Heliyon 10 (2024) e31922

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Unlocking the combined action of *Mentha pulegium* L. essential oil and Thym honey: *In vitro* pharmacological activities, molecular docking, and *in vivo* anti-inflammatory effect

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ARTICLE INFO

Keywords:
Human and medicine
Cardiovascular disease
Preventive medicine
Synergistic effect
Anti-inflammatory
Dermatoprotection
Antioxidant

ABSTRACT

Mentha pulegium L., a plant widely embraced for its therapeutic properties by populations worldwide, including Morocco, has long been recognized for its potential in treating various ailments. This study aims to comprehensively evaluate the antioxidant, anti-inflammatory, and dermatoprotective properties of essential oil derived from *M. pulegium*, and thyme honey as well as their combined effects. To unravel the chemical composition, a rigorous GC-MS analysis was conducted. Subsequently, we examined their antioxidant potential through three distinct assays: DPPH●, hydrogen peroxide assay, and xanthine oxidase assay. The anti-inflammatory properties were scrutinized through both in vitro and *in vivo* experiments. Simultaneously, the

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https://doi.org/10.1016/j.heliyon.2024.e31922
Received 25 February 2024; Received in revised form 22 May 2024; Accepted 23 May 2024
Available online 28 May 2024

dermatoprotective efficacy was investigated in vitro by evaluating tyrosinase inhibition. Our findings revealed that pulegone constitutes the predominant compound in *M. pulegium* essential oil (MPEO), constituting a remarkable 74.82 % of the composition. Significantly, when the essential oil was combined with thym honey, it exhibited superior anti-inflammatory and dermatoprotective effects across all *in vivo* and in vitro tests. Moreover, our in silico molecular docking analysis hinted at the potential role of cyclohexanone, 3-methyl, an element found in the MPEO, in contributing to the observed outcomes. While this study has unveiled promising results regarding the combined in vitro, *in vivo* and in silico biological activities of the essential oil and honey, it is imperative to delve further into the underlying mechanisms through additional experimentation and alternative experimental methods. Understanding these mechanisms in greater detail will not only enhance our comprehension of the therapeutic potential but also pave the way for the development of innovative treatments and applications rooted in the synergy of these natural compounds. Furthermore, it would be advantageous to test different possible combinations using experimental design model. Moreover, it would be better to test the effect of single compounds of MPEO to clearly elucidate their efficiency. MPEO alone or combined with thyme honey may be a useful for the development of novel biopharmaceuticals.

1. Introduction

For centuries, people have recognized the therapeutic benefits of plants, and plant-based medicines have proven effective in treating various illnesses [1,2]. These medications are typically administered as plant-derived mixtures or potent extracts, preserving their essential components. However, many global health challenges, such as diabetes, chronic inflammation, infections, degenerative diseases, and cancer, still lack adequate solutions within modern medicine [3,4]. Nevertheless, the process of exploring new drugs is complex and requires a thorough evaluation of different aspects of natural and synthetic compounds, including their pharmacokinetics, safety, and effectiveness, during the screening phase [5,6]. While modern medicine has taken precedence over traditional practices in treating and managing human ailments [3], there has been a recent surge in the utilization of herbal remedies for health enhancement and ailment treatment across numerous countries, especially in developing societies [7]. Natural products present a promising reservoir of therapeutic elements that could lead to innovative drug discoveries [8–11], offering potential molecules with diverse pharmacological and biological attributes beneficial for addressing various human and animal diseases [12,13].

Remarkably, natural entities, especially essential oils (EOs), stand as an auspicious wellspring of biologically dynamic compounds. EOs showcase significant biological and pharmacological attributes, encompassing different biological functionalities [14,15]. These precious EOs predominantly reside within medicinal and aromatic plants, substantiating pivotal physiological roles within the plant's lifecycle. Furthermore, honey, a biogenic creation of bees, emerges as an additional pivotal repository of biologically potent molecules with their origins entrenched in medicinal plants. Indeed, honey compositions can be discerned in a contextual manner, reflective of the botanical sources the bees forage upon, such as *Eucalyptus*, *Thymus*, and *Origanum*.

Recent scholarly investigations attest that honey encompasses a plethora of bioactive molecules, particularly flavonoids and phenolic acids, thereby endowing it with substantial biological and pharmacological attributes. Meanwhile, Imtara et al. comprehensively explored the antioxidant properties as well as wound-healing ramifications exhibited by Tulkarm honey and *Thymus vulgaris* honey [16]. In a parallel vein, Elamine et al. undertook an examination of the physicochemical traits and antioxidant capabilities inherent in Moroccan Zantaz honey [17]. In recent research, Assaggaff and his team investigated the individual and combined effects of Eucalyptus globulus essential oil and honey as agents with anti-inflammatory, antioxidant, dermatoprotective, and antimicrobial properties [18]. El-Guendouz et al. also conducted an assessment, exploring the antioxidant and diuretic roles of both Capparis spinosa honey and propolis [19]. Likewise, Botoub et al. [20] focused their work on examining the antioxidant characteristics and enzyme inhibitory capabilities of two different types of Euphorbia honey [20]. Given honey's classification as a consumable, its inherent biological and pharmacological traits bestow a prophylactic modality against a spectrum of maladies [21,22]. Conversely, the amalgamation of essential oils (constituting inedible and modestly soluble molecules) with honey could potentially ameliorate essential oil solubility and enhance their intestinal absorptivity [23]. In this context, the exploration of synergistic amalgamations between discrete essential oil specimens and honey has recently captured scientific attention [24].

It is crucial to note, however, that the scope of research pertaining to the anti-inflammatory properties of Moroccan honey remains relatively limited. It is noteworthy that research investigating the synergistic interplay between honey and essential oils (EOs), has been relatively scarce [25–27]. However, none of these studies explored the potential synergistic impact of combining honey with EOs to address inflammation. The current research is to evaluate the anti-inflammatory properties inherent in the amalgamation of *M. pulegium* essential oil (MPEO) and Moroccan thyme honey. This investigation is directed towards evaluating for the first time the impact of the combined treatment of *M. pulegium* essential oil (MPEO) and Thym honey on two distinct types of edemas induced within Wistar rats, also its in vitro anti-inflammatory, antioxidant and the dermatoprotective abilities. Consequently, this study serves as a pioneering effort, being the first to delve into the *in vivo* anti-inflammatory and the in vitro anti-inflammatory, antioxidant, and the dermatoprotective capabilities of a concoction comprised of thyme honey and peppermint essential oil.

2. Material and methods

2.1. Chemicals and reagents

Acarbose, quercetin, ascorbic acid, 2,2′-diphenyl-1-picrylhydrazyl (DPPH●), H_2O_2, xanthine oxidase (XO), NaCl, 5-Lipoxygenase (5-LOX), linoleic acid, ethanol, methanol, quercetin, tyrosinase and L-DOPA were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). All other elements were of analytical grade.

2.2. Essential oil extraction and honey samples

Honey samples of *Thymus vulgaris* L. leaves were provided by beekeepers in Taza (in March 2023), while the plant material of *Mentha pulegium* L. was collected from its natural habitat in the Taounate region (34° 32′ 09″ N, 4° 38′ 24″ W) in June 2023. The botanical identification was performed at the Scientific Institute, Mohammed V University in Rabat, under a voucher specimen RAB 113370. The extraction procedure of *M. pulegium* essential oil was done using hydro-distillation technique in a Clevenger-type device. Briefly, 200 g of the dry plant was placed in a balloon filled to 2/3 with distilled water; the whole was brought to boil for 170 min. The obtained oil was recovered and conserved at appropriate conditions (4 °C), pending experimental uses. The honey samples were kept at 25 °C, and the experiments were performed at the 3 months of collection date.

2.3. Chemical analysis of volatile compounds

The composition of *M. pulegium* essential oil (MPEO) was determined using gas chromatography coupled with mass spectrometry (GC/MS) analysis following the methods outlined by Mekkaoui et *al.* [28]. To provide a summary, we utilized an HP6890 GC instrument connected to an HP5973 MS, featuring a 5 % phenylmethyl silicone HP−5 MS capillary column (30 m in length, 0.25 mm in diameter, and a film thickness of 0.25 μm) for the GC analysis. The column temperature was initially set at 50 °C for 5 min and then increased to 200 °C at a rate of 4 °C per minute. Helium was used as the carrier gas at a flow rate of 1.5 mL/min, employing a split mode (flow rate: 112 mL/min, split ratio: 1/74.7). The column was held at the final temperature for 48 min, while the injector and detector temperatures were maintained at 250 °C. The operation of the instrument was controlled by the computer system "HP ChemStation," which managed its functions and allowed us to monitor the progress of chromatographic analyses. We manually injected diluted samples (1/20 in methanol) of 1 μL. The mass spectrometer operated with an ionization voltage of 70 eV, an ion source temperature of 230 °C, and a scanning range of 35–450 (*m/z*). To determine the qualitative and quantitative composition of the various compounds, we relied on the percentage area of each peak in the sample compounds and confirmed their identities through reference to the NIST/EPA/NIH mass spectral library version 2.0 (built on July 1, 2002).

2.4. PASS, pharmacokinetics, and toxicity predictions

In this specific investigation, we employed the and pkCSM web servers to analyze the physicochemical characteristics, drug similarity, and pharmacokinetic properties of the compounds [29,30]. To assess toxicity levels, we used the Protox II webserver to provide information on LD_{50} values, toxicity classification, organ toxicity and various toxicological endpoints [31]. Employing these methods and tools yielded significant insights into the potential therapeutic uses and potential adverse effects associated with the primary chemical compounds identified in MPEO.

2.5. In vitro antioxidant potential

The antioxidant capacity of the MPEO, thyme honey and their mixture was evaluated through three well-established in vitro assays: DPPH●, H_2O_2, and xanthine oxidase (XO) tests, following the protocols established by the research team in previous studies [32–34]. Each experiment was repeated three times, using allopurinol and ascorbic acid as positive controls. For each test, the concentrations of sample that resulted in a 50 % inhibition (IC_{50}) were determined and expressed in μg/mL units (concentrations between 2×10^3 and 0.49 μg/mL).

2.6. Anti-inflammation assays

2.6.1. In vivo anti-inflammatory activity

The research involved 72 adult Wistar rats, with weights ranging from 200 to 250 g and ages between 3 and 5 months. These rats were sourced from the central animal facility at the Faculty of Medicine and Pharmacy in Rabat. The animal housing facility was kept at a constant temperature of 20 °C, with a lighting cycle of 12 h of light followed by 12 h of darkness [35,36]. The rats were divided into six groups, each consisting of six rats. These groups were structured to mimic the same anti-inflammatory test models as described in Mekkaoui et al. study [37].

a. For the carrageenan-induced paw edema test

In all experimental groups, edema was induced by injecting 0.05 mL of carrageenan, which had been diluted to a 1 % concentration

in a 9 % NaCl solution, into the plantar aspect of the left paw of the rats. To assess changes in volume for both legs of each rat, we utilized a LE 7500 digital plethysmometer, controlled by SeDaCOM software. Measurements were taken just before the carrageenan injection, as well as at 1 h and 30 min, 3 h, and 6 h after the induction of edema. The untreated right hind paw served as the control in this experiment [38].

- **Group I:** Rats in this group were given daily feedings of TH for two months (10 mg/kg).
- **Group II:** Rats in this group were given daily feedings of MPEO for two months (10 mg/kg).
- **Group III:** This group received daily treatment for two months with a combination of thyme honey (TH) and MPEO (10 mg/kg).
- **Group IV:** The positive control group was administered indomethacin (10 mg/kg) 1 h before the induction of edema.
- **Group V:** Control

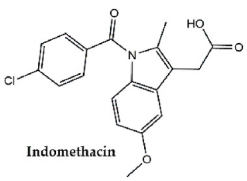
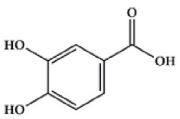
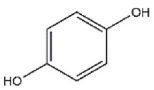
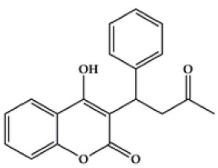
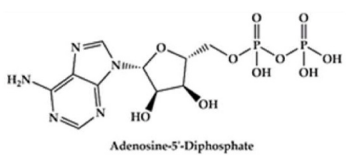
b. For experimental trauma-induced hind paw edema test

Inflammation was induced by applying a 50 g weight to the back of the left hind paw of all the animals, following the method described in Ref. [39]. We evaluated edema and computed the edema inhibition percentage using the same procedures as in the previous experiment. The anti-inflammatory effectiveness was quantified as the percentage of edema thickness inhibition (% Inh) in treated animals compared to the control group, with VL representing the volume of the left paw and VR representing the volume of the right paw.

$$\%Inh = \frac{(Mean\ (V_L\ -\ V_R))\ control\ -\ (Mean\ (V_L\ -\ V_R))\ treated}{(Mean\ (V_L\ -\ V_R))\ control} \times 100$$

During the study, three distinct groups were established for experimentation. Group I was administered daily doses of TH for two-month duration (10 mg/kg). Group II administered daily doses of MPEO for two-month duration (10 mg/kg). In contrast, Group III was subjected to daily treatment for a period of two months, receiving a mixture of thyme honey (TH) and the essential oil of *M. pulegium* (MPEO) (10 mg/kg). Group IV was designated as the positive control group and was provided with indomethacin (20 mg/kg) 1 h

Table 1
Molecular docking simulation parameters.

Proteins/Resolution	PDB ID	Grid Box Size	Grid Box Center	Native Ligand/Inhibitor	Ref.
Active Site of COX-2/3.00 Å	**1DDX**	size_x = 40 size_y = 40 size_z = 40	center_x = 42.270 center_y = 33.808 center_z = 36.342	 Indomethacin	[45, 46]
TIR domain of Toll-like receptor 6 (TLR6)/2.20 Å	**4OM7**	size_x = 40 size_y = 40 size_z = 40	center_x = 10.36 center_y = 5.678 center_z = −18.687		[47]
Lipoxygenase/2.10 Å	**1N8Q**	size_x = 40 size_y = 40 size_z = 40	center_x = 22.455 center_y = 1.2930 center_z = 20.362	 Protocatechuic acid	[43]
Tyrosinase/2.20 Å	**5I3B**	size_x = 40 size_y = 40 size_z = 40	center_x = 17.728 center_y = 96.860 center_z = 32.239	 Hydroquinone	[48]
CYP2C9/2.55 Å	**1OG5**	size_x = 40 size_y = 40 size_z = 40	center_x = −19.823 center_y = 86.686 center_z = 38.275	 Warfarin	[49]
NADPH Oxidase/1.80 Å	**2CDU**	size_x = 40 size_y = 40 size_z = 40	center_x = 18.997 center_y = −5.777 center_z = −1.808	 Adenosine-5'-Diphosphate	[50]

before the edema induction process.

2.6.2. In vitro dermatoprotective and anti-inflammatory activities

The evaluation of 5-Lipoxygenase (5-LOX) inhibition was conducted through in vitro testing. To assess the lipoxygenase inhibitory activity of thyme honey and MPEO, we followed a method based on the oxidation of linoleic acid at 234 nm, as described by Andrade et al. [40], with some modifications. In brief, the procedure involves pre-incubating each essential oil (20 μL, dissolved in Tween 80) (concentrations between 1×10^3 and 0.49 mg/mL) along with 5-LOX from Glycine max (100 U/mL, 20 μL) in 200 μL of phosphate buffer (0.1 M, pH 9) at room temperature for 5 min. The reaction was initiated by adding 20 μL of linolenic acid (4.18 mM in ethanol) and monitored for 3 min at 234 nm. The reported results represent the average value ± standard error of the mean (SEM) derived from three independent experiments, each conducted in triplicate. Quercetin was used as the positive control in this experiment. For the honey sample, 5 g were diluted in 10 mL of distilled water on the day of the assay and thoroughly mixed for 5 min using a vortex. The assay involved the utilization of 150 μL of this prepared solution [20]. Furthermore, tyrosinase inhibitory potential was conducted to examine the dermatoprotective effect of MPEO adopting to the previous works [41].

2.7. Molecular docking

The molecular docking analysis was conducted following previously established methods [42–44]. Four target proteins related to anti-inflammatory effects, including lipoxygenase from *Glycine* max (LOX, PDB ID: 1N8Q) [43], the Active Site of COX−2 from *Mus musculus* (PDB: 1DDX) [45,46], the Human TIR domain of Toll-like receptor 6 (TLR6) (PDB: 4OM7) [47], and Tyrosinase from *Bacillus megaterium* (PDB ID: 5I3B) for dermatoprotective evaluation [48], were selected. Additionally, two proteins associated with antioxidant properties, Human CYP2C9 (PDB ID: 1OG5) [49], and NADPH Oxidase from *Fructilactobacillus sanfranciscensis* (PDB ID: 2CDU) [50], were chosen (Table 1), grid box sizes were retrieved from relevant literature. Ligands derived from the essential oil of *M. pulegium* were prepared by obtaining sdf (3D conformer) files from PubChem database, and converting them to pdb files using PyMol software. The 3D crystallographic structures of the selected proteins were retrieved from the Protein Data Bank website using their respective PDB IDs. These structures were analyzed using Discovery Studio 4.1 software, which enables a comprehensive examination of protein structures. Prior to docking, protein preparation wa carried out as depicted in previous works [42–44]. The binding energies (ΔG) of the ligand complexes were determined and expressed in Kcal/mol. Visual representations of 2D and 3D molecular interactions were generated and analyzed using Discovery Studio 4.1 software to identify binding interactions between proteins and ligands. Furthermore, prior research was consulted to identify relevant target proteins, shedding light on potential mechanisms of action of the compounds found in both essential oils.

2.8. Statistical analysis

The results were presented as the average value ± the standard deviation, calculated from three separate analyses for each measurement. To assess differences in the observed parameters among the samples, we employed analysis of variance (ANOVA). All statistical data processing was conducted using the SPSS software package.

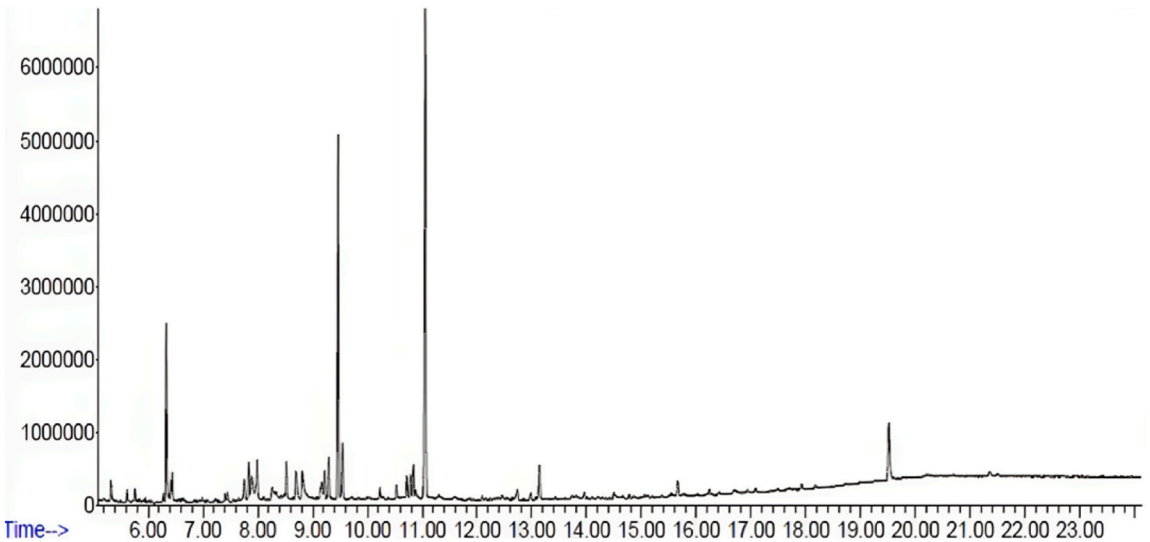


Fig. 1. Chromatogram of gas chromatography (GC) analysis of MPEO.

3. Results and discussion

3.1. Chemical composition of MPEO

The chemical constituents of MPEO were determined using GC-MS analysis (Fig. 1). The results are summarized in Table 2. It represents the percentage of each volatile compound, elution order, the chemical formula, structural subclass, and retention index (RI). A total of 15 components were characterized in MPEO (Fig. 2) (representing 96.00 % of total oil), belonging particularly to oxygenated monoterpenes (88.8 %), with a low rate of monoterpene hydrocarbons (4.7 %). Pulegone is the main identified compound in MPEO with a high concentration of 73.8 %. Other compounds, including carvone (6.0 %), dihydrocarvone (4.6 %), menthone (2.1 %) and *p*-mentha-3,8-diene (2.0 %) were also detected in interesting amounts.

Similar chemical constituents were identified for Algerian, Turkish and Iranian *M. pulegium* EO samples, which highly rich in pulegone with a respective content of 70.5 %, 71.4 % and 49.0 % [53–55]. Nevertheless, other investigations which focused on the volatile components of *M. pulegium* have detected other chemotypes, including menthol chemotype, piperitone and piperitenone with a small concentration of pulegone [56]. One of main factors responsible for these chemical variations may be polymorphism.

Pulegone was also identified as a major compound with different percentages in Moroccan *M. pulegium* samples. In fact, this compound may attaint an amount of more than 80 %. Moreover, a comparative study carried out in Marrakech region has shown the predominance of pulegone (57,0–62.8 %), followed by menthone (9.5–15.1 %) and D-limonene (5,0–7.0 %) [57]. *M. pulegium* EO from Rabat region was characterized by the presence of pulegone (73.00 %), menthone (8.63 %), and α-pinene (1.70 %) [58]. Other compounds were identified as lowest percentages <1.0 %. Furthermore, several investigations carried out in the different areas worldwide have revealed pulegone as the main component, in Bulgaria pulegone (45.5 %); from Uruguay pulegone (73.4 %); from Egypt pulegone (43.5 %); from Tunisia pulegone (41.8 %), [6]; and from Algeria pulegone (38.815 %), menthone (19.240 %), piperitenone (16.528 %) [59,60].

Interestingly, Domingues and Santos [61] in their comprehensive review have stated that *M. pulegium* EOs show significant chemical fluctuation, varying both in nature and number of constituents. This difference are potentially related to plant polymorphism [54], but can also be attributed to seasonal variation [62], and geographical origin [63,64]. In addition, the authors have also indicated that the plant section and maturity, extraction method used as well as drying the plant material may slightly affect the chemical constituents of mint EOs [65]. These events may modulate and/or alter the key enzymatic pathways implicated in the synthesis of different volatile components.

3.2. Physiochemical and pharmacokinetic properties (ADME) of MPEO

3.2.1. Physicochemical characteristics of MPEO's compounds

In contemporary drug discovery research, there has been notable increase in the utilization of various approaches. Nevertheless, the challenge of unsuccessful identification of new drug candidates persists, primarily due to issues related to their pharmacokinetics or bioavailability [66,67]. Within the realm of pharmacology, Computer-Aided Drug Design (CADD) emerges as an efficient tool that

Table 2
Phytoconstituents identified from *M. pulegium* EO using GC-MS analysis.

No.[a]	Compounds[b]	Molecular formula	Chemical class	RI[c]	RI lit[d]	%Relative peak area
						M. pulegium EO
1	*α*-Pinene	$C_{10}H_{16}$	Monoterpene hydrocarbons	937	939	0.7
2	Cyclohexanone-3-methyl	$C_7H_{12}O$	Other	952	952	0.4
3	*ß*-Pinene	$C_{10}H_{16}$	Monoterpene hydrocarbons	974	980	0.5
4	Myrcene	$C_{10}H_{16}$	Monoterpene hydrocarbons	992	991	0.1
5	Octanol-3	$C_8H_{18}O$	Other	995	993	2.1
6	2-Carene	$C_{10}H_{16}$	Monoterpene hydrocarbons	1003	1001	tr
7	Limonene	$C_{10}H_{16}$	Monoterpene hydrocarbons	1030	1031	1.4
8	*p*-Mentha-3,8-diene	$C_{10}H_{16}$	Monoterpene hydrocarbons	1071	1072	2.0
9	Menthone	$C_{10}H_{18}O$	Oxygenated monoterpenes	1150	1154	2.1
10	Pinocarvone	$C_{10}H_{14}O$	Oxygenated monoterpenes	1166	1162	1.0
11	Isomenthol	$C_{10}H_{20}O$	Oxygenated monoterpenes	1182	1182	0.3
12	Menthol	$C_{10}H_{20}O$	Oxygenated monoterpenes	1171	1158	0.6
13	Dihydrocarvone	$C_{10}H_{16}O$	Oxygenated monoterpenes	1193	1193	4.6
14	Pulegone	$C_{10}H_{16}O$	Oxygenated monoterpenes	1236	1237	**73.8**
15	Carvone	$C_{10}H_{14}O$	Oxygenated monoterpenes	1240	1242	6.0
Total identified %						**96.0 %**
Monoterpene hydrocarbons						4.7
Oxygenated monoterpenes						88.8
Other						2.5

[a] In order of elution on HP-5Ms.
[b] Compounds identified based on RI and MS.
[c] Retention index calculated from alkanes series on HP-5 MS capillary column (C9–C31).
[d] Retention indices from literature [51,52].

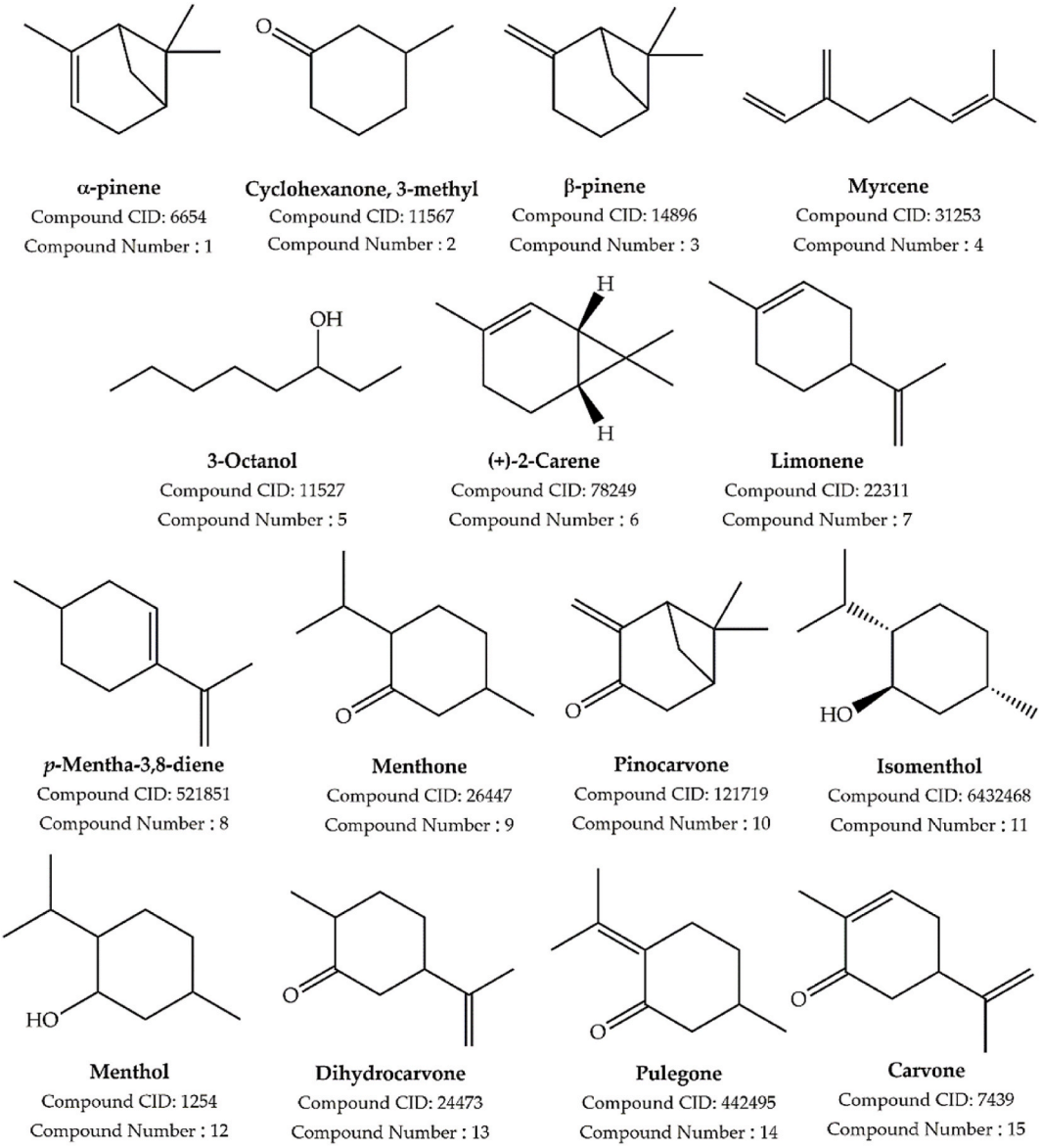


Fig. 2. Phytochemical structures of the identified compounds in of *M. pulegium* EO.

can save time and resources. This study, in particular, employed in silico pharmacokinetic analysis (Absorption, Distribution, Metabolism, and Excretion) as an alternative method to explore the bioactive compounds present in the studied essential oil (MPEO). This approach enables the prediction of a wide range of parameters critical for drug discovery.

Table 3 provides an analysis of the physicochemical properties and drug-likeness evaluation for 15 key compounds identified in MPEO. This comprehensive assessment covers various parameters, including Hydrogen-Bond Donors, Hydrogen-Bond Acceptors, Topological Polar Surface Area, distribution coefficient, and solubility. Furthermore, we applied both Lipinski's Rule of Five and the Veber filter to assess their suitability as potential drug candidates.

The findings indicate that all 15 compounds exhibit moderate to high solubility and meet the criteria outlined by Lipinski's Rule of Five and the Veber filter. In general, a majority of these compounds demonstrate favorable solubility levels and moderate to high permeability across the blood-brain barrier, suggesting their potential for brain penetration. These results suggest that these specific compounds hold promise for therapeutic applications owing to their advantageous physicochemical properties and alignment with drug-likeness criteria. Nonetheless, further in vitro and *in vivo* investigations are imperative to validate their efficacy and safety.

3.2.2. Pharmacokinetic properties of the identified compounds

Regarding absorption characteristics, the compounds exhibit favorable solubility, a crucial factor influencing the availability and effectiveness of drugs. To assess the likelihood of these molecules being absorbed through the intestinal barrier, we employed in silico

Table 3
Physiochemical and drug-likeness analysis of the compounds identified in MPEO. **(1)** α-Pinene, **(2)** Cyclohexanone, 3-methyl, **(3)** β-Pinene, **(4)** Myrcene, **(5)** 3-Octanol, **(6)** (+)-2-Carene, **(7)** Limonene, **(8)** *p*-Mentha-3,8-diene, **(9)** Menthone, **(10)** Pinocarvone, **(11)** Isomenthol, **(12)** Menthol, **(13)** Dihydrocarvone, **(14)** Pulegone, **(15)** Cavone.

Compound Number	HBD	HBA	TPSA (Å^2)	Log Po/w (WLOGP)	Log S (SILICO S-IT)	Lipinski's Rule of Five	Veber filter
1	0	0	0.00	3.00	−2.23 (+++)	Yes; 1 violation: MLOGP>4.15	Yes
2	0	1	17.07	1.77	−1.53 (+++)	Yes; 0 violation	Yes
3	0	0	0.00	3.00	−2.48 (+++)	Yes; 1 violation: MLOGP>4.15	Yes
4	0	0	0.00	3.48	−2.42 (+++)	Yes; 0 violation	Yes
5	1	1	20.23	2.34	−2.12 (+++)	Yes; 0 violation	Yes
6	0	0	0.00	3.00	−2.23 (+++)	Yes; 1 violation: MLOGP>4.15	Yes
7	0	0	0.00	3.31	−2.26 (+++)	Yes; 0 violation	Yes
8	0	0	0.00	3.31	−2.26 (+++)	Yes; 0 violation	Yes
9	0	1	17.07	2.65	−2.17 (+++)	Yes; 0 violation	Yes
10	0	1	17.07	2.18	−2.38 (+++)	Yes; 0 violation	Yes
11	1	1	20.23	2.44	−1.48 (+++)	Yes; 0 violation	Yes
12	1	1	20.23	2.44	−1.48 (+++)	Yes; 0 violation	Yes
13	0	1	17.07	2.57	−2.18 (+++)	Yes; 0 violation	Yes
14	0	1	17.07	2.71	−2.38 (+++)	Yes; 0 violation	Yes
15	0	1	17.07	2.49	−2.16 (+++)	Yes; 0 violation	Yes

HBD stands for Hydrogen-Bond Donors, HBA for Hydrogen-Bond Acceptors, Log Po/w represents the distribution coefficient P, and Log S indicates Solubility. Solubility is denoted as (+++) to signify high solubility.

Caco-2 permeability measurements (expressed as log Papp in 10^{-6} cm/s) [68]. As indicated in Table 3 above, the phytoconstituents generally demonstrate high Caco-2 permeability (in theory, a molecule with Log Papp >0.9 is considered as highly permeable through the intestines). In summary, these molecules tend to possess good intestinal absorption potential and moderate skin permeability. Notably, Cyclohexanone, 3-methyl was found to have a high probability to cross skin barrier (with a theoretical value of log Kp = −2.543 < −2.5). In the context of absorption prediction, it is imperative to determine whether these molecules function as substrates or inhibitors of the prominent ABC transporters, specifically P-glycoprotein. Notably, none of the molecules are identified as substrates for P-glycoprotein, except Cyclohexanone, 3-methyl, Limonene, *p*-Mentha-3,8-diene, and pulegone. However, none of the molecules served as an inhibitor for both P-glycoprotein I and II.

In the evaluation of these compounds, various factors impact their distribution, including the volume of distribution at steady-state, blood-brain barrier permeability, and central nervous system permeability, as outlined in Table 4 and Fig. 2. Generally, these compounds are characterized by a VDss within the range of low to moderate, suggesting their effective distribution within the bloodstream. However, it's crucial to highlight that these phytochemicals do not demonstrate the ability to penetrate the central nervous system.

In the realm of drug development, it is essential to assess the anticipated activity of a molecule and its interactions with CYP isozymes in order to predict potential drug metabolism or toxicity outcomes [69]. In the context of this study, all the compounds were identified to be non-substrates for both CYP2D6 and CYP3A4. In contrast, Myrcene (4) was predicted to inhibit CYP3A4, thereby increasing the risk of adverse effects, including the potential for drug-drug interactions (DDIs), as presented in Table 5.

The essential aspect of drug disposition involves renal clearance, which is influenced by excretion processes primarily mediated by major organic cation transporters such as Renal OCT2. In this context, none of the compounds found in MPEO were determined to be substrates for these transporters [70]. The comprehensive evaluation of total clearance for these compounds is achieved by combining

Table 4
Absorption prediction of MPEO.

Absorption parameters	Water solubility	Caco-2 permeability	Intestinal absorption	Skin permeability (Log Kp)	P-gp substrate	P-gp I inhibitor	P-gp II inhibitor
Units	Log mol/L	log Papp in 10^{-6} cm/s	%	cm/s	Categorical (Yes/No)		
α-Pinene	−3.733	1.38	96.04	−1.827	No	No	No
Cyclohexanone, 3-methyl	−1.344	1.49	97.18	−2.543	Yes	No	No
β-Pinene	−4.191	1.38	95.52	−1.653	No	No	No
Myrcene	−4.497	1.40	94.69	−1.043	No	No	No
3-Octanol	−2.252	1.48	92.73	−1.716	No	No	No
(+)-2-Carene	−3.580	1.39	95.97	−1.782	No	No	No
Limonene	−3.568	1.40	95.89	−1.721	Yes	No	No
***p*-Mentha-3,8-diene**	−3.568	1.40	95.89	−1.721	Yes	No	No
Menthone	−2.668	1.22	97.32	−1.872	No	No	No
Pinocarvone	−2.818	1.47	95.98	−2.193	No	No	No
Isomenthol	−2.217	1.37	95.25	−1.919	No	No	No
Menthol	−2.217	1.37	95.25	−1.919	No	No	No
Dihydrocarvone	−2.385	1.41	97.55	−2.131	No	No	No
Pulegone	−2.587	1.51	96.77	−2.172	Yes	No	No
Carvone	−2.324	1.41	97.70	−2.145	No	No	No

Table 5
Distribution characteristics prediction of MPEO. **(1)** α-Pinene, **(2)** Cyclohexanone, 3-methyl, **(3)** β-Pinene, **(4)** Myrcene, **(5)** 3-Octanol, **(6)** (+)-2-Carene, **(7)** Limonene, **(8)** *p*-Mentha-3,8-diene, **(9)** Menthone, **(10)** Pinocarvone, **(11)** Isomenthol, **(12)** Menthol, **(13)** Dihydrocarvone, **(14)** Pulegone, **(15)** Cavone.

Distribution Parameters	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
VDss (human)	0.667	0.105	0.685	0.363	0.142	0.519	0.396	0.396	0.174	0.458	0.137	0.137	0.182	0.18	0.17
BBB permeability (Log BB)	0.79	0.13	0.81	0.78	0.62	0.75	0.73	0.73	0.60	0.78	0.58	0.58	0.58	0.55	0.58
CNS permeability (Log PS)	−2.20	−2.61	−1.85	−1.90	−2.29	−2.25	−2.37	−2.37	−2.15	−2.26	−2.11	−2.11	−2.47	−2.41	−2.47

hepatic and renal clearance, as described in Table 6 [30].

Fig. 3 depicts bioavailability radars for the identified compounds, with the pink region delineating the oral bioavailability criteria that a molecule's graph must entirely encompass to be regarded as possessing drug-like properties. These criteria take into account six key physicochemical properties: lipophilicity, size, polarity, solubility, flexibility, and saturation, all of which are crucial for achieving optimal oral bioavailability. In this study, it is evident from the figure that all the phytocompounds conform to the criteria for oral bioavailability (see Fig. 4).

The BOILED-Egg model serves as an initial tool for assessing both intestinal absorption and blood-brain barrier permeability, primarily relying on lipophilicity and polarity as key parameters [71]. Within this model, the white region signifies compounds with robust intestinal absorption, while the yolk (yellow area) designates a high level of BBB permeability, as illustrated in Fig. 5. Distinguished by color-coded dots on the graph, the molecules are categorized into P-glycoprotein substrates and non-substrates. In this specific context, the phytocompounds are identified as possessing robust intestinal absorption and excellent blood-brain barrier penetration capabilities. Importantly, they are also recognized as non-substrates for P-glycoprotein.

3.3. In silico toxicity prediction (using pro-Tox II)

One of the primary hurdles in drug discovery revolves around identifying the toxic endpoints of molecules. In this regard, in silico predictions have garnered substantial attention among drug developers as an efficient alternative for assessing the toxicity profiles of potential drug candidate molecules [72]. Table 7 diplays for each molecule 7 estimated parameters such as LD_{50}, toxicity class, and their probability to cause hepatotoxic, carcinogenic, immunotoxic, mutagenic and cytotoxic effects.

In accordance with Table 8, the determination of the toxicity class is based on the median lethal dose values as outlined in the GHS (Globally Harmonized System of Classification and Labeling of Chemicals). Among the listed phytoconstituents, Pulegone (14) stands out as having the lowest toxicity value, with a median lethal dose of 470 mg/kg, categorized as class 4 ("harmful if swallowed"). This classification suggests a heightened risk of acute toxicity associated with Pulegone (14). Conversely, several other phytoconstituents, namely 2, 5, 9, 11, 12, and 15, fall into the "harmful if swallowed" category (class 4), with median lethal doses ranging from 300 mg/kg to 2000 mg/kg. In contrast, phytoconstituents 1, 3, 4, 6, 7, 10, and 13 are classified as "may be harmful if swallowed" in class 5. Furthermore, the analysis extends to safety considerations, including the absence of cytotoxic effects in HepG2 cell lines and the absence of drug-induced liver injury (DILI). Additionally, the table highlights the presence or absence of specific toxicological effects such as carcinogenicity, immunotoxicity, mutagenicity, and cytotoxicity. Notably, the majority of the tested phytoconstituents are categorized as "Inactive" for these effects, suggesting a lower level of concern in these aspects. However, it is essential to note that one particular compound, namely *p*-Mentha-3,8-diene (8), is classified as "Active" for mutagenicity, indicating the potential for genetic toxicity. This discovery emphasizes the imperative for more comprehensive examination and prudence when contemplating the utilization of this compound. While flavonoids and terpenoids have been acknowledged for their various advantageous effects on organisms, it is important to recognize that they may also exhibit certain risks or adverse properties, particularly under specific conditions such as mutagenicity or carcinogenicity [73,74].

3.4. In vitro antioxidant activity

Free radicals are unstable molecules naturally produced during cellular metabolism or in response to environmental factors. When they accumulate excessively, they can damage cells, proteins, DNA, and lipids, thereby contributing to premature aging and various diseases, including cardiovascular diseases, cancer, and neurodegenerative diseases.

The assessment of the antioxidant activity of MPEO, thyme honey (TH) and their mixture (TH-MPEO) was conducted using three commonly employed complementary in vitro tests, namely DPPH●, H_2O_2, and xanthine oxidase (XO) methods. The results are summarized in Table 9, with MPEO IC_{50} values of 21.13 ± 0.01 μg/mL, 29.53 ± 0.01 μg/mL, and 16.49 ± 0.05 μg/mL for the DPPH●, H_2O_2, and xanthine oxidase (XO) tests, respectively. Thyme honey has also shown interesting antioxidant activity with IC_{50} values ranging between 14.67 ± 0.05 and 29.53 ± 0.01 μg/mL. This antioxidant activity has been enhanced when used the mixture of MPEO with thyme honey (TH-MPEO) with IC_{50} values of 15.45 ± 0.05, 14.67 ± 0.05 and 22.06 ± 0.03 for the DPPH●, H_2O_2, and XO tests, respectively. These values are lower than those of the positive controls (ascorbic acid for the DPPH● and H_2O_2 tests, and allopurinol for the xanthine oxidase test).

By comparing these results, it can be observed that MPEO appears to exhibit variable antioxidant efficacy depending on the test used. It seems to be more effective in the xanthine oxidase test, with a lower IC50, suggesting a strong ability to inhibit this enzyme.

Table 6
Metabolism parameters prediction of MPEO's compounds. **(1)** α-Pinene, **(2)** Cyclohexanone, 3-methyl, **(3)** β-Pinene, **(4)** Myrcene, **(5)** 3-Octanol, **(6)** (+)-2-Carene, **(7)** Limonene, **(8)** *p*-Mentha-3,8-diene, **(9)** Menthone, **(10)** Pinocarvone, **(11)** Isomenthol, **(12)** Menthol, **(13)** Dihydrocarvone, **(14)** Pulegone, **(15)** Cavone.

Metabolism parameters	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
CYP2D6 substrate	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
CYP3A4 substrate	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
CYP2D6 inhibitor	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
CYP3A4 inhibitor	No	No	No	**Yes**	No	No	No	No	No	No	No	No	No	No	No

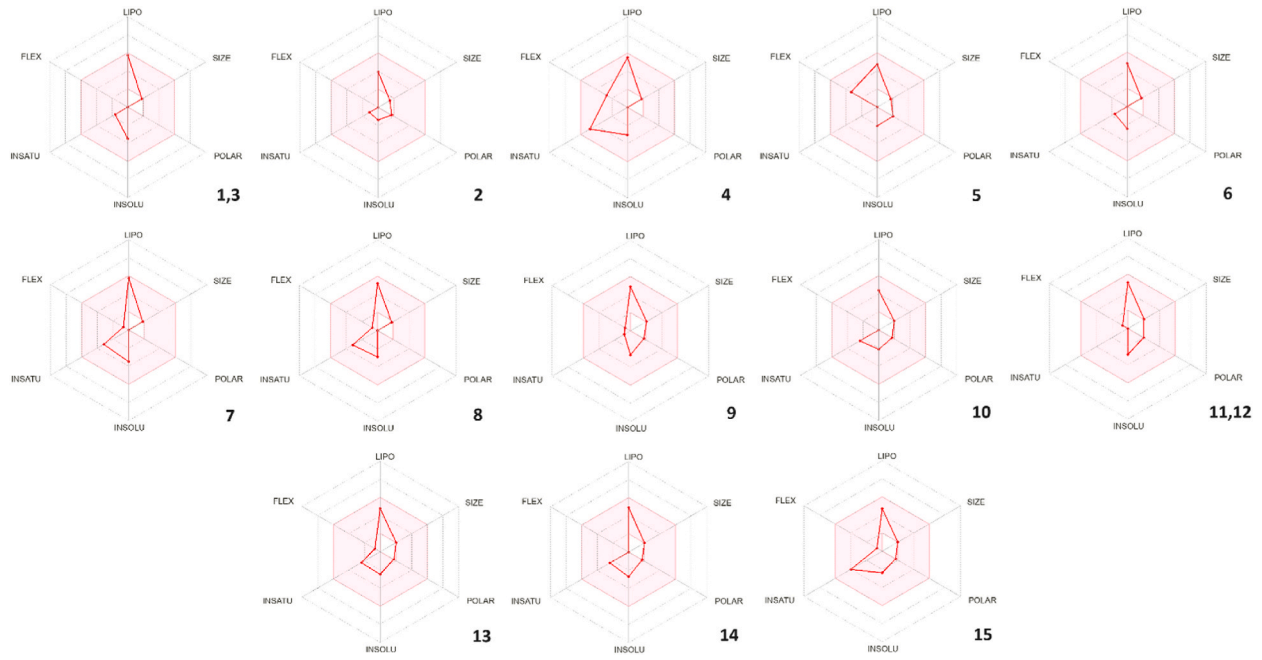


Fig. 3. Bioavailability radars of MPEO's compounds. **(1)** α-Pinene, **(2)** Cyclohexanone, 3-methyl, **(3)** β-Pinene, **(4)** Myrcene, **(5)** 3-Octanol, **(6)** (+)-2-Carene, **(7)** Limonene, **(8)** *p*-Mentha-3,8-diene, **(9)** Menthone, **(10)** Pinocarvone, **(11)** Isomenthol, **(12)** Menthol, **(13)** Dihydrocarvone, **(14)** Pulegone, **(15)** Cavone.

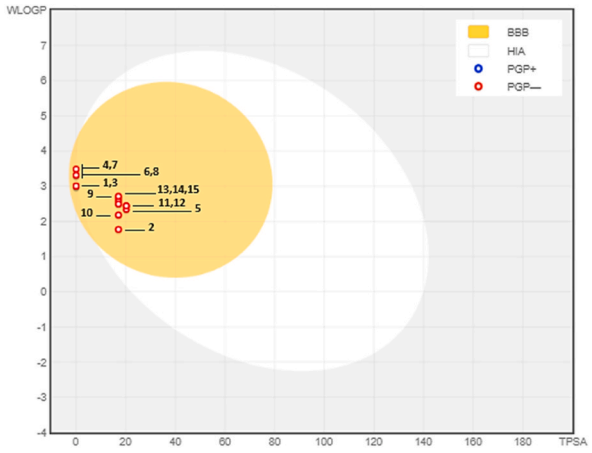


Fig. 4. BOILED-Egg method for evaluating blood-brain barrier permeability, gastrointestinal absorption, substrates and inhibitors of P-glycoprotein for MPEO chemical composition. **(1)** α-Pinene, **(2)** Cyclohexanone, 3-methyl, **(3)** β-Pinene, **(4)** Myrcene, **(5)** 3-Octanol, **(6)** (+)-2-Carene, **(7)** Limonene, **(8)** *p*-Mentha-3,8-diene, **(9)** Menthone, **(10)** Pinocarvone, **(11)** Isomenthol, **(12)** Menthol, **(13)** Dihydrocarvone, **(14)** Pulegone, **(15)** Cavone.

However, its efficacy is slightly reduced in the H2O2 test compared to the DPPH● test (Fig. X). The IC_{50} results from in vitro tests indicate that the essential oil of *Mentha pulegium* exhibits notable activity. However, these findings are not in agreement with previous research results. Indeed, prior studies have revealed that the oil of *M. pulegium* from another region of Morocco exhibited an IC_{50} of 7.659 mg/mL, and even for the essential oil from Iran, an IC_{50} of 14.736 g/mL for antioxidant activity against the DPPH● radical [75, 76]. These studies demonstrated that these oils displayed a rather modest antioxidant activity compared to the essential oil used in our study. The antioxidant capacity of essential oils is frequently shaped by the interplay among their primary constituents, along with the synergistic effects that arise from the interactions between major and minor components [77]. The essential oil of *Mentha pulegium* is rich in pulegone, a natural compound found in certain plants such as peppermint. Pulegone is known to possess antioxidant properties, signifying its ability to neutralize free radicals and reduce oxidative damage in the organism [78]. Other minor molecules may also have direct or synergistic effects to enhance the antioxidant activity of the studied essential oil [79].

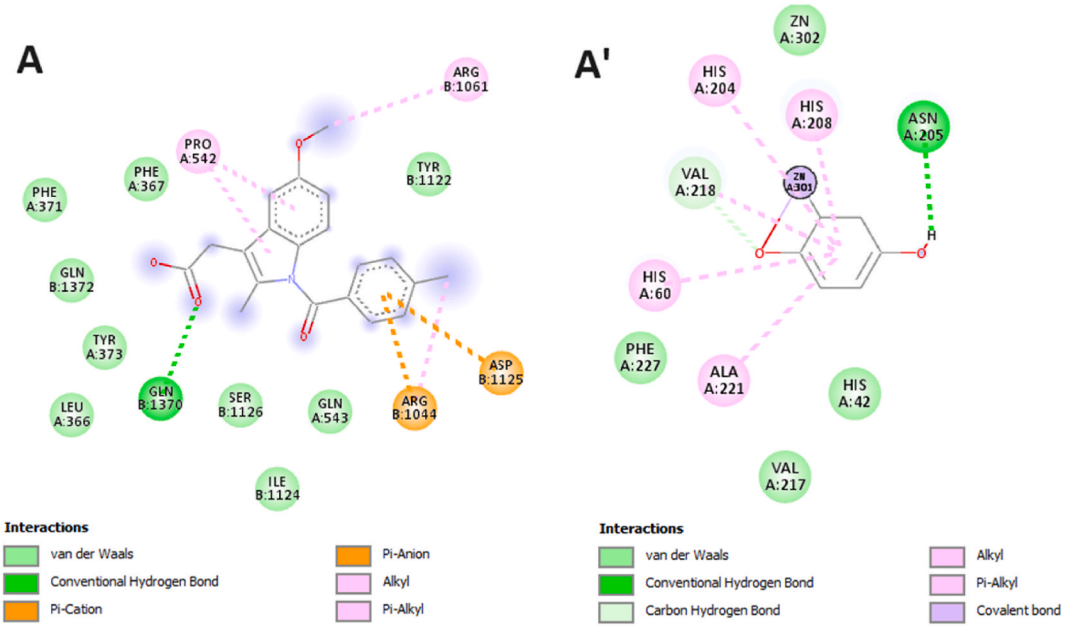


Fig. 5. Schem of the 2D structure of the NSAID Indomethacin (A), and MPEO compound (cyclohexanone, 3-methyl) (A′), bounded to the pocket region of COX-2 (PDB ID: 1DDX).

Table 7
Excretion parameters prediction of MPEO. **(1)** α-Pinene, **(2)** Cyclohexanone, 3-methyl, **(3)** β-Pinene, **(4)** Myrcene, **(5)** 3-Octanol, **(6)** (+)-2-Carene, **(7)** Limonene, **(8)** *p*-Mentha-3,8-diene, **(9)** Menthone, **(10)** Pinocarvone, **(11)** Isomenthol, **(12)** Menthol, **(13)** Dihydrocarvone, **(14)** Pulegone, **(15)** Cavone.

Excretion Parameters	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Total clearence (Log mL/min/Kg)	0.043	0.25	0.03	0.43	1.57	0.045	0.21	0.21	0.24	0.03	1.18	1.18	0.27	0.19	0.22
Renal OCT2 substrate	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No

3.5. *In vitro dermatoprotective and anti-inflammatory activities*

Lipoxygenase plays a crucial role in instigating inflammatory processes, making its inhibition a pivotal therapeutic objective for managing chronic inflammation. In this investigation, we delved into the anti-inflammatory potential of thyme honey and its combination with a mixture, employing the 5-LOX enzyme inhibition assay. The results, as presented in Table 10, reveal that MEPO and thyme honey (TH) exhibited enzyme inhibition with an IC_{50} of 45.18 ± 0.02 and 89.23 ± 0.04 mg/mL, respectively. Intriguingly, the combination of thyme honey (TH) and the mixture displayed a notably significant synergistic effect, yielding an IC_{50} of 22.93 ± 0.04 mg/mL. These findings hold considerable promise when juxtaposed with the reference compound, quercetin (IC_{50} = 3.17 ± 0.02 mg/mL). We examined the dermatoprotective impact of both thyme honey and a mixture of thyme honey by assessing their ability to inhibit tyrosinase, the pivotal enzyme in melanogenesis. Results in table showed that thyme honey presented a significant inhibitory activity against this enzyme (IC_{50} = 69.82 ± 0.01 mg/mL). Whereas the combination between thyme honey and the mixtures exhibited a significant synergistic effect with an IC_{50} of 53.96 ± 0.05 mg/mL. These results were encouraging compared with quercetin (IC_{50} = 25.41 ± 0.06 mg/mL) used as the standard molecule.

Several studies have mentioned the biological applications of Moroccan honey [17,19,80]. However, only a few works have investigated the anti-inflammatory activity of Moroccan honey and the association between honey and essential oils or plant extracts [28,81]. Therefore, the main objective of the present work is to evaluate the anti-inflammatory effects of Moroccan thyme honey and thyme honey with the mixture using *in vivo* and in vitro tests.

Furthermore, we investigated the dermatoprotective potential of both thyme honey and its combination with mixtures by assessing their inhibitory effects on tyrosinase activity, a pivotal enzyme involved in skin degradation that reduces the skin's cellular maintenance properties. Our findings demonstrated that both thyme honey on its own and in combination with mixtures exhibited significant inhibitory activity against this enzyme.

These results align with a study conducted by Habib et al. [82], which examined the tyrosinase inhibition potential of 16 honey samples in vitro. The authors observed that honey sourced from arid regions exhibited the highest tyrosinase inhibitory activity. Additionally, Qasem et al. [83], reported that honey derived from Matricaria chamomilla displayed noteworthy tyrosinase inhibition,

Table 8
Toxicity characteristics prediction for MPEO phytoconstituents**. (1)** α-Pinene, **(2)** Cyclohexanone, 3-methyl, **(3)** β-Pinene, **(4)** Myrcene, **(5)** 3-Octanol, **(6)** (+)-2-Carene, **(7)** Limonene, **(8)** *p*-Mentha-3,8-diene, **(9)** Menthone, **(10)** Pinocarvone, **(11)** Isomenthol, **(12)** Menthol, **(13)** Dihydrocarvone, **(14)** Pulegone, **(15)** Carvone. **Ina.:** Inactive; **Act.:** Active.

Molecules	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
LD_{50} (mg/Kg)	3700	500	4700	5000	1000	4800	4400	7000	500	5000	940	940	5000	470	1640
Class	5	4	5	5	4	5	5	6	4	5	4	4	5	4	4
Hepatotoxicity	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.
Carcinogenicity	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.
Immunotoxicity	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.
Mutagenicity	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	**Act.**	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.
Cytotoxicity	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.	Ina.

Table 9
In vitro antioxidant activity of MPEO.

EO/Positive control	DPPH● IC_{50} (μg/mL)	H_2O_2 IC_{50} (μg/mL)	**Xanthine Oxidase IC_{50} (μg/mL)**
MPEO	21.13 ± 0.01^{c}	29.53 ± 0.01^{d}	16.49 ± 0.05^{d}
TH	19.58 ± 0.03^{c}	20.04 ± 0.02^{c}	23.84 ± 0.1^{c}
TH-MPEO	15.45 ± 0.05^{b}	14.67 ± 0.05^{b}	22.06 ± 0.03^{c}
Ascorbic acid	9.83 ± 0.06^{a}	7.73 ± 0.02^{a}	–
Allopurinol	–	–	6.35 ± 0.02^{a}

Table 10
In vitro anti-inflammatory and dermatoprotective activity.

Assays	IC_{50} (mg/mL)			Control
	MPEO	TH	TH-MPEO	Quercetin
5-Lipoxygenase	$45.18 \pm 0.02^{****}$	$89.23 \pm 0.04^{****}$	$22.93 \pm 0.04^{****}$	3.17 ± 0.02
Tyrosinase	$73.37 \pm 0.03^{****}$	$69.82 \pm 0.01^{****}$	$53.96 \pm 0.05^{****}$	25.41 ± 0.06

Values are expressed as mean ± S.E.M. ($n = 6$), $^{****}p < 0.01$.

with an IC_{50} value of 81.53 ± 0.01 μg/mL.

As suggested by certain researchers, the inhibition of tyrosinase can be attributed to the presence of hydroxyl groups within the phenolic compounds found in honey samples. These hydroxyl groups have the potential to form hydrogen bonds with specific enzyme sites, thereby diminishing enzyme activity [84,85]. Additionally, flavonoids have been identified as compounds capable of interacting with copper ions located in the tyrosinase active site, consequently leading to enzyme activity inhibition [86,87].

Tyrosinase, as a bi-functional enzyme, plays a pivotal role in the oxidation of phenols. Its inhibition can result in a reduction in melanin secretion [82], which explains its application in skincare products. Thus, it is conceivable that formulations incorporating Moroccan thyme honey may hold promise for mitigating age-related senescence markers and alleviating inflammatory conditions.

3.6. In vivo anti-inflammatory, activity

The body's anti-inflammatory response plays a critical role in safeguarding against serious diseases, including diabetes, cancer, and neurodegenerative disorders [88]. However, the use of synthetic anti-inflammatory drugs has been linked to various adverse effects on human health. Consequently, researchers have shifted their focus towards natural remedies as an alternative to synthetic anti-inflammatory medications. Natural products, notably honey and essential oils, are recognized as valuable sources of bioactive compounds possessing anti-inflammatory properties without accompanying side effects [89]. With this perspective in mind, our study was designed to investigate the anti-inflammatory potential of Thyme honey (TH), MEPO and their mixtures in a carrageenan-induced acute inflammation model. Table 11 and Table 12 present the impact of thyme honey, MEPO and mixtures on carrageenan-induced inflammatory edema in the left paw of rats. Following the subplantar injection of carrageenan in the control group, there was a notable increase in the volume of the carrageenan-injected left paw, peaking at 180 min post-induction (0.581 ± 0.060 mL). This clearly demonstrates that the carrageenan injection elicited an acute inflammatory response in the left hind paw of the experimental animals.

Thyme honey, MPEO as well as the mixture, exhibited a reduction in edema volume during different phases of the inflammatory response, with statistical significance ($p < 0.05$). Notably, their effects on carrageenan-induced edema were contingent on time, as demonstrated in the table. Thyme honey and MPEO displayed a moderate level of anti-inflammatory activity, with respective inhibitions of edema at 1h30, 3h, and 6h, measuring 9.77 %, 17.56 %, and 15.92 % for thyme honey and 3.21 %, 8.0 % and 7.42 % for MPEO. In contrast, when Thyme honey was used in conjunction with the mixture, it demonstrated a more substantial inhibition of 23.24 % after 3h, surpassing the effect observed with Thyme honey alone (17.56 %). It's worth noting that the reference drug indomethacin, administered at a dose of 10 mg/kg p.o., exhibited a stronger anti-edematous effect across all time points, with percentage inhibitions of 69.92 %, 74.70 %, and 63.54 % at 1h30, 3h, and 6h, respectively (Table 12).

Table 11
Effect of TH, MPEO and TH mixed with MPEO on carrageenan-induced rat paw edema.

Treatment groups	Mean volume of edema (mL)		
	90 min	3h	6h
Gr I (TH)	$0.351 \pm 0.02^{*}$	$0.479 \pm 0.01^{*}$	$0.396 \pm 0.06^{*}$
Gr II (MPEO)	$0.482 \pm 0.01^{*}$	$0.500 \pm 0.06^{*}$	$0.498 \pm 0.02^{*}$
Gr III (TH-MPEO)	$0.320 \pm 0.11^{*}$	$0.446 \pm 0.09^{*}$	$0.391 \pm 0.04^{*}$
Gr IV (Indomethacin)	$0.117 \pm 0.05^{*}$	$0.147 \pm 0.01^{*}$	$0.167 \pm 0.03^{*}$
Gr V (Control)	0.389 ± 0.09	0.581 ± 0.06	0.471 ± 0.07

Values are expressed as mean ± S.E.M. ($n = 6$); $^{*}P < 0.05$ statistically significant compared to the control and reference drug (Indomethacin).

Table 12
Percentage of inhibition of inflammation of TH, MPEO and their mixture using carrageenan-induced rat paw edema.

Treatment groups	Inhibition of Inflammation Induced By carrageenan (%)		
	90 min	3h	6h
Gr I (TH)	09.77*	17.56*	15.92*
Gr II (MEPO)	03.21*	08.00*	07.42*
Gr II (TH-MPEO)	17.74*	23.24*	16.99*
Gr III (Indomethacin)	69.92	74.70	64.54

Values are expressed as mean ± S.E.M. ($n = 6$); these results compared with standard drug (Indomethacin, 10 mg/kg *p.o.*) were administered by the oral route.

In our *in vivo* analysis, both pure thyme honey and thyme honey combined with other constituents displayed significant reductions in paw thickness in rats following inflammation induction, contrasting with the positive control group. Particularly noteworthy was the superior efficacy exhibited by the combination of thyme honey and the mixtures in diminishing inflammation volume when compared to thyme honey used in isolation.

Our in vitro assessments further underscored these observations by revealing a substantial inhibition of 5-Lipoxygenase activity. Notably, this inhibitory effect was most pronounced when honey was employed in conjunction with the mixture. These results align with prior research conducted by Alzubier et al., in 2011, suggesting that honey's anti-inflammatory properties may be attributed to specific chemical components, particularly flavonoids [90]. These flavonoids have garnered recognition for their capacity to hinder the cyclooxygenase and lipoxygenase pathways within arachidonate metabolism. Additionally, various studies have corroborated this by highlighting how flavonoids and phenolic acids found in honey can downregulate the expression of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β, achieved through the suppression of phosphorylated IKK expression [91,92]. Moreover, they have demonstrated the ability to mitigate the expression of nitric oxide synthase and curtail the generation of reactive oxygen species (ROS) [93,94].

In a separate investigation centered on the analysis of thyme honeys sourced from diverse regions in Greece, it was discovered that carvacrol [95], a unique compound exclusively present in thyme honey, activates PPAR α and γ while concurrently repressing COX-2 expression [96,97]. Collectively, these findings provide compelling evidence of thyme honey's anti-inflammatory attributes, making it a promising candidate for inflammation mitigation.

3.7. Molecular docking

Molecular docking is a highly regarded and extensively utilized technique within the realm of structure-based drug design (SBDD). Its significance lies in its exceptional proficiency in precisely forecasting the manner in which small-molecule ligands assume their conformations within the intricately defined binding sites of their respective target proteins [98]. This technique, often referred to as molecular docking (MD), has emerged as a cornerstone in the field of drug discovery and has significantly advanced drug development efforts since its inception in the 1980s, coinciding with the development of the initial algorithms that underpin this methodology [99]. Molecular docking stands as a powerful tool in the realm of bioinformatics, offering a key to unlocking the intricate complexities of

Table 13
Heat map of the binding affinity (Kcal/mol) values of the identified components in MPEO.

N°	Compounds	1DDX	4OM7	1N8Q	5I3B	1OG5	2CDU
			Free Binding energy (Kcal/mol)[a]				
–	Native Ligand	−6,9	−5,4	−6	−5,5	−6,6	−8,6
1	α-Pinene	−5,1	−5,5[a]	−5,4	−4,7	−5,2	−5,5
2	Cyclohexanone, 3-methyl	−7,9[a]	−8,1[a]	−7,7[a]	−5,5[a]	−7,7[a]	−4,8
3	β-Pinene	−5	−5,2	−5,2	−4,6	−5,2	−5,6
4	Myrcene	−4,3	−4,7	−5,5	−4,7	−4,7	−4,8
5	3-Octanol	−4,8	−4,3	−4,4	−3,9	−4,5	−4,2
6	(+)-2-Carene	−5,5	−5,2	−5,7	−4,9	−5,7	−5,8
7	Limonene	−5,1	−5,5[a]	−5	−4,9	−5,5	−5,6
8	*p*-Mentha-3,8-diene	−5,1	−5,5[a]	−5,6	−4,7	−5,4	−5,8
9	Menthone	−5,2	−5,1	−5	−4,8	−5,2	−5,4
10	Pinocarvone	−5,5	−5,5[a]	−5,3	−5	−5,4	−6
11	Isomenthol	−5,5	−5,6[a]	−5,5	−5,1	−5,2	−5,5
12	Menthol	−5,5	−5,5[a]	−5,6	−4,9	−5,3	−5,8
13	Dihydrocarvone	−5,3	−5,9[a]	−5,8	−5,3	−5,5	−6
14	Pulegone	−5,9	−5,4	−5,4	−5,3	−5,5	−5,8
15	Carvone	−5,6	−5,8[a]	−5,4	−5,5[a]	−5,9	−6,7

[a] In each column, the color scale spans from red, which corresponds to the native ligand ΔG, transitioning through yellow (mid-point), and culminating in green (native ligand ΔG + 4 kcal/mol). The proteins under investigation include anti-inflammatory targets: 1DDX (COX-2), 4OM7 (TIR domain of Toll-like receptor 6, TLR6), 1N8Q (Lipoxygenase), and 5I3B (Tyrosinase enzyme), as well as antioxidant targets: 1OG5 (CYP2C9) and 2CDU (NADPH oxidase).

ligand-receptor interactions. This method provides invaluable insights into how small molecules, known as ligands, intricately conform to and interact with their designated target proteins. It serves as an indispensable resource, enabling researchers to delve into critical molecular events, such as understanding the precise binding mechanisms and the specific intermolecular interactions that bolster the stability of the ligand-receptor complex [91]. In the context of our study, molecular docking served as an instrumental technique in unraveling the potential mechanisms of action underlying the components identified within MPEO. Through the examination of binding affinity values, where a reduction in binding energy typically signifies enhanced compound affinity, our results could shed light on the precise manner in which these molecules engage with a specific target. This comparison is made with a native ligand, a known inhibitor against the target protein.

To conduct this molecular docking analysis, we followed the established protocols outlined in previous references [42–44]. The objective was to explore the potential mechanisms related to anti-inflammatory, dermatoprotective, and antioxidant properties of the phytocompounds within MPEO. We selected the protein structures of four anti-inflammatory target proteins, which included lipoxygenase (LOX, 1N8Q) [43], the Active Site of Human COX−2 (1DDX) [45,46], the TIR domain of Toll-like receptor 6 (TLR6) (4OM7) [47], and Tyrosinase for the dermatoprotective assessment [48]. Additionally, two proteins associated with antioxidant functions were chosen: CYP2C9 (1OG5) [49], and NADPH Oxidase (2CDU) [50]. These selections formed the basis for our comprehensive evaluation of the potential mechanisms of action of the compounds found within MPEO.

The docking scores were visualized in a heatmap-style table using a color scheme that ranged from red, which represented the lowest energy values (often corresponding to the docking score of the native ligand), to green, indicating the highest energy values (Table 13.). This approach facilitated the identification of a set of compounds that consistently exhibited potential inhibitory properties by comparing their lowest scores to that of the native ligand for a specific target. Compounds with docking scores equal to or lower than the score of the native ligand were marked with an asterisk (*).

3.7.1. Molecular docking simulations for the anti-inflammatory activity and the dermatoprotective activities

we conducted a molecular docking study to assess the interactions between the compounds identified within MPEO, determined via GC-MS analysis. These compounds displayed moderate affinity parameters, exhibiting binding energy values ranging from −4.3 to −7.9 kcal/mol, as they engaged with the COX-2 (cyclooxygenase-2) protein structure (PDB ID: 1DDX). COX-2 plays a pivotal role in our in silico investigations, particularly when predicting the anti-inflammatory potential of potential drug candidates, due to its central role in the inflammatory cascade [45,46]. COX-2 is responsible for facilitating the synthesis of prostaglandins, which have a critical function in inflammation, pain, and fever. Elevated COX-2 levels have been implicated in numerous inflammatory conditions, including arthritis, cancer, and cardiovascular diseases [100,101]. It's worth noting that among the tested compounds, cyclohexanone, 3-methyl, exhibited the most favorable outcome, with a free binding energy of −7.9 kcal/mol, outperforming the NSAID indomethacin, which achieved a docking score of −6.9 kcal/mol. Both compounds formed a conventional hydrogen bond, the first ligand with the amino acid residue GLN B:1370 and the second with an amino acid from the active site pocket ASN A:205. For detailed information, please refer to Table 13, Fig. 5A, A'.

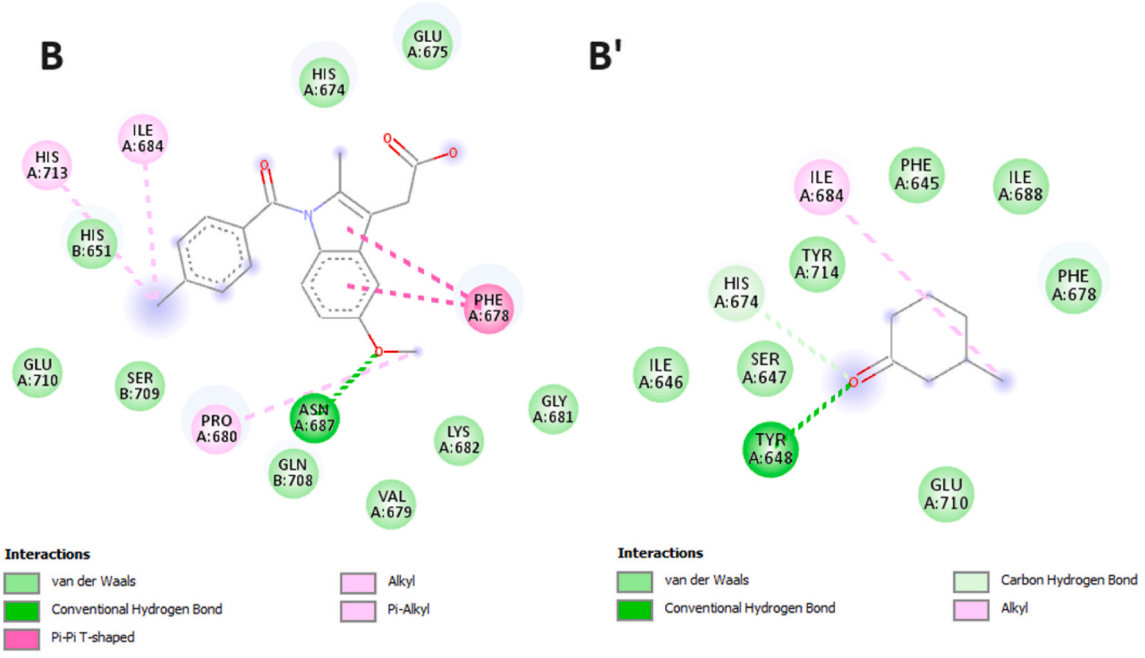


Fig. 6. Schem of the 2D structure of the NSAID Indomethacin (B), and MPEO compound (cyclohexanone, 3-methyl) (B'), bounded to the pocket region of TLR-6 (PDB ID: 4OM7).

Inflammation is a critical component of the immune response initiated by Toll-Like Receptors (TLRs) when they encounter pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) [102,103]. Upon activation, TLR6 triggers a signaling cascade resulting in the production of pro-inflammatory cytokines and chemokines, which in turn attract immune cells to sites of infection or tissue damage [103]. In this context, our attention was focused on Toll-Like Receptor 6 (TLR6, PDB ID: 4OM7), an anti-inflammatory protein. Identifying compounds capable of binding to TLR6 and influencing its activity carries significant implications for the development of anti-inflammatory agents. Our docking analysis revealed nine potent TLR6 inhibitors within MPEO, exhibiting binding energies surpassing that of Indomethacin (−5.4 kcal/mol, as shown in Fig. 1.). These compounds encompass (1) α-Pinene, (2) Cyclohexanone, 3-methyl, (7) Limonene, (8) *p*-Mentha-3,8-diene, (10) Pinocarvone, (11) Isomenthol, (12) Menthol, (13) Dihydrocarvone, and (15) Cavone. Notably, Cyclohexanone, 3-methyl (2), stands out as the most potent inhibitor, forming a single conventional hydrogen bond with the amino acid residue Tyr A:648 within the protein's active site (refer to Fig. 6. B′). These findings strongly support the notion that the anti-inflammatory properties of the two essential oils can be attributed to the diverse array of compounds they contain.

Excessive production of components involved in the arachidonic acid (AA) cascade, particularly those associated with the cyclo-oxygenase (COX) and lipoxygenase (LOX) pathways, is a significant contributor to severe inflammatory diseases in humans [104]. Lipoxygenase (LOX) serves as a critical enzyme that limits the conversion of arachidonic acid into leukotriene (LT), a key mediator of inflammation. Inhibiting LOX activity can reduce the levels of LT, thereby exerting an anti-inflammatory effect [104]. Among the compounds found in MPEO, only one ligand demonstrated strong inhibitory potential, specifically, cyclohexanone, 3-methyl (Fig. 7. C), with a binding energy of −7.7 kcal/mol, surpassing protocatechuic acid with a binding energy of −5.5 kcal/mol (Fig. 7. C').

Tyrosinase plays a central role as the primary regulatory enzyme in the melanin biosynthesis pathway, particularly in the initial two stages: (i) the conversion of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and (ii) the oxidation of DOPA to dopaquinone [20]. In the docking investigations and calculation of binding free energy, both cyclohexanone, 3-methyl (Fig. 8 D′), and carvone (Fig. 8 D″) exhibited the highest interaction energy with tyrosinase (PDB ID: 5I3B) at −5.5 kcal/mol for each ligand, which is equivalent to the binding energy observed with the natural inhibitor of tyrosinase, hydroquinone (which also exhibited a free binding energy of −5.5 kcal/mol) (Fig. 8 D).

3.7.2. Molecular docking for the antioxidant activity

A group of enzymes known as lipoxygenases, containing metallic elements, plays a significant role in catalyzing the oxidation of polyunsaturated free fatty acids via a redox mechanism. This process results in the formation of oxygen-centered fatty acid hydroperoxide radicals, which have implications for various adverse health conditions [105,106]. In this context, we directed our attention toward a specific lipoxygenase enzyme, cytochrome P450 (PDB: 1OG5), recognized for its involvement in cellular lipid metabolism and its potential as a target for drug development to address conditions linked to fatty acid peroxidation. In our evaluation of cytochrome P450 (PDB: 1OG5), none of the compounds exhibited robust inhibitory effects, except for compound 2 (cyclohexanone, 3-methyl), which displayed a noteworthy free binding energy of −7.7 kcal/mol. This performance surpassed that of the natural ligand for CYP2C9, warfarin, which had a binding energy of −6.6 kcal/mol. Fig. 9 provides a visual representation of the 2D interactions between the compound and the amino acid residues within the binding pocket.In light of this, we focused on a specific lipoxygenase enzyme, cytochrome P450 (PDB: 1OG5), which has been identified as a key player in cellular lipid metabolism and may offer novel targets for drug development aimed at addressing conditions associated with fatty acid peroxidation. Regarding cytochrome P450 (PDB: 1OG5), none of the compounds exhibited strong inhibitory effects except for compound 2 (cyclohexanone, 3-methyl) (Fig. 8 E'), which demonstrated a notable free binding energy of −7.7 kcal/mol. This performance surpassed that of the natural ligand for CYP2C9, warfarin (Fig. 9 E), which had a binding energy of −6.6 kcal/mol. The 2D interactions between the compound and the amino acid residues within the binding pocket are visually represented in Fig. 9.

E NADPH oxidase is a pivotal enzyme that plays a central role in the intricate process of generating reactive oxygen species (ROS) within cells, a phenomenon that lies at the heart of oxidative stress [107]. Elevated levels of ROS have been strongly associated with the onset of oxidative damage, a phenomenon implicated in a wide array of pathological conditions, including but not limited to neurodegenerative disorders, cardiovascular diseases, and various forms of cancer. In the context of our meticulous investigation, we embarked on a comprehensive analysis of the active site of the NADPH Oxidase Protein, which bears the distinctive PDB ID: 2CDU. Our endeavor involved an in-depth examination of the microenvironment surrounding this protein's active site, which is defined by the presence of critical amino acids such as GLY158, TYR159, ILE160, GLY180, HIS181, TYR188, VAL214, CYS242, and GLY244. However, our research yielded a significant and noteworthy finding: none of the compounds subjected to molecular docking exhibited a free binding energy that was either equivalent to or surpassed the benchmark set by the native ligand adenosine 5′-diphosphate (ADP), which is characterized by an energy value of −8.6 kcal/mol (as meticulously detailed in Table 13). This compelling observation leads us to an unequivocal conclusion: the compounds under scrutiny within MPEO do not exhibit the inhibitory potential necessary to effectively target and modulate the activity of the NADPH Oxidase Protein. This discovery, while providing crucial insights into the molecular interactions and potential inhibitory properties of these compounds, underscores the need for further exploration and investigation in our quest to uncover novel therapeutic agents against oxidative stress-related conditions.

4. Conclusions

The exploration into the various biological activities of *M. pulegium* essential oil (MPEO), honey, and their combined formulation has yielded compelling evidence affirming the pharmacological promise held by this plant. The GC-MS analysis identified pulegone as major compound of MPEO with a 73.8 %, followed by carvone (6.0 %). Moreover, as revealed by the antioxidant assays, including

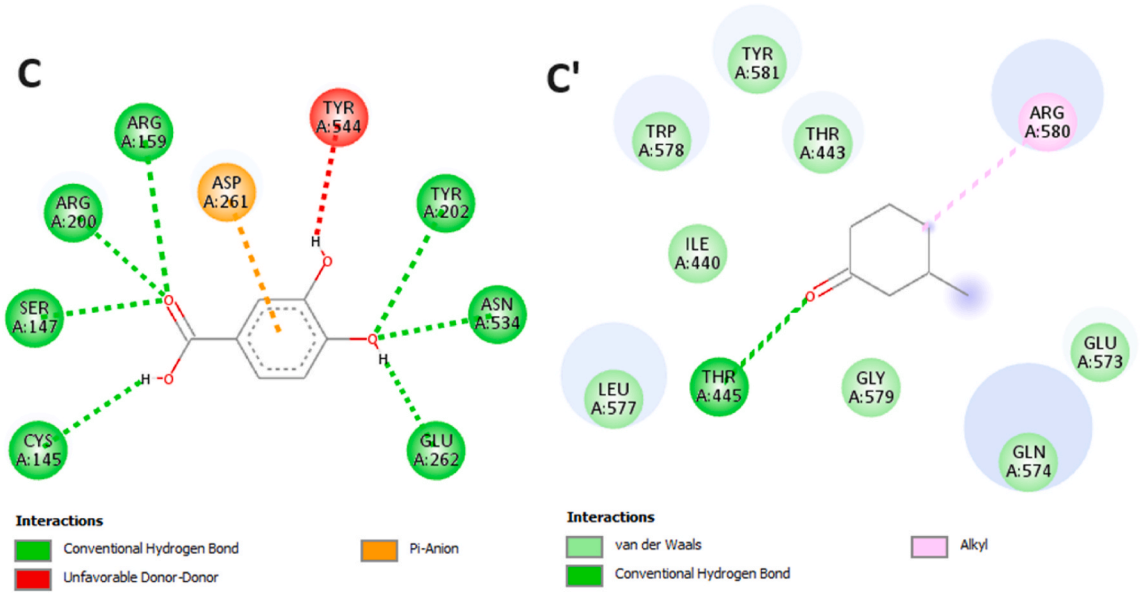


Fig. 7. Representation of the 2D structure of the protocatechuic acid (native ligand) (C), and MPEO compound (cyclohexanone, 3-methyl) (C′), bounded to the pocket region of Lipoxygenase (PDB ID: 1N8Q).

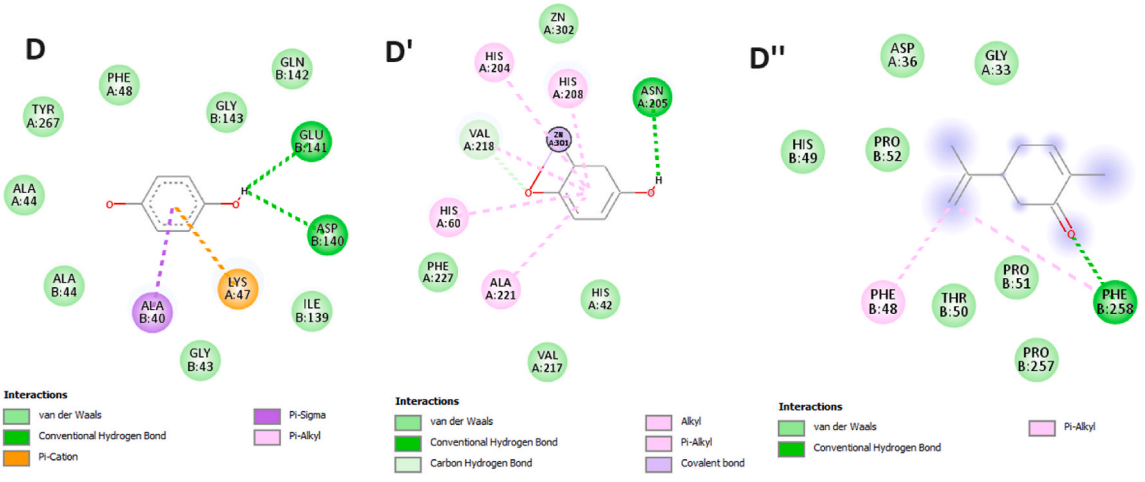


Fig. 8. Representation of the 2D structure of the native ligand, hydroquinone (D), and MPEO compounds (cyclohexanone, 3-methyl) (D′), and carvone (D″), bounded to the pocket region of Tyrosinase active site (PDB ID: 5I3B).

DPPH, H202, and XO, MPEO possess a valuable antioxidant potential, especially when combined with thyme honey. Furthermore, MPEO alone and combined with thyme honey have showed to inhibit LOX-5 (45.18 ± 0.02 and 22.93 ± 0.04, respectively) and tyrosinase (73.37 ± 0.03 and 53.96 ± 0.05, respectively). Moreover, in silico molecular docking analysis hinted at the potential role of cyclohexanone, 3-methyl, an element found in the MPEO, in contributing to the observed outcomes.

These findings underscore its robust antioxidant, anti-inflammatory, and dermatoprotective attributes, positioning it as a highly encouraging candidate for the development of alternative therapies targeting degenerative diseases. These multifaceted biological activities, ranging from antioxidant and anti-inflammatory properties to dermatoprotection prowess, collectively highlight the versatile and promising nature of *M. pulegium* as a potential source for the development of novel drugs and therapeutic approaches.

4.1. Strengths and limitations

This investigation reported the Synergistic effect of *Mentha pulegium* L. essential oil and Thym honey on antioxidant, dermatoprotective, and anti-Inflammatory properties using binary combination. However, it would be advantageous to test different possible combinations using experimental design model. Moreover, it would be better to test the effect of single compounds of this oil to clearly

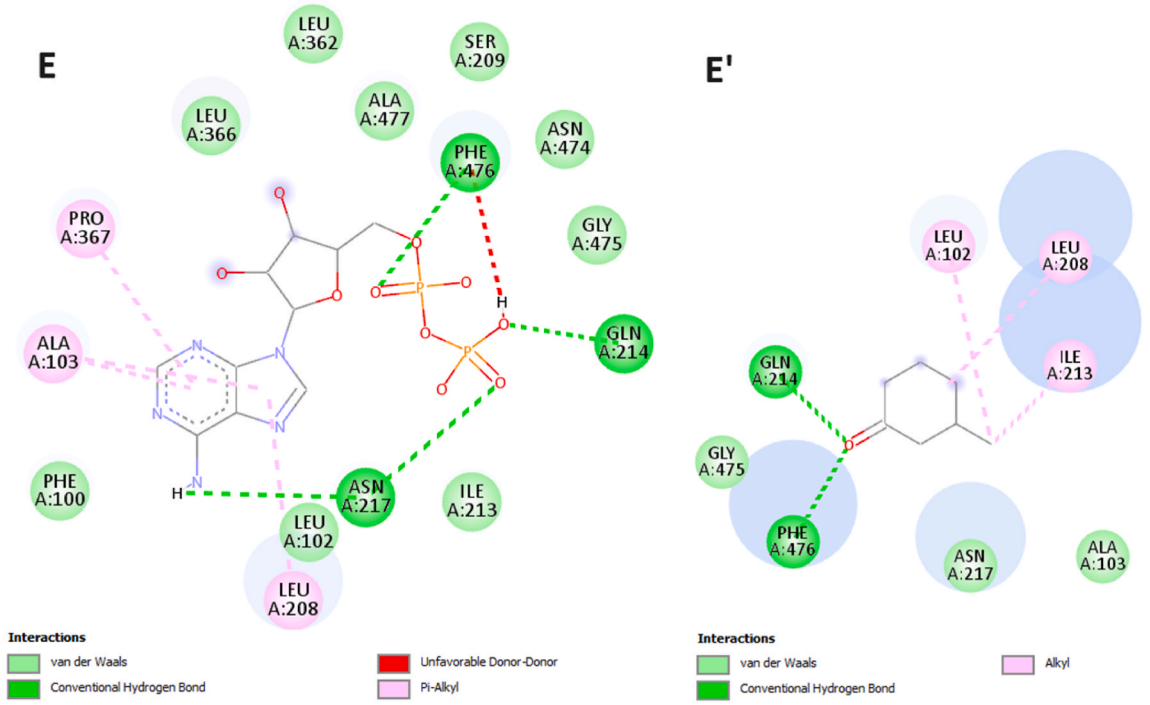


Fig. 9. Representation of the 2D structure of the warfarin (E), and MPEO compound (cyclohexanone, 3-methyl) (E′), bounded to the pocket region of Cytochrome P450 (PDB ID: 1OG5).

elucidate their efficiency. Moreover, this study provides only the in vitro, *in vivo* and in silico biological effects of the tested oil alone and combined with Thym honey without details about the molecular mechanisms underlying these effects.

The in silico simulation indicates clear evidence about the safety of the chemical compounds present in *Mentha pulegium* EO. However, in order to complete the toxicity profile of this EO, in depth investigations should be carried out to examine its toxicity over time using animal models.

Ethical approval

This experiment was carried out on normal male Albino rats weighing between 25 and 30 g were housed under standard control conditions at the animal facility of the Faculty of Science, Sidi Mohamed Ben Abdellah University, Fez. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This approval is registered under the reference number L.25. USMBA-SNAMOPEQ 2023-03.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Hamza Assaggaf: Writing – review & editing, Supervision, Software, Project administration, Methodology. **Naoufal El Hachlafi:** Writing – original draft, Formal analysis, Data curation. **Amine Elbouzidi:** Writing – review & editing, Resources, Project administration, Investigation. **Mohamed Taibi:** Writing – original draft, Validation, Software, Resources. **Nesrine Benkhaira:** Writing – original draft, Software, Resources, Project administration. **Fatima El Kamari:** Writing – original draft, Software, Project administration, Investigation, Conceptualization. **Sulaiman Mohammed Alnasseri:** Writing – review & editing, Validation, Project administration, Methodology. **Wafa Laaboudi:** Writing – original draft, Resources, Project administration. **Abdelhakim Bouyahya:** Writing – review & editing, Validation, Supervision, Investigation, Formal analysis, Conceptualization. **Chrismawan Ardianto:** Writing – original draft, Supervision, Resources, Methodology, Investigation, Conceptualization. **Khang Wen Goh:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Long Chiau Ming:** Writing – review & editing, Validation, Resources, Project administration. **Hanae Naceiri Mrabti:** Writing – review & editing, Visualization, Validation, Formal analysis, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors extend their appreciation to the Deanship for Research & Innovation, Ministry of Education in Saudi Arabia for funding this research work through the project number: IFP22UQU4310026DSR074.

List of abbreviations

Mentha pulegium essential oil (MPEO)
thyme honey (TH)
2,2′-diphenyl-1-picrylhydrazyl (DPPH●)
xanthine oxidase (XO)
5-Lipoxygenase (5-LOX)
chromatography coupled with mass spectrometry (GC/MS)
standard error of the mean (SEM)
Cyclooxygenase-2 inhibitors (COX-2)
Toll-like receptor 6 (TLR6)
retention index (RI)
Computer-Aided Drug Design (CADD)
structure-based drug design (SBDD)
drug interactions (DDIs)
drug-induced liver injury (DILI)
Absorption, Distribution, Metabolism, and Excretion (ADEM)
pathogen-associated molecular patterns (PAMPs)
danger-associated molecular patterns (DAMPs)
3,4-dihydroxyphenylalanine (DOPA)
reactive oxygen species (ROS)
5′-diphosphate (ADP)

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