Chemical composition and skin inflammation protective profile of pulegone rich essential oil of *Mentha arvensis* L.

Anil Kumar Maurya1,5, Janak Raj Bahl2, Anju Yadav3, Om Prakash1, Archana Saxena1, Anirban Pal1, Chandan Singh Chanotiya3, Feroz Khan3 & Dnyaneshwar Umrao Bawankule1,6*

1Molecular Bioprospection Division; 2CIMAP Research Centre, Pantnagar; 3Laboratory of Aromatic Plants and Chiral Separation; and 4Molecular and Structural Biology Division, CSIR-Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow-226 015, Uttar Pradesh, India. 
5Department of Biotechnology, Sai Nath University Ranchi–835 219, Jharkhand, India
6CSIR-Academy of Scientific and Innovative Research, New Delhi-110 025, India

Received 17 November 2016; revised 22 March 2018

The mint oil obtained from *Mentha arvensis* L. is an important ingredient of ointments, pain balms, lozenges, syrups and various cosmetic preparations. Using half sib progeny selection method, CSIR-CIMAP, Lucknow, India has developed a new chemotype (MAC/BS-11) of *Mentha arvensis*. Essential oil extracted from the aerial shoots of this chemotype (MaP) is rich in pulegone. Here, we conducted a blind pharmacological study using MaP to evaluate its therapeutic profile against skin inflammation using in vivo and in silico assays. Results of this study conclude that MaP significantly (*P* <0.05) reduced the skin thickness, ear weight and pro-inflammatory cytokines production in 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced mouse ear inflammation model. In vivo toxicity profiles indicate that it is safe for topical application on skin. Molecular docking study also revealed its strong binding affinity to the active site of the pro-inflammatory proteins. These findings suggest that MaP, a pulegone rich essential oil of *Mentha arvensis*, could be a potential therapeutic candidate for the treatment of skin inflammation.

**Keywords:** Itching, Mint oil, Skin irritation

Essential oils have received considerable attention as source of potential bioactive compounds in food processing and manufacturing due to their flavouring and preservatives properties and also in pharmaceutical industry for their pharmacological properties. *Mentha arvensis* L. (Lamiaceae), commonly called corn mint or wild mint, is well-known for its fragrance and flavour compositions. *M. arvensis* is native to the temperate regions of Asia and Europe and is used as a carminative, anti-spasmodic, antipeptic ulcer agent, and used in traditional medicine to treat indigestion, skin diseases, coughs and cold. Pharmacological studies have demonstrated that it improves cognitive performance and increases alertness apart from its antioxidant, antifungal, antibacterial, antiallergic and neuroprotective effects. *M. arvensis* has its unique importance among mint family because of its high content of menthol. Menthol mint oil is an important ingredient of pain balms, creams, ointments, syrups and various cosmetic preparations and has also been used to increase the shelf life of grains and edible products.

Skin plays a central role in host immunological defences. However, regulation of these defence mechanisms is also crucial, as inappropriate or misdirected immune activity is implicated in the pathogenesis of a large variety of acquired inflammatory skin disorders. Inflammatory skin diseases have a significant impact on the quality of life in human beings. It is estimated that globally 1-3% of the human population is exposed to a number of chemical mutagens and carcinogens, accidentally or occupationally and suffers psoriasis and the prevalence is even higher for chronic eczematous conditions. The skin diseases have three characteristic events common: an unbalance between the local immune response and its down regulatory mechanisms, an exaggerated inflammatory response and an increased epidermal turnover. During inflammation, the inflammatory region is infiltrated with mononuclear cells, such as monocytes, macrophages and lymphocytes, producing a wide range of Inflammatory mediators, such as interleukin-1
beta (IL-1β), interlukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) play important roles in inflammatory diseases. Extensive experimental evidence has shown that exposure of skin to 12-O-tetradecanoylphorbol-13-acetate (TPA) induces a pleiotropic tissue response encompassing a strong Inflammatory reaction similar to that observed in a number of skin diseases.

In the present study, the potential benefits of essential oil extracted from aerial shoots of new chemotype (MAC/BS-11) of Mentha arvensis L. which is rich in pulegone in skin inflammation. Here onwards, we refer to the pulegone rich essential oil as MaP. We conducted a blind pharmacological study using MaP with special emphasis on inflammation in (TPA)-induced skin inflammation in mice. We have further confirmed our finding in in silico system through molecular docking interaction study.

Materials and Methods

Plant material

A new chemotype (MAC/BS-11) of Mentha arvensis with unique aroma was developed through half sib progeny selection at research farm of CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India. The aerial shoots of this chemotype were collected at 110 days of plant growth for extraction of essential oil. The extracted essential oil was coded as MaP to conduct blind pharmacological study.

Isolation of essential oil from M. arvensis

The fresh plant material (200 g) of the selected chemotype was subjected to hydrodistillation in triplicate in a Clevenger-type apparatus for 3 h for extraction of essential oil. The essential oil was measured directly in extraction burette, and the oil yield was calculated as volume (mL) of essential oil per 100 g of fresh plant material. The oils were dried over anhydrous Na2SO4 and kept at 4°C prior to analysis.

Gas chromatography (GC) analysis

Column oven programme parameters

GC analysis was done using Supelcowax 10 capillary column (30 m x 0.32 mm i.d., film thickness is 0.25 μm) on Agilent 4890D gas chromatograph. The column oven was programmed from 40-120°C at the rate of 2.5°C/min with hold time of 9 min., then to 140°C at the rate of 2°C/min, and with 2 min hold in 250°C at the rate of 5°C/min with final hold time of 2 min. H2 gas was used as a carrier gas at constant column pressure 7 psi with split flow of 100 mL/min.; and the Injector and detector (FID) temperatures were 250°C.

Identification of essential oil constituents

Characterization was achieved on the basis of retention time, elution order, relative retention index using a homologous series of n-alkanes (C8-C25 hydrocarbons, Polyscience Corp. Niles IL), co-injection with standards in a GC-FID capillary column supplied from Sigma Aldrich and Fluka.

Experimental animals

Male BALB/c Mice (25-35 g) of 8-10 week old and female New Zealand white rabbits (1500±250 g body wt.) of two years old housed at 22±2°C (60–80% humidity) under a 12 h light/dark cycle and with access to food and water ad libitum, were used in these experiments that were performed during the light phase of the cycle. The animals were allowed to adapt to the laboratory for at least 2 h before testing and were used only once. Experiments were performed according to the ethical guidelines suggested by the Institutional Animal Ethics Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India.

In vivo study

TPA-induced mouse ear inflammation

Topical inflammation was induced in right ears of male BALB/c mice by the topical application of TPA (2.5 μg) dissolved in acetone (20 μL). A volume of 10 μL was delivered to both the inner and outer surface of the ear. MaP was diluted in acetone in the ratio of 0.1% and 1.0% for topical anti-inflammatory study. MaP was applied to the right ears at the dose of 20 μL/ear/time 30 min after TPA administration. For comparison, two other groups were treated with TPA (vehicle control and dexamethasone 200 μg/ear).

Ear edema and tissue weight measurement

Edema was expressed as the increase in ear thickness due to the inflammatory challenge. Ear thickness was measured before and after induction of the inflammatory response using a electronic digital micrometer (Aerospace Instruments). The micrometer was applied near the tip of the ear just distal to the cartilaginous ridges and the thickness was recorded. To minimize variation due to technique, a single investigator performed the measurements throughout any one experiment. At 6 h, when TPA-induced inflammation was maximal, animals were euthanized.
by ether asphyxiation and 1.0 cm diameter punch of ear tissue wet weight was taken for quantification of inflammatory mediators from tissue homogenate. These tissues were quickly placed in a beaker containing ice-cold Tris- hydrochloride buffer (pH 7.4) and minced into small pieces on ice and homogenized immediately in tissue homogenizer (Pro Scientific Inc, USA). The homogenate (5%) was frozen and stored at –80°C until used for biochemical estimation. The tissue homogenate was processed for estimation of pro-inflammatory cytokines.

Quantification of pro-inflammatory cytokine

Expression pro-inflammatory cytokines (TNF-α and IL-6) were quantified from tissue homogenate using commercially available mouse specific enzyme immune assay (EIA) kits (BD Biosciences, USA) as per the manufactures instruction.

Primary skin irritation

Primary skin irritation study was performed for the topical application of MaP in four healthy New Zealand white rabbits (1500±250 g body wt.) as per the approved protocol by the Institutional Animal Ethical Committee of CSIR-CIMAP, Lucknow, India. MaP (1%) was applied to the back skin of rabbits on one inch square area. Normal saline (1%) was applied as vehicle control at opposite side of skin. Observations were made at 1, 4, 24, 48 and 72 h to assess individual erythema and edema. The primary irritation index (PII) was determined using the following formula; PII = Test site score – Control site score

Grading score scale for primary skin irritation test in rabbit is depicted as Table 1.

<table>
<thead>
<tr>
<th>Skin Irritation Score</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythema formation</td>
<td></td>
</tr>
<tr>
<td>No erythema</td>
<td>0</td>
</tr>
<tr>
<td>Very slight erythema</td>
<td>1</td>
</tr>
<tr>
<td>Well defined erythema</td>
<td>2</td>
</tr>
<tr>
<td>Moderate to severe erythema</td>
<td>3</td>
</tr>
<tr>
<td>Severe erythema</td>
<td>4</td>
</tr>
<tr>
<td>Oedema formation</td>
<td></td>
</tr>
<tr>
<td>No edema</td>
<td>0</td>
</tr>
<tr>
<td>Very slight oedema</td>
<td>1</td>
</tr>
<tr>
<td>Moderate oedema</td>
<td>2</td>
</tr>
<tr>
<td>Moderate oedema</td>
<td>3</td>
</tr>
<tr>
<td>Severe oedema</td>
<td>4</td>
</tr>
</tbody>
</table>

In silico study

Molecular docking experiment

The two dimensional structures of the molecules were constructed with the ChemDraw Ultra. Energy minimization of the compounds was performed with Discovery studio 3.5. The 3D chemical structure of known dexamethasone was retrieved from the PubChem compound database at NCBI (http://www.pubchem.ncbi.nlm.nih.gov). Crystallographic 3D structures of target proteins (TNF-α: (PDB: 1A8M) and IL6: (PDB: 1N26) were retrieved from the Brookhaven Protein Databank (http://www.pdb.org)17. Hydrogen atoms were added to the protein targets to achieve the correct ionization and tautomeric states of amino acid residues such as His, Asp, Ser, and Glu. Molecular docking of the compounds against selected target was achieved using the ‘AutoDock Vina’18.

Statistical analysis

Data were expressed as Mean±SEM. For statistical analysis, one-way ANOVA followed by Tukeys test was used. Probability (P) values less than 0.05 were considered significant.

Results

Composition of essential oil isolated from M. Arvensis

Constituents of leave essential oil of Mentha arvensis are listed in Fig. 1. A total of 92.3% constituents were identified. Monoterprenoids dominate the essential oil. Pulegone (64%) was found as an exclusive component of the oil. Other identified constituents were α-pinene (0.3%), β-pinene (0.4%), sabinene (0.1%), β-myrcene (0.4%), limonene (1%), menthone (2.5%), isomenthone (0.6%), menthyl acetate (5.3%), neomenthol (1%), menthol (15.3%) and piperitone (1.4%). The GC chromatogram is depicted as Fig. 1.

In vivo study

Ear thickness and ear weight

Ear edema was observed in all TPA-treated animals at 6 h after treatment. In animals treated with vehicle (Acetone), initial ear thickness was 0.24±0.05 mm. Ear thickness increased to 0.48±0.04 mm by 6 h after TPA treatment. MaP treated experimental mice showed significant reduction of ear edema when compared with vehicle treated mice. MaP and dexamethasone treatment exhibited the significant reduction in ear edema when compared with the mice treated with vehicle (Fig. 2A). Edema was also indicated by changes in ear punch masses at 6 h, and...
the treatment effects were similar to the changes in ear thickness. Ear punch biopsy weight at 6 h was significantly lower in MaP and dexamethasone treated mice groups compared to the TPA treated vehicle control group (Fig. 2B).

Pro-inflammatory cytokines

The mice treated with vehicle in TPA-induced ear inflammation showed the significant ($P < 0.05$) increased in pro-inflammatory cytokines (TNF-α and IL-6) production when compared with the untreated mice treated with vehicle, an acetone alone whereas MaP showed the inhibition of pro-inflammatory cytokines levels. Significant ($P < 0.05$) inhibition of pro-inflammatory cytokines production was observed in 1.0% MaP and dexamethasone treated mice (Fig. 3).

Primary skin irritation

Skin irritation test was conducted to determine the primary skin irritation 72 h after the application of MaP on rabbit skin. Significant erythema and edema formation were observed in MaP treatment site compared to vehicle treated site (Table 2). Pigmentation, blood oozing and rough skin were also not observed in both the control and treatment site. According to Federal Hazardous Substances Act (FHSA) regulations, a material with a PII of less than 5.00 is generally not considered a primary irritant to the skin. The PII result concluded that the application of MaP was not irritant to the rabbit skin.
In silico study

In this study, molecular interaction of pulegone with TNF-α and IL-6 has been reported. The binding affinity obtained in the docking experiment allowed the activity of the ‘pulegone’ to be compared to that of the standard anti-inflammatory compound dexamethasone (Table 3). The docking results were analyzed with binding pocket containing the most stable poses of dexamethasone. Pulegone showed binding affinity (negative docking energy) against TNF-α (−5.9 kcal/mol) and IL-6 (−5.3 kcal/mol). When we compared the binding site pocket amino acid residues, we found in case of TNF-alpha that no amino acids were in share with dexamethasone. While, in case of IL-6, PRO-46, LEU-69, SER-72, LEU-90, VAL-91, ASP-92, SER-122, LEU-123, THR-124 amino acids were in share with dexamethasone.

Discussion

Using half sib progeny selection method, the CSIR-CIMAP, Lucknow has developed a new chemotype (MAC/BS-11) of Mentha arvensis. The gas chromatographic study revealed that the essential oil extracted from aerial shoots of this chemotype is rich in pulegone. Pulegone rich essential oil was coded as MaP and we conducted blind pharmacological study with special emphasis on skin inflammation. Treatment of most inflammatory skin diseases are dominated by topical or oral corticosteroids. They exert some side effects on skin, including immunosuppression, hyperglycemia. Essential oil has generated considerable interest in chemical and pharmaceutical industries, mainly in cosmetics to overcome the side effects of topically used anti-inflammatory drugs. In the in vivo study, we examined the therapeutic role of

Table 3 — Molecular Docking study of pulegone on inflammatory target protein: TNF-α and IL-6

<table>
<thead>
<tr>
<th>Compound</th>
<th>TNF-α (PDB: 1A8M)</th>
<th>IL-6 (PDB: 1N26)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Binding affinity</td>
<td>Binding pocket</td>
</tr>
<tr>
<td></td>
<td>(Kcal/mol)</td>
<td>amino acids</td>
</tr>
<tr>
<td>Common residues</td>
<td>No common residues found</td>
<td>No common residues found</td>
</tr>
</tbody>
</table>
topical application MaP on ear thickness, ear weight and production of pro-inflammatory cytokine in skin inflammation induced by topical application of 12-O-tetradecanoylphorbol-13-acetate (TPA). The results of study showed that the topical application of TPA significantly increased the ear thickness, ear weight and pro-inflammatory cytokine production (TNF-α and IL-6) when compared to the untreated mice. The induction of ear skin inflammation in mice by TPA represents a promising animal model for elucidating the mechanism of clinical dysfunction and for evaluating the efficacy of topical anti-inflammatory agents.

Increased skin thickening is often the first hallmark of skin irritation and local inflammation. It is widely recognized that the secretions of cytokines by macrophages in response to injury are key mediators of the inflammatory response. Topical application of MaP is able to reduce the ear thickness, ear weight and pro-inflammatory cytokine production when compared to the vehicle treated mice and primary skin irritation study in rabbits revealed that MaP is safe for topical application on skin. Previous reports are also supporting to our hypothesis that essential oil extracted from aromatic plants exhibiting the anti-inflammatory property against the skin inflammation. The monoterpenic compound pulegone present in many aromatic plants has shown significant suppression of pro-inflammatory cytokines (IL-6, IL-8, and TNF-α) through the cytokine-cytokine receptor interaction (CCRI), MAPK, and the Toll-like receptor (TLR) signaling pathways. The anti-inflammatory activity of MaP, an essential oil extracted from Mentha arvensis may be due to high content of pulegone in essential oil.

In vivo anti-inflammatory profile of MaP was further confirmed by molecular docking study. The aim of the molecular interaction study was to explore the interaction of pulegone, a one of the major compound present in MaP with pro-inflammatory cytokine receptors. The interaction study was compared with dexamethasone, a standard steroidal anti-inflammatory drug. Molecular interaction study of pulegone with pro-inflammatory cytokines through docking showed high binding affinity i.e. low docking energy with pro-inflammatory cytokines. Earlier reports also conclude that the molecules having high binding affinity with targeted protein exhibited therapeutic efficacy.

Conclusion
These results demonstrate that topical application of MaP, the essential oil extracted from aerial shoots of new chemotype (MAC/BS-11) of Mentha arvensis, is an effective anti-inflammatory treatment in the TPA-induced skin inflammation. This study suggests the suitability of MaP as a drug like candidate for further studies to obtain a suitable prototype drug for chronic skin inflammatory disorders.

Acknowledgment
We acknowledge the CSIR-Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Lucknow-226 015, India for financial support through institutional project (OLP-17).

References


