Absorbance of the tested sample; A blood = Absorbance of the control.

Sodium diclofenac was used as standard anti-inflammatory drug being processed in a similar manner with the PEO. All determinations were performed in triplicate and the values are expressed as mean ± standard deviation (SD).

2.2.2.2. Carrageenan-induced paw edema *in vivo*

The anti-inflammatory test was evaluated by the carrageenan-induced paw edema in the mice, according to the method of Niu et al. [20]. Male Swiss mice (25.28±1.75 g) were briefly anesthetized with ethyl ether and injected sub-plantarly into the left hind paw with 0.1 mL of suspension of carrageenan (0.2 mg/mL) in isotonic saline. The right hind paw was injected with 0.1 mL of saline and used as a control. PEO at doses of 2, 20, or 200 µL/kg and vehicle (isosaline NaCl 0.9%) were administrated orally 30 min before administering the carrageenan. Sodium diclofenac (50 mg/kg, orally) was used as the reference drug. The mice were sacrificed 4 hours later. The difference in weight between right untreated and left treated hind paws...
was calculated and results are expressed as the increase in paw weight (mg). The percentage inhibition of the inflammatory response was estimated by comparison to the negative control and calculated by using this formula:

\[
\% \text{ Inhibition of edema} = \left(1 - \frac{\Delta P_t}{\Delta P_c}\right) \times 100
\]

where \(\Delta P_t\) is the difference in paw weight in the drug-treated group, and \(\Delta P_c\) is the difference in paw weight in the control group.

2.2.2.3. Xylene-induced ear edema in vivo

Topical anti-inflammatory activity was assessed as inhibition of xylene-induced ear edema in mice [20]. The animals were divided into groups of five. Thirty minutes after the dermal application of PEO at different doses (2, 20, or 200 mg/kg), vehicle (sweet almond oil) and a reference drug (Ketoprofen, 2.5%), 0.03 mL of xylene was applied to the posterior and anterior surfaces of the right ear. The left ear was considered as control. Four hours after xylene application, the animals were sacrificed by cervical dislocation 30 min later and the plug (5 mm in diameter) was detached and removed with a stainless steel punch from both the treated right ear and the untreated left ear. The difference in weight between the two plugs was considered as a measure of inflammation and edematous responses. PEO anti-inflammatory potential was expressed as percentage of the edema weight reduction in treated mice in comparison to the control group and calculated using the following formula:

\[
\% \text{ Inhibition of topical edema} = \left(1 - \frac{\Delta W_t}{\Delta W_c}\right) \times 100
\]

where \(\Delta W_t\) is the change in weight of ear tissue in the treated mice, and \(\Delta W_c\) the change in weight of ear tissue in the control mice (vehicle).

2.2.2.4. Morphological analysis of mouse ear tissue

The resulting inflammatory response was checked and monitored by measurement of edema formation and by microscopic observation. For morphological examination of cutaneous inflammation, biopsies from control and treated ears of animals were collected at the end of the test. Biopsies were fixed in 10% neutral formalin, routinely processed, and sectioned at 5 \(\mu\)m using a microtome (Leica RM, Nussloch, Germany). Sections were stained with Hematoxylin & Eosin (H&E) and length was evaluated using light microscopy. The tissues were observed with a light microscope and graded as mild (+), moderate (++), and severe (+++) for inflammation phase. Infiltration and polymorphonuclear (PMN) cells’ accumulations were also assessed [21].

2.2.3. In vivo wound healing activity

2.2.3.1. Preparation of test samples for bioassay

An excision wound model was used to determine the wound healing property. For the in vivo wound models, PEO was incorporated in a topical cream formulation (0.5% w/w) (Table 1). The above cream was prepared by exactly weighing the lipophilic and aqueous phase ingredients and taking in a beaker separately and heating. The lipophilic phase was prepared by melting the waxes and emulsifiers and mixing the ingredients regularly. The aqueous phase was prepared by dissolving the water-soluble ingredients in deionized water. The two phases were warmed to 65°C until all ingredients were dissolved. When the water and oil phase were at the same temperature, the aqueous phase was then slowly mixed with the lipophilic
phase with agitation till the cream congealed and cooled. The topical emulsion was cooled to laboratory
temperature to form a semisolid cream base.

A quantity of each test cream was applied topically on the wounded position directly after a wound
was created with a surgical blade. The rats in the negative group (vehicle) were treated with the cream base
only, whereas those in the positive control group were treated with Madecassol® cream.

<table>
<thead>
<tr>
<th>Table 1. Topical cream preparation with 0.5% of peppermint essential oil.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ingredients</strong></td>
</tr>
<tr>
<td>Lipophilic phase</td>
</tr>
<tr>
<td>Sweet Almond Oil</td>
</tr>
<tr>
<td>Beeswax</td>
</tr>
<tr>
<td>Stearic Acid</td>
</tr>
<tr>
<td>Cetyl Alcohol</td>
</tr>
<tr>
<td>Ceteareth-20</td>
</tr>
<tr>
<td>Peppermint (<em>Mentha piperita</em> L.)</td>
</tr>
<tr>
<td>Aqueous phase</td>
</tr>
<tr>
<td>Deionized water</td>
</tr>
<tr>
<td>Glycerin</td>
</tr>
<tr>
<td>Trolamine</td>
</tr>
</tbody>
</table>

2.2.3.2. Circular excision wound model

A circular excision model was employed to monitor wound closure and wound contraction times. Each
group of rats was anesthetized with 0.01 mL of Ketamine® (Ketamine chlorhydrate, 50 mg/mL, El Kendi
Pharmaceutical, Algiers, Algeria). The back hairs of the animals were removed by shaving. A circular wound
was formed on the dorsal inter-scapular region of each rat by excising the skin with a 2 cm biopsy punch;
wounds were left open [5].

The PEO cream, the reference drug (Madecassol® 1% cream, containing *Centella asiatica* extract as an
active ingredient), and the vehicle cream bases were administered topically once a day till the wound was
completely healed (day 15). The progressive changes in wound area were checked by using transparent
tracing paper every day. Wound area was estimated by retracing the wound on a millimeter scale graph paper
and then weighing the paper to estimate the areas. In addition, wound area was measured using an AutoCAD
program. Wound contraction was expressed as the percentage of the reduction in wounded area using the
following formula:

\[ \text{% wound contraction} = \left(1 - \frac{\text{Wound area on corresponding day}}{\text{wound area on zero day}} \right) \times 100 \]

At the end of his experiment, a specimen sample of cutaneous tissue was collected from the healed skin of
each group of rats for histological examination.

2.2.3.3. Histopathology analysis

The skin samples from each group were isolated at the end of the experiment on day 15. Samples were
fixed in 10% buffered formalin, processed, and blocked with paraffin and then sectioned into 5 µm sections
and stained with H&E stains. Skin tissues were observed with a light microscope (Olympus CX41) and
graded as mild (+), moderate (++), or severe (+++) for epidermal or dermal remodeling. Re-epithelization or
ulcer in the epidermis, fibroblast production, polymorphonuclear cells and neo-vascularization in the dermis
were examined to score the epidermal or dermal remodeling [5].

### 2.2.4. Acetic acid-induced writhing test

The anti-nociceptive activity of PEO was assessed using the writhing test (abdominal constriction test), according to the method of Niu et al. [20]. Mice were randomly separated into five groups (5 animals per group). The solution of acetic acid (10 mL/kg, 0.6%) was injected intraperitoneally (i.p.), and the contraction and constriction of the abdominal muscles together with stretching of the hind limbs was calculated over a period of 10 minutes, starting immediately after the injection of acetic acid solution. The PEO (2, 20 and 200 µL/kg, orally), phloroglucinol (Spasfon® 80 mg/kg, orally) as the standard drug or positive control, and water (0.5 mL, orally) as the negative control were administered 30 min before the acetic acid injection. Mice with a decrease in the number of twists were protected by the respective dose of PEO. Anti-nociceptive activity was estimated as the percentage of inhibition of abdominal contractions between the control and the treated groups using the following formula:

\[
\text{Inhibition} (\%) = \left( \frac{G_c - G_t}{G_c} \right) \times 100
\]

where \( G_c \) is the average of the negative control group stretches and \( G_t \) is an average of stretches of the treated group.

### 2.3. Statistical analysis

The results obtained in our study were presented as mean±SD where each value represents a minimum of 6 animals. One way analysis of variance (ANOVA) was carried out to determine the variability among different groups. Significant differences among groups were estimated and calculated using Tukey’s multiple comparison tests in which the results were compared with that of the control group. The results were considered statistically significant at \( p<0.05 \). XLSTAT2014 software (Addinsoft, Paris, France) was used for all statistical analysis.

### 3. RESULTS AND DISCUSSION

#### 3.1. Chemical composition of peppermint essential oil

The quantitative and qualitative compositions of the EO obtained from the aerial part of peppermint are presented in Table 2. Twenty-three compounds are identified in the EO from Algerian peppermint with the main chemical components as menthol (53.29%), menthone (16.41%), menthyl acetate (6.82%) and 1,8-cineole (4.74%). Other chemical compounds were detected but were less than 4% (Table 2). Also, PEO showed a high content of oxygenated monoterpene compounds (92.75%) and low amounts of monoterpeno hydrocarbons (3.96%).

The peppermint samples analyzed in our research meet the requirements of the European Pharmacopoeia which establishes that PEO should contain between 30-55% menthol, 14-32% menthone and between 2.8-10% menthyl acetate [22]. It has been reported that PEO is characterized by significant variations in the amounts of menthol, menthone, menthofuran, menthyl acetate, and 1,8-cineole, being with the menthol/menthone chemotype the most commonly identified [17]. A published paper related to the analysis of PEO cultivated in the Republic of Srpska (Bosnia and Herzegovina) and isolated by hydrodistillation on semi-industrial scale has revealed very similar results to the ones obtained in this article. Menthol was the main component in both oils [15]. Some previous reports also revealed menthol and menthone as the most
dominant chemical compounds in PEO [13]. Therefore, in the case of Iranian PEO the most important components were menthol (53.3%), followed by menthyl acetate (15.1%) and menthofuran (11.2%) [23].

<table>
<thead>
<tr>
<th>Number</th>
<th>Retention time (min)</th>
<th>Compound</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13.051</td>
<td>α-Pinene</td>
<td>0.59</td>
</tr>
<tr>
<td>2</td>
<td>15.526</td>
<td>Sabinene</td>
<td>0.39</td>
</tr>
<tr>
<td>3</td>
<td>15.815</td>
<td>β-Pinene</td>
<td>0.76</td>
</tr>
<tr>
<td>4</td>
<td>18.491</td>
<td>α-Terpinene</td>
<td>0.14</td>
</tr>
<tr>
<td>5</td>
<td>19.034</td>
<td>p-Cymene</td>
<td>0.26</td>
</tr>
<tr>
<td>6</td>
<td>19.413</td>
<td>Limonene</td>
<td>1.48</td>
</tr>
<tr>
<td>7</td>
<td>19.637</td>
<td>Eucalyptol</td>
<td>4.74</td>
</tr>
<tr>
<td>8</td>
<td>21.455</td>
<td>γ-Terpinene</td>
<td>0.26</td>
</tr>
<tr>
<td>9</td>
<td>23.422</td>
<td>Terpinolene</td>
<td>0.08</td>
</tr>
<tr>
<td>10</td>
<td>24.613</td>
<td>Linalool</td>
<td>0.28</td>
</tr>
<tr>
<td>11</td>
<td>28.877</td>
<td>Menthone</td>
<td>16.41</td>
</tr>
<tr>
<td>12</td>
<td>29.275</td>
<td>Menthofurane</td>
<td>3.85</td>
</tr>
<tr>
<td>13</td>
<td>29.399</td>
<td>Isomenthone</td>
<td>2.17</td>
</tr>
<tr>
<td>14</td>
<td>29.781</td>
<td>neo-isomenthol</td>
<td>3.39</td>
</tr>
<tr>
<td>15</td>
<td>30.974</td>
<td>Menthol</td>
<td>53.29</td>
</tr>
<tr>
<td>16</td>
<td>31.707</td>
<td>α-Terpineol</td>
<td>0.19</td>
</tr>
<tr>
<td>17</td>
<td>34.664</td>
<td>cis-Isopulegone</td>
<td>1.16</td>
</tr>
<tr>
<td>18</td>
<td>35.773</td>
<td>Piperitone</td>
<td>0.45</td>
</tr>
<tr>
<td>19</td>
<td>38.472</td>
<td>Menthyle acetate</td>
<td>6.82</td>
</tr>
<tr>
<td>20</td>
<td>44.710</td>
<td>β-Bourbonene</td>
<td>0.31</td>
</tr>
<tr>
<td>21</td>
<td>47.139</td>
<td>β-Caryophyllene</td>
<td>1.73</td>
</tr>
<tr>
<td>22</td>
<td>51.138</td>
<td>Germacrene D</td>
<td>0.99</td>
</tr>
<tr>
<td>23</td>
<td>52.078</td>
<td>γ-Elemene</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Table 2. Chemical composition of the peppermint essential oil using a GC-MS method.

Nevertheless, important differences in quantitative composition could also happen, as in some case of Korean PEO. It has a considerably different chemical profile in comparison to the aforementioned EOs with linalyl acetate (28.2%) as the most dominant compound [24]. Usually, quantitative and qualitative composition of PEO varies commonly, among other factors, it depends on the climate of plant cultivation, time of harvest, extraction methods, storage conditions, environmental and ecological conditions [13, 18, 25].

3.2. In vitro and in vivo anti-inflammatory activities

3.2.1. Irritation test in red blood cell (RBC) system cellular model in vitro

Exposure of RBC to hypotonic condition results in the lysis of the membranes, with the hemolysis and oxidation of hemoglobin. Membrane stabilization is correlated with the prevention of leakage of serum protein into the tissues and limiting the inflammatory response [26]. The membrane stabilizing activity of the PEO at different concentrations is presented in Table 3. Except the higher dose of PEO, in all tested concentrations it was observed that the protection of erythrocyte membrane was similar to the positive control.
For example, at lower concentration (0.4 mg/mL), PEO showed a membrane protection (92.253±0.203%) comparable with diclofenac (92.913±0.221%) at 0.003 mg/mL.

Table 3. Effect of peppermint essential oil on stabilization of RBC membrane in vitro.

<table>
<thead>
<tr>
<th>Treatment (s)</th>
<th>Concentration (mg/mL)</th>
<th>Absorbance (660 nm)</th>
<th>RBC membrane protection (%)</th>
<th>IC\textsubscript{50} (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (PBS)</td>
<td></td>
<td>0.568</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PEO</td>
<td>6</td>
<td>0.367</td>
<td>35.387±4.867 \textsuperscript{BC}</td>
<td>4.649±2.210 \textsuperscript{B}</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.051</td>
<td>91.021±1.031 \textsuperscript{A}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>0.044</td>
<td>92.253±0.508 \textsuperscript{A}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>0.041</td>
<td>92.781±0.101 \textsuperscript{A}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.044</td>
<td>92.253±0.203 \textsuperscript{A}</td>
<td></td>
</tr>
<tr>
<td>Sodium diclofenac</td>
<td>30</td>
<td>0.460</td>
<td>19.014±12.707 \textsuperscript{B}</td>
<td>1.198±0.735 \textsuperscript{A}</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.411</td>
<td>27.552±3.354 \textsuperscript{B}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>0.045</td>
<td>92.165±0.419 \textsuperscript{A}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>0.041</td>
<td>92.693±0.227 \textsuperscript{A}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.003</td>
<td>0.040</td>
<td>92.913±0.221 \textsuperscript{A}</td>
<td></td>
</tr>
</tbody>
</table>

Each value represents the mean ± SD. PEO: Peppermint essential oil. IC\textsubscript{50}: Median inhibitory concentration, PBS: Phosphate-buffered saline. Means within the same column followed by different letters are significantly different (\(p<0.05\)) according to ANOVA one-way analysis followed by Tukey’s post hoc multiple comparison tests.

The PEO demonstrated RBC membrane stabilization activity by inhibiting hypotonicity induced lysis of erythrocyte membrane. The RBC membrane is similar to the lysosomal membrane and its stabilization suggests that the PEO may as well stabilize the membranes of lysosomes which is imperative in preventive the inflammatory response by inhibiting the discharge of lysosomal constituents of activated neutrophil such as proteases and bactericidal enzymes, which cause additional tissue inflammation and damage [26]. Although the exact mode of action of the RBC membrane stabilization by the PEO is not identified yet, hypotonicity induced hemolysis may arise from decrease of the cells due to osmotic loss of fluid components and intracellular electrolyte. The PEO may prevent the processes, which may stimulate or enhance the efflux of these intracellular molecules [19].

3.2.2. In vivo anti-inflammatory activity using carrageenan-induced paw edema test

Carrageenan-induced paw edema is frequently used to assess the anti-inflammatory activity of different EOs and phytochemicals. The anti-inflammatory activity of orally administered PEO (2, 20, and 200 µL/kg) was determined using the paw edema model. As shown in Table 4 and Figure 1, PEO showed a potent anti-inflammatory potential. At 4 hours after oral administration of PEO, the weight of treated left hind paw was similar for 20 µL/kg and 200 µL/kg (0.133±0.0075 g and 0.129±0.0058 g, respectively) with edema inhibition values of 12.27±3.94% and 9.29±3.94%. This level of edema inhibition was comparable to the level observed using 50 mg/kg of the standard reference drug (11.43±6.07%).

Investigations on the anti-inflammatory action of the PEO are limited. Only one research article suggested the ability of PEO to decrease the carrageenan-induced paw edema in animals at higher dose (200 mg/kg) [27]. Besides a decrease of prostaglandin concentration in the damaged tissue structure, it is probable that the EOs were also able to impact the first phase of this inflammatory model, probably by preventing the release of additional pro-inflammatory mediators [27].
Figure 1. In vivo anti-inflammatory effect of PEO using carrageenan-induced paw edema assay.

Groups of mice (n = 5/group) were pretreated with vehicle (NaCl, 0.9%), NSAID: non-steroidal anti-inflammatory drug (Sodium diclofenac, 50 mg/kg, orally). PEO: Peppermint essential oil at doses of 2, 20, and 200 µL/kg (orally). ns: no significant difference (p>0.05); *: significant difference (p<0.05) according to ANOVA one-way analysis followed by Tukey’s post hoc multiple comparison test.

Table 4. In vivo anti-inflammatory effect of PEO using carrageenan-induced paw edema assay.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weight Left hind paw (g) #</th>
<th>% Inhibition of edema</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control (Vehicle)</td>
<td>0.130±0.0089 B</td>
<td>/</td>
</tr>
<tr>
<td>Positive control (NSAID)</td>
<td>0.147±0.0116 A</td>
<td>11.43±6.07</td>
</tr>
<tr>
<td>PEO 200</td>
<td>0.129±0.0058 A</td>
<td>12.27±3.94</td>
</tr>
<tr>
<td>PEO 20</td>
<td>0.133±0.0075 A</td>
<td>9.29±3.94</td>
</tr>
<tr>
<td>PEO 2</td>
<td>0.137±0.0072 AB</td>
<td>6.45±4.94</td>
</tr>
</tbody>
</table>

Groups of mice (n = 5/group) were pretreated with vehicle (NaCl, 0.9%), NSAID: non-steroidal anti-inflammatory drugs (Sodium diclofenac, 50 mg/kg, orally). PEO: Peppermint essential oil at doses of 2, 20, and 200 µL/kg (orally). #: means within the same column followed by the same capital letter are not significantly different (p>0.05) according to ANOVA one way analysis followed by Tukey’s post hoc multiple comparison test.

Previous studies suggest that numerous peripheral mechanisms could be responsible for the anti-inflammatory activity of EOs [13, 21]. Recently, several papers have reported oxygenated monoterpenes and their hydrocarbon derivatives as the principal components of EOs, which have an active anti-inflammatory potential [28]. In our study, menthol and menthone have been found to be the major compounds in PEO. It appears that menthol can be partly associated with the observed anti-inflammatory effect, but it is not apparent if the other oxygenated monoterpenes (menthone, menthy acetate and 1.8-cineole) can also potentiate this activity [29]. Our data are in agreement with those published for other EOs rich in menthol that demonstrated a potent and strong anti-edematogenic effect [13, 27].

3.2.3. In vivo anti-inflammatory activity using xylene-induced ear edema assay

Because the PEO demonstrated an anti-inflammatory effect through carrageenan-induced paw edema assay, this property was further assessed by estimating the degree of inhibition of xylene-induced ear edema
in mice. Topical application of xylene on the right ears caused noticeable edema as indicated by the augmentation in the ear plug weight of the right ear compared with the untreated left ear (Table 5 and Figure 2).

The topical application of PEO was capable of reducing inflammation in xylene-induced ear acute edema in a dose-dependent manner. In comparison with positive control (Ketoprofen topical gel), PEO exhibited a powerful and effective anti-inflammatory activity in our experimental animal model. Ketoprofen gel produced a 38.09% inhibition of xylene-induced edema, and this effect was statistically similar to those observed with the doses of PEO. PEO reduced the inflammatory response by 38.09% for 200 mg/kg, and by 36.50 for 20 mg/kg. To the best of our knowledge, this is the first research to demonstrate that the Algerian PEO possesses a significant topical anti-inflammatory activity in vivo.

Table 5. Peppermint EO prevents xylene-induced ear edema in mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weight (mean, mg) ± SD</th>
<th>% inhibition of edema</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control (Vehicle)</td>
<td>12.6±1.341</td>
<td>3.4±1.140 B</td>
</tr>
<tr>
<td>NSAID (Ketoprofen 2.5%)</td>
<td>7.8±1.095</td>
<td>0.4±0.707 A</td>
</tr>
<tr>
<td>PEO 200 µL/kg</td>
<td>7.8±0.836</td>
<td>1±0.577 A</td>
</tr>
<tr>
<td>PEO 20 µL/kg</td>
<td>8±0.707</td>
<td>1±0.5 A</td>
</tr>
<tr>
<td>PEO 2 µL/kg</td>
<td>8.4±1.140</td>
<td>0.2±0.707 A</td>
</tr>
</tbody>
</table>

Data are presented as Mean (mg) ± Standard Deviation (SD) (n = 5/group). NSAID: non-steroidal anti-inflammatory drugs. PEO: Peppermint essential oil at doses of 2, 20, and 200 µL/kg (topical application). Vehicle: Sweet almond oil. #Means within the same column followed by the same capital letter are not significantly different (p>0.05) according to ANOVA one way analysis followed by Tukey’s post hoc multiple comparison test.

Figure 2. Topical anti-inflammatory activity of peppermint oil using xylene-induced ear edema test.

Data are presented as Mean (mg) ± Standard Deviation (SD) (n = 5/group). NSAID: non-steroidal anti-inflammatory drugs. PEO: Peppermint essential oil at doses of 2, 20, and 200 µL/kg (topical application). ns: no significant difference (p>0.05); *: significant difference (p<0.05) according to ANOVA one-way analysis followed by Tukey’s post hoc multiple comparison tests.
PEO is important oil with various health benefits comprising its aptitude to diminish inflammation. Consistent with our data, topical application PEO has shown anti-inflammatory properties in mouse model because of its inhibitory effect on the production of nitric oxide and prostaglandin E2 [13]. Furthermore, Atta and Alkofohi [30] have revealed that the ethanol extracts of peppermint induced an anti-inflammatory and a dose-dependent pain-reducing protective activity against both proliferative and exudative inflammation.

3.2.4. Examining the mouse ear tissue morphology

We investigated H&E-stained ear sections from xylene-induced animals (Figure 5). By histological comparison, topical application of PEO decreased ear thickness and associated pathological indicators (Figure 3.C1-C3) to an extent comparable to the positive control (Diclofenac gel) (Figure 3.B). These findings directly demonstrate the properties of PEO within the target tissue, providing additional confirmation that PEO ameliorates xylene-induced contact dermatitis.

![Figure 3. Histopathology sections of mice ear biopsies showing keratin, epidermal, dermal layers, muscle and cartilage.](image-url)

**Negative control (Gx100)** - Edema (++); inflammation phase (+++); inflammatory cell infiltration (+++) in epidermal and dermal layers, muscle and cartilage.

**Positive control treatment (Ketum gel® 2.5%w) (Gx40)** - Edema (±); inflammatory cell infiltration (+), inflammation phase (±).

**Peppermint essential oil treatment with different doses** (C1: PEO 2 µL.Kg (Gx100), C2: PEO 20 µL.Kg (Gx100), C3: PEO 200 µL.Kg (Gx40)) = edema (±); inflammatory cell infiltration (+), inflammation phase (±).

Microscopic investigation showed the valuable anti-inflammatory activity of the topical application with PEO. Compared to the negative control group, edema was dramatically reduced by the previous topical treatment with PEO (Figure 3 A vs Figure 3 C1-C3). To the best of our knowledge, this is the first study to reveal that Algerian PEO possesses a significant topical anti-inflammatory activity, which is confirmed by histopathology examination. It has been published that the topical application of EO delivers satisfactory efficacy in both molecular and pathological phases and thus recommended it for the preparation of natural
creams and ointments [21]. Knowing that oxygenated monoterpenes have outstanding anti-inflammatory properties [28, 29], the anti-inflammatory effect of PEO could be partially explained by the presence of oxygenated terpenes, such as menthol, menthone and eucalyptol (1,8-cineole).

3.3. Pharmacological evaluation of wound healing activity

3.3.1. Effect of PEO on wound area and percent wound contraction

In this work, the wound healing activity PEO was assayed using animal model of excision. This model is useful for the estimation of wound epithelialization and contraction, and to measure the formation of granulation tissue [5]. The wound area (mm$^2$) in all animal (rat) groups was measured and estimated on day 1, 3, 6, 9, and 15 (Figure 4). The results of the present investigation indicate that topical treatment with PEO (0.5%) exhibits significant wound healing activity. This was demonstrated by the decrease in wound area rate between the 6th (1.67±0.14 mm$^2$) and the 9th (0.49±0.22 mm$^2$) days of treatment and enhanced epithelialization of the excision wound when compared with the vehicle (2.32±0.77 mm$^2$; $p<0.05$) and Madecassol 0.1% (2.23±0.35 mm$^2$; $p<0.05$) creams.

![Figure 4. Effect of PEO cream formulation treatment on wound area (mm$^2$) in rats.](image)

*significant difference ($p<0.05$) according to ANOVA analysis followed by Tukey’s post hoc multiple comparison test.

3.3.2. Histopathological examination

Histopathological examinations were also consistent with the data of the excision experimental method. For demonstration of the wound healing process, representative photomicrographs (Figure 5), stained with H&E, were also studied. Phases in wound healing progressions with variable degrees were examined within the experimental groups. Stages in wound healing processes (proliferation, inflammation, and remodeling) were verified and recorded (Table 6).

Wound healing progressions delayed in the vehicle group, while faster remodeling in different degrees was observed in the PEO group. The topical application of PEO rich in menthol accelerates the wound repair, which was confirmed by histological analysis (Table 6). Proliferation of collagen, fibrous tissue, and capillaries with epidermal covering at the margin of wound were observed. Examination showed a better
epithelialization, fibroblast population and collagen deposition in animals treated with PEO cream (Figure 5.BE) when compared to those treated with the vehicle (Figure 5.AD) or Madecassol 0.1% dermal creams (Figure 5.CF), after 8 and 15 days of wounding, respectively. Treatment with PEO cream formulation resulted in decreased inflammation, increased rate of tissue perfusion and proliferation as well as remodeling, along with re-epithelization. In early studies, menthol was reported to be an important monoterpene in the wound healing process [31]. Therefore, high amount of menthol in PEO could promote the wound healing activity [28, 31].

Table 6. Wound healing processes and healing phases of the vehicle, PEO cream, and Madecassol® cream administered to rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>S</th>
<th>U</th>
<th>RE</th>
<th>FP</th>
<th>CD</th>
<th>PMN</th>
<th>NV</th>
<th>I</th>
<th>P</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>+++</td>
<td>++</td>
<td>−/+</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>−/+</td>
</tr>
<tr>
<td>PEO (0.5%)</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Madecassol®</td>
<td>+/+</td>
<td>−</td>
<td>++</td>
<td>+++</td>
<td>−/+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
</tbody>
</table>

Hematoxylin & Eosin stained sections were scored as mild (+), moderate (++) and severe (+++ for epidermal and/or dermal remodeling.


Figure 5. Histopathological view of wound healing and epidermal/dermal re-modeling tissue. Photomicrographs of sections of skin from rats stained with H&E (X40).

Skin microscopic image of (A) wound control rat (B) PEO cream formulation treated and (C) Positive control rat. S: Scab; re: Re-Epithelialization; S: Scab; F: Fibroblast; nv: Neovascularization; PMN: Neutrophil polymorphonuclear cells; Gs: Sebaceous gland; U: Ulcus.
Our findings suggest that PEO cream formulation might be useful for the fast healing of acute wounds by decreasing the inflammatory cells as well as by increasing connective tissue formation in the repaired tissue. Several studies have described that fibroblast cells contribute in the synthesis of collagen to contraction of the wound edge through wound healing steps and also have intense effects on keratinocyte production and deposition of basement proteins [5, 8].

3.4. Analgesic activity using acetic acid-induced writhing test

The *in vivo* anti-nociceptive activity of PEO was assessed through experimental models of animal pain stimuli using the writhing test. Figure 6 shows that the PEO showed significant and dose-dependent anti-nociceptive effects, reducing the number of writhes at all the three tested doses, which were similar to the reference drug (Phloroglucinol). An injection of acetic acid produced 77.8 ± 5.4 writhes in the vehicle control group. The EO at doses of 2, 20, and 200 µL/kg (i.p.) presented 22.87%, 28.53% and 32.13% of anti-nociceptive activity, respectively. The administration of the reference drug (Phloroglucinol, 80 mg/kg) resulted in a 28.53% inhibition in writhing when compared with the vehicle group.

![Figure 6. Anti-nociceptive activity of PEO in the acetic acid-induced writhing test *in vivo*.](image)

The effect of the PEO (2, 20 and 200 µL/kg, *i.p.*), and Phloroglucinol (Spasfon® 80 mg/kg, *i.p.*) in the acetic-acid-writhing-induced nociception test in mice. Values are expressed as mean ± SD (n = 6, per group); ns: not significant (*p* >0.05), * significantly different (*p*<0.05) from the control group, according to ANOVA, followed by Tukey’s test.

In our study, PEO was more active in decreasing the number of abdominal contortions at all tested doses. These results corroborate the possible anti-inflammatory mechanism of PEO resulting in its anti-nociceptive action. The anti-nociceptive effect of the major compounds found in the EO, menthol and menthone, are known [7]. Anti-nociceptive action has also been found in 1,8-cineole, and this effect is not antagonized by the administration of naloxone administration [32]. In addition, patients with neuropathic pain have been reported to also exhibited increased analgesic response induced by menthol [33].

4. CONCLUSION

Current findings largely support the anti-inflammatory, tissue remodeling, analgesic and wound-healing properties of peppermint essential oil. We suggest that PEO, with menthol as the major active
component, is a favorable candidate for use in skin care products with anti-inflammatory and wound-healing properties. The data of our research may offer an experimental base for additional systematic studies and clinical application of peppermint resources.

**Authors’ Contributions:** Study concept and design: SK, MNB and MAF; Experiments: SK, AB and MNB; Analysis and interpretation of data: SK, AB, MNB and WS; Drafting of the manuscript: MNB and WS; and Critical revision of the manuscript for important intellectual content: MNB and WS. All authors critically revised the Article for important intellectual content and approved the final version.

**Conflict of Interest:** The authors declare that they have no conflict of interest.

**Ethical Approval:** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All applicable national (Research and Development Center, Saidaal Pharmaceutical, Algiers, Algeria), and/or institutional guidelines (European Convention for the Protection of Animals Used for Experimental and Other Scientific Purposes n°2010/63/EU) for the care and use of animals were followed.

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**REFERENCES**


