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Research article

Petroselinum crispum L., essential oil as promising source of bioactive compounds, antioxidant, antimicrobial activities: *In vitro* and *in silico* predictions

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ABSTRACT

This exploratory study aims to identify the volatile compounds in PC-Eo (*Petroselinum crispum* L. essential oil) and evaluate its antioxidant and antimicrobial properties *in vitro*. Molecular docking, drug-likeness prediction, and pharmacokinetics (absorption, distribution, metabolism, excretion, and toxicity—ADMET) were among the *in silico* simulations that were used to explain the biological properties observed *in vitro*. For PC-Eo's chemical screening, gas chromatography-mass spectrophotometry (GC-MS) was employed. The antioxidant activity of PC-Eo was evaluated using five *in vitro* complementary techniques, including 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical scavenging activity, β-Carotene bleaching test (BCBT), reducing power (RP), and phosphomolybdenum assay (TAC). GC-MS analysis revealed that the primary components of PC-Eo are apiol (49.05 %), Myristicin (21.01 %), and 1-allyl-2,3,4,5-tetramethoxybenzene (13.14 %). The results of the *in vitro* antioxidant assays indicate that PC-Eo exhibits a superior antioxidant profile. The *in vitro* antimicrobial activity of PC-Eo was assessed against five strains, including 2 g-positive bacteria, 2 g-negative bacteria, and one fungal strain (*Candida albicans*). The disc-diffusion assay revealed significant antibacterial and antifungal activities against all strains, with zones of inhibition exceeding 15 mm. The microdilution test highlighted the lowest MIC and MBC values with gram-positive bacteria, ranging from 0.25 to 0.5 % v/v for MIC and 0.5–1.0 % v/v for MBC. For the fungal strain, MIC was recorded at 1.25 % and MFC at 2.5 % v/v. PC-Eo demonstrates bactericidal and fungicidal activity based on the MBC/MIC and MFC/MIC ratios. According to the ADMET study, the primary PC-Eo compounds have advantageous pharmacokinetic characteristics. These

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findings provide empirical support for the traditional uses of this plant and indicate its possible use as a natural remedy.

1. Introduction

With over 3000 species spread across over 400 genera, the Apiaceae (Umbelliferae) family is one of the largest families of flowering plants [1,2]. It is regarded as a significant supplier of raw materials for the flavor, perfume, cosmetics, and pharmaceutical industries. Depending on seed varieties, genetic sources, and environmental factors, this family's chemical composition varies significantly, but it is rich in proteins, fibers, carbohydrates, and essential oils (EOs) [3]. EOs and extracts from Apiaceae species have been used in natural therapies, pharmaceuticals, alternative medicine, and food preservation [4]. Investigating these plants scientifically is essential to understand their potential as sources of natural agrochemicals and their biological activities, contributing to the improvement of healthcare quality.

The Apiaceae possesses some of the widely employed vegetables and aromatic herbs such as anise (*Pimpinella anisum* L.), wild carrot (*Daucus carota* L.), caraway (*Carum carvi* L.), celery (*Apium graveolens*), coriander (*Coriandrum sativum*), cumin (*Cuminum cyminum*), dill (*Anethum graveolens* L.), fennel (*Foeniculum vulgare* Mill.), Gotu kola (*Centella asiatica*), parsley (*Petroselinum crispum* L.), parsnip (*Pastinaca sativa* L.), Toothpick weed; khella (*Ammi visnaga* (L.) Lam.) [5,6].

Petroselinum crispum (Mill.) Fuss belongs to the Apiaceae family and is also recognized by various names, including *Petroselinum sativum* Hoffm., *Petroselinum vulgare* Lagasca, *Petroselinum crispum* (Mill.) Nyman ex A.W. Hill, *Apium petroselinum* L., *Carum petroselinum* (L.) Benth., Hook. f., and *Petroselinum hortense* Hoffm. This plant holds significance in the food, cosmetic, and pharmaceutical industries due to its aromatic, culinary, and medicinal properties [7].

Microorganisms' resistance poses a threat to human health and food production, which is why researchers are always looking for new natural antimicrobial agents [8]. While synthetic fungicides are typically effective and affordable, concerns have been raised about their residual toxicity, potentially leading to carcinogenic and teratogenic side effects [9]. Additionally, there is a growing demand for natural products to control microorganisms, prompting the re-evaluation of potential medicinal plants for disease control.

Essential oils are a mixture typically comprise a diverse array of volatile compounds, including mono- and sesquiterpenes, as well as aromatic and aliphatic components derived from phenols [10].

Essential oils are recognized for their multifaceted properties that extend beyond their traditional roles as natural food additives and fragrances. In addition to their well-documented antibacterial [11], antifungal [12], and anti-inflammatory activities [8], essential oils exhibit various other beneficial properties [13]. Furthermore, numerous essential oils have been substantiated to possess antioxidant activity [14].

The literature on the medicinal properties of parsley essential oil, particularly with emphasis on its status in Morocco, is currently limited. While there have been studies demonstrating the antioxidant and antimicrobial activities of parsley essential oil, comprehensive research specifically focusing on its medicinal properties in the context of Moroccan cultivation is scarce [15].

By examining the available literature, researchers can better understand the current knowledge gaps and identify areas for further investigation. Therefore, future studies should aim to explore the medicinal properties of parsley essential oil in the Moroccan context and contribute to filling this gap in the literature [16].

The current study was conducted to explore the therapeutic potential of Moroccan parsley. The objectives included the determination of volatile compounds in *P. crispum* essential oil, assessment of its *in vitro* antimicrobial and antioxidant properties, and *in silico* simulations, encompassing molecular docking and pharmacokinetics absorption.

2. Materials and methods

2.1. Plant material and Eo extractions

Petroselinum crispum L. seeds were harvested in the Sefrou region (33° 410 4500 N,4° 220 1800 W) in July 2022, according to the most recent WHO guidelines [17]. Botanical authentication was conducted at the Scientific Faculty, Sidi Mohammed Ben Abdellah University in Fez, Morocco, under voucher specimen RAB40104. The extraction of PC-Eo was carried out through hydro-distillation using a Clevenger-type apparatus. In brief, 100 g of dry seeds was placed in a 2L flask with 1L of distilled water and boiled for 3 h. The resulting oil was collected and stored at a temperature of 4 °C until the subsequent assays. At least three separate extractions were performed, and the mean yield and standard deviation were calculated.

2.2. Essential oil chemical characterization using GC/MS

Thermo-Fisher Scientific, Waltham, MA, USA, used mass spectrometry (Quadrapole, PolarisQ, S/N 210729) in conjunction with gas chromatography (TRACE GC-ULTRA, S/N 20062969) for the chemical composition analysis of PC-Eo. For the analysis, a capillary column (HP-5MS) measuring 50 m in length, 0.32 mm in internal diameter, and 1.25 μm in film thickness was used. With a 5 °C per minute increment, the temperature program covered a range of 40–280 °C. Ionization took place in electron-impact mode (EI) at 70 eV, with the injector and detector (Polaris Q) maintained at 250 °C and 200 °C, respectively.

The carrier gas was helium, with a split ratio of 1:40 and a flow rate of 1 mL/min. A volume of 1 μL of the Eo was introduced for

analytical purposes. The determination of component percentages was made possible by the identification of Eo components through a comparison of their retention times with those recorded in the NIST-MS Search Version 2.0 library.

2.3. Antioxidant activity

2.3.1. DPPH scavenging capacity

Using previously published techniques, the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical was used to measure the free radical scavenging activity [18]. 825 mL of DPPH was combined with a volume of 50 μL of Eo that had been dissolved in ethanol at various concentrations. The absorbance at 517 nm was measured after the reaction mixture was left in the dark for 60 min. The standard used was butylated hydroxytoluene (BHT). Every determination was made three times. The IC_{50} values were calculated from the graph by % inhibition using the following equation (Equation (1)).

$$\text{Inhibition } (\%) = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100 \quad (1)$$

2.3.2. ABTS radical scavenging activity

Using an ABTS free radical cation decolorization assay, PC-Eo's capacity to scavenge free radicals was ascertained [19]. To summarize, 825 μL of the ABTS radical cation solution was mixed with 50 μL of different dilutions of each ethanolic Eo and Trolox, which was used as a positive control. For 6 min, these solutions were allowed to sit at room temperature in the dark. The absorbance measurements were then taken with a UV/Vis spectrophotometer at 734 nm.

As a negative control for comparison, a blank sample with an equal volume of ethanol and ABTS solution was utilized. Equation (1) was utilized to compute the percentage inhibition of absorbance, and the half-maximal inhibitory concentration (IC_{50}) values were expressed in micrograms per milliliter (μg/mL) and graphically determined. All analyses were conducted in triplicate.

2.3.3. Linoleic acid/β -carotene bleaching assay (BCBT)

The β-carotene linoleic acid test was utilized to evaluate the inhibition of lipid peroxidation capacity, adhering to the guidelines provided by El Hachlafi et al. To put it briefly, 1 mg of β-carotene was dissolved in 5 mL of chloroform to create a stock solution, which was then mixed with 10 mg of linoleic acid and 100 mg of Tween-80. A rotary evaporator was used to evaporate the chloroform at 45 °C and 100 rpm. The residue was then combined with 50 mL of distilled water. Next, 100 μL of PC-EO at different concentrations was combined with 1 mL of β-carotene solution. Following a 100-min incubation period in boiling water at 50 °C, the test tubes' β-carotene absorbance was measured at 470 nm in comparison to a blank. Equation (2) was used to calculate the antioxidant properties based on residual color inhibition relative to the control.

$$\text{Inhibition } (\%) = \frac{\text{Abs}_{\,(t=100\ \text{min})}}{\text{Abs}_{\,(t=0)}} \times 100 \quad (2)$$

where Abs (t = 100 min) represents the absorbance of β-carotene after 100 min of experimentation in the presence of PC-Eo, and Abs (t = 0) represents the absorbance of β-carotene at the start of the assay [20].

2.3.4. Reducing power (RP)

By following established procedures, the decision to reduce power was made [21]. Specifically, 200 μL of 0.2 M sodium phosphate buffer (pH 7.6) and 200 μL of 1 % potassium ferricyanide were mixed with 50 μL of PC-Eo. Subsequently, the mixture was incubated for 20 min at 50 °C to promote the conversion of ferricyanide to ferrocyanide. The reaction was then stopped by adding 200 μL of 10 % trichloroacetic acid. For 10 min, the mixture was centrifuged at 500 g of force. At last, the supernatant was combined with 120 μL of 0.1 % ferric chloride and 600 μL of distilled water. The plotting of absorbance at 700 nm against sample concentration resulted in a linear relationship. The results were expressed in μg/mL and were defined as the concentration of the extract at which the absorbance reached 0.5 (EC_{50}). Each experiment was conducted in triplicate. An increase in absorbance in the reaction mixture signifies an enhancement in reducing power. Ascorbic acid was used as a positive control at various concentrations.

2.3.5. Total antioxidant activity (TAC)

The phosphomolybdenum method, described by Nouioura et al., was used to evaluate the overall antioxidant capacities of all extracts. In this procedure, 1 mL of a reagent solution consisting of 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate was combined with 50 μL of PC-Eo or a standard reference substance (ascorbic acid). Using a PerkinElmer Lambda 40 UV/Vis spectrophotometer, the sample was incubated for 90 min at 95 °C in a water bath. The absorbance of the sample was measured at 695 nm, and this measurement was compared to a blank. As a mean of three triplicate measurements ± standard deviation (SD), the results were reported in milligrams of ascorbic acid equivalent (mg AAE) per gram of Eo (mg AAE/g Eo). Quercetin and BHT were employed as standards in this analysis [22].

2.4. Antimicrobial activity

2.4.1. Microbial strains

In these exploratory studies, a total of four bacterial strains were used: one fungal strain, *Candida albicans*, 2 g-negative bacteria,

Escherichia coli ATCC 25922, and *Salmonella enterica serotype* Typhi, and 2 g-positive bacteria, *Staphylococcus aureus* ATCC 29213 and *Bacillus subtilis* ATCC 6633. The present study's bacterial strains were all obtained from the Laboratory of Microbial Biotechnology and Bioactive Molecules at the Faculty of Sciences in Fez, Morocco.

2.4.2. Disc-diffusion assay

Using some reforms and minor adjustments as outlined elsewhere [23], the agar disc-diffusion method was used to assess the antimicrobial efficacy of PC-Eo. Briefly put, bacterial culture suspensions were added to LB agar (bacterium medium) and Yeast Extract-Peptone-Dextrose Agar (*Candida* species) mediums, respectively. Sterile paper discs measuring 6 mm in diameter were impregnated with 10 μL of pure PC-Eo before being put on the agar plates. The standards for bacteria and fungi were the discs saturated with fluconazole (10 μg/disc) and fusidic acid (10 μg/disc), respectively. For 24 h, bacterial cultures were incubated at between thirty and 35 °C. After the incubation period, the inhibitory zone diameters were measured precisely in millimeters. The mean value ± standard error of the mean based on three independent experimental repetitions was presented as the result.

2.4.3. Minimum inhibitory concentration assay (MIC)

The MIC of PC-Eo was determined following a previously documented method [20], with minor adjustments. Serial two-fold dilutions of the PC-Eo were prepared, spanning concentrations from 8.0 to 0.0625 % (v/v) in Mueller–Hinton broth, supplemented with 5 % dimethyl sulfoxide (DMSO). It is worth noting that prior studies have established that DMSO concentrations up to 7.8 % do not exert a significant impact on the viability of microbial cells [24], and our preliminary experiments confirmed that 5.0 % DMSO does not affect microbial growth. Subsequently, 5 μL of a calibrated bacterial suspension was added to each well containing 100 μL of the serially diluted PC-Eo, and 95 μL of sterile LB broth was introduced into all the wells. Negative controls, which consisted of all components without the bacterial suspension, were included, along with positive controls, (Fusidic acid). Following the designated incubation period, 20 mL of 2 mg/mL of *p*-iodonitrotetrazolium chloride (INT) was introduced into all micro-tubes. The plates were subjected to an additional 30-min incubation period, during which the presence of microorganism growth was signified by the appearance of a purple-red color, a consequence of the reduction of INT into formazan. The MIC was determined as the lowest concentration of the extract capable of inhibiting microbial growth following 24 h (bacteria) or 48 h (*Candida* species) of exposure to the PC-Eo.

2.4.4. MBC and MFC assessment

LB agar medium was used to sub-culturing the test dilutions in order to determine the Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC). These plates were then further incubated for a duration of either 48 h for *Candida* species or 24 h for bacteria. The MBC or MFC was the highest dilution at which no single bacterial colony was visible on the plates. Furthermore, MBC/MIC and MFC/MIC ratios were also computed [20].

2.5. Drug-likeness of PC-Eo

To evaluate the safety of the chemical compounds extracted from the *Petroselinum crispum* essential oil and their similarities to candidate drugs, *in-silico* predictions of Lipinski physicochemical characteristics and pharmacokinetic features of absorption, distribution, metabolism, excretion, and toxicity (ADMET) were carried out to discover the potential resemblances of the essential oil's chemical components to therapeutic drugs, so that they could be used as a novel resource of medicinal treatments without causing any undesirable effects in terms of hepatotoxicity and skin sensitivity [25,26]. For this goal, Swiss ADME and pKcsm online servers were used appropriately to achieve the intended results.

2.6. Molecular docking simulations

To investigate the inhibition mechanism of molecules extracted from *Petroselinum crispum* essential oil towards the protein targets, the molecular docking technique was performed using Discovery Studio 2021 [27] and Autodock 4.2 software [28], based on the standard protocol of (ligand-protein) complex preparation as clearly stated and recognized in the literature [29], in which the major compounds of PC-Eo were docked to NADPH oxidase from *Lactobacillus Sanfranciscensis* [30], sterol 14-alpha demethylase (CYP51) from *Candida Albicans* [31], and DNA Gyrase-B from *Escherichia-Coli* [32], as targeted receptors coded in proteins data bank (PDB) basis by 2CDU.pdb, 5TZ1.pdb, and 6F86.pdb, which were extracted by X-ray diffraction method with good resolutions of 1.80 Å, 2.00 Å, and 1.90 Å, respectively.

2.7. Statistical analysis

Every experiment was conducted using three separate tests ($n = 3$), and the results were expressed as mean ± standard deviations (SD). The statistical program GraphPad Prism 8.0 was used to analyze the data. The means were compared using One-way analysis of variance (ANOVA), and a Tukey test was then run. A p-value of less than 0.05 was considered statistically significant.

Table 1
Principal constituents of organic parsley essential oils from Morocco and their relative percentages of total chromatogram area, and Kovats Index.

No	RT[a] (min)	Molecular Formula	Compounds	Kováts Indexes		Peak Area%
				RI[c]	RI lit	
1	1.30	$C_{10}H_{16}$	α -Pinene	927	933	3.95
2	1.72	$C_{10}H_{16}$	β -Pinene	978	980	4.70
3	2.75	$C_{10}H_{16}$	β -Phellandrene	997	1001	1.77
4	7.16	$C_{10}H_{14}O$	Myrtenal	1188	1193	0.23
5	19.64	$C_{11}H_{12}O_3$	Myristicin	1528	1526	21.01
6	20.49	$C_{12}H_{16}O_3$	Elemicin	1550	1554	5.36
7	21.10	$C_{13}H_{18}O_4$	1-allyl-2,3,4,5-tetramethoxybenzene	1590	1591	13.14
8	22.45	$C_{12}H_{14}O_4$	Apiol	1684	1685	49.05
9	23.35	$C_{12}H_{14}O_4$	Dillapiole	1623	1633	0.79
			Chemical classes			
			Phenylpropanoids			89 0.35
			Monoterpene hydrocarbons			10.42
			Oxygenated monoterpenes			0.23
			Total			100 %

[a] Retention times.
[c] Retention index (HP-5MS column), RIL: literature retention index [10].

3. Results and discussion

3.1. Phytochemical profile of Petroselinum crispum essential oil

The essential oil yield of *Petroselinum crispum* was approximately 2.32 ± 0.13 % (V/W), and nine compounds were identified using an HP-5MS capillary column. Gas chromatographic analysis revealed the predominance of monoterpenoids and phenylpropanoids in the essential oil of *P. crispum*, with apiol (49.05 %), myristicin (21.01 %), and 1-allyl-2,3,4,5-tetramethoxybenzene (13.14 %) as major constituents making the total identified components in the essential oil of *P. crispum* 100 % (Table 1 and Figs. 1 and 2).

These findings align with previous research by Maroufpoor et al., who reported the presence of apiol (10.086 %), myristicin (17.184 %), and D-limonene (18.829 %) as major components in Iranian *P. crispum* [33]. Similarly, parsley from Spain was found to have the highest concentration of Apiole, followed by β-phellandrene, *p*-mentha-1,3,8-triene, and myristicin. Another study by Linde et al., revealed that the major compounds in Brazilian parsley were apiol (50.3 %) and myristicin (14.0 %) [8]. Apiol and myristicin have consistently been among the major constituents of parsley essential oil worldwide [34]. Therefore, the quantities of apiole in our samples were similar to those described in the literature.

However, there have been limited studies on various species within the Apiaceae family. Previous findings from Tunisian species, specifically *Foeniculum vulgare* seeds, have identified several compounds shared with those found in PC-Eo. These include phenylpropanoids such as estragole as the predominant compound with a high percentage of 76.2 %, followed by α-thujone (9.6 %) and limonene (8.6 %) [35].

Khammassi et al., obtained comparable findings in their study on the volatile constituents of Tunisian *Deverra tortuosa* (Desf.). Three phenylpropanoid derivatives, namely apiole, methyl eugenol, and elemicin, were detected in low concentrations. Apiole, a characteristic metabolite of the Apiaceae family, was exclusively found in umbels [6].

The diverse chemical variation observed in parsley essential oil could be attributed to genetic and environmental factors affecting the plant's secondary metabolism, the timing of harvesting at different developmental stages, interactions with microorganisms and insects, and various post-harvest techniques, as suggested by previous research [36]. Additionally, the aging of parsley seeds led to a reduction in the concentration of various compounds, such as apiole [13]. Drying also caused decreases in the concentrations of several volatile components, particularly apiole, which was halved compared to fresh samples [14]. Oven drying at 45 °C and freeze-drying resulted in the most significant losses of volatiles. It is worth noting that *p*-mentha-1,3,8-triene is susceptible to photooxidation, and losses of this compound may contribute to aroma changes in parsley following drying or storage [37].

3.2. Antioxidant activity

In today's context, there is a growing interest in assessing the antioxidant activity of natural products and their extracts, particularly within the food, pharmaceutical, and cosmetic industries. An essential aspect of measuring the antioxidant capacity of these active ingredients lies in the selection of appropriate methods. To comprehensively evaluate different mechanisms of action, the antioxidant activity of the parsley samples under investigation was analyzed using five widely recommended *in vitro* assays: DPPH, ABTS, BCBT, RP, and TAC.

Colorimetric methods commonly employed to evaluate the antioxidant activity of various constituents present in plants, foods, beverages, or natural extracts include the DPPH and ABTS assays, which assess free radical scavenging activity. These assays operate on the principle of chemical radical reduction in the presence of antioxidant agents that donate hydrogen [22]. The stability and commercial availability of the DPPH radical have contributed significantly to the recognition of these methods as legitimate, simple, rapid, accurate, reliable, and cost-effective approaches for assessing the free radical scavenging potential of natural antioxidants [38].

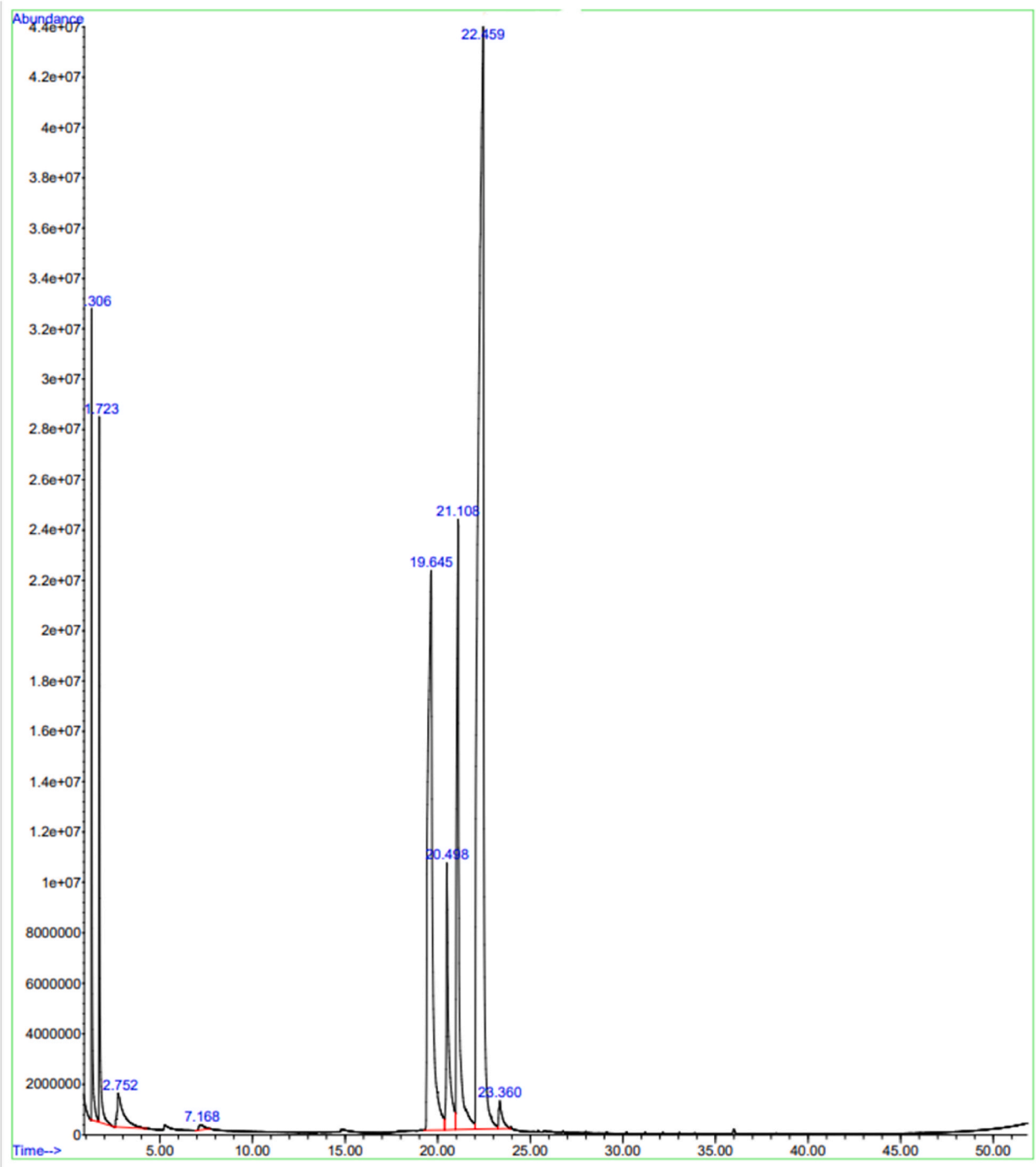

Fig. 1. GC-MS chromatogram of essential oil extracted from *Petroselinum crispum* seeds.

In the DPPH assay, the mechanism of action involves measuring the free radical scavenging activity of the examined extracts by reducing the DPPH$^{\bullet}$ radical to its non-radical form, DPPH-H [39]. Conversely, the ABTS test primarily relies on the chemical interaction between specific antioxidant molecules and the ABTS radical cation (ABTS$^{\bullet+}$). This interaction leads to the quenching of the ABTS$^{\bullet+}$ nitrogen atom by a hydrogen atom from the antioxidant, resulting in the decolorization of the mixture. These methods offer valuable insights into the antioxidant potential of the tested parsley samples by assessing their ability to neutralize free radicals and, consequently, their potential applications in various industries [40].

The method, commonly referred to as "β-carotene bleaching," involves the oxidation of both β-carotene and linoleic acid. This

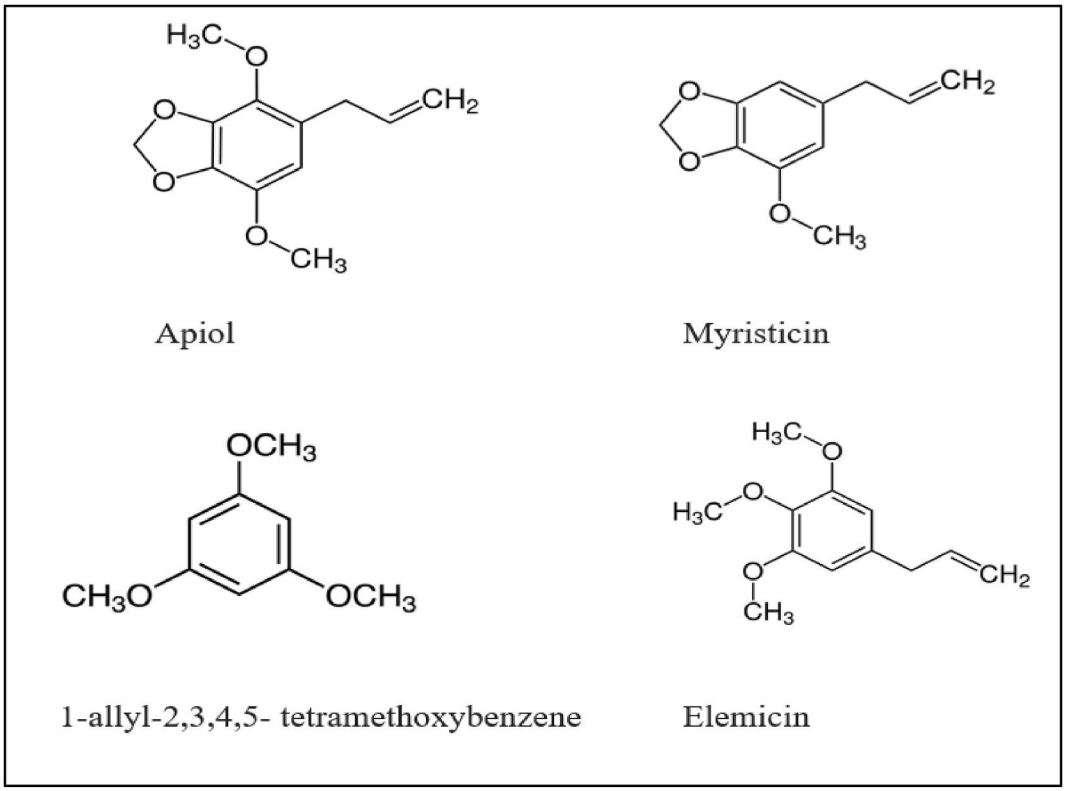

Fig. 2. Molecular structures of phytochemical compounds in PC-Eo.

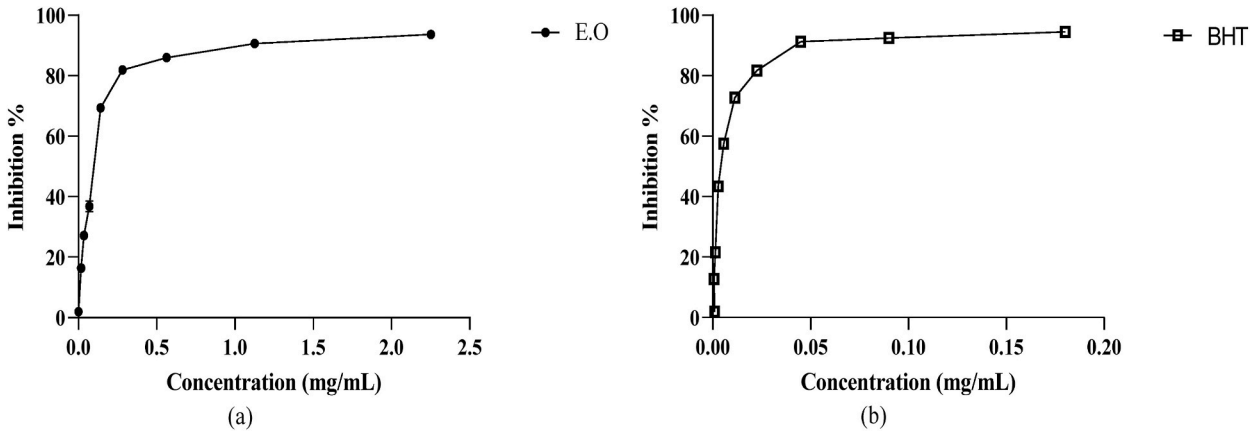

Fig. 3. Antioxidant activity of PC-Eo using DPPH assay (a, b).

technique assesses the ability of antioxidant compounds present in plant extracts to neutralize the linoleic acid peroxide radical, which, in turn, prevents the oxidation of β-carotene during the emulsion phase. In the absence of antioxidants, β-carotene rapidly undergoes discoloration, attributed to the attack by the free linoleic acid radical, leading to the breakage of the molecule's double bonds [41].

Another method employed in this study, RP, is a colorimetric assay that capitalizes on the ability of antioxidant molecules to facilitate the reduction of ferric ions (Fe^{3+} to Fe^{2+}). This reduction is achieved through the action of electron-donating antioxidants [42].

A more comprehensive approach involves employing a spectrum of assessments for individual antioxidants and indicators of oxidative damage, with total antioxidant capacity potentially serving as one of these evaluations. A diminished TAC level might indicate the presence of oxidative stress or an elevated vulnerability to oxidative harm [43].

As depicted in (Fig. 3, Table 2), PC-Eo exhibited substantial antioxidant activity, with notable differences among the assays. In the

Table 2
Antioxidant activities of PC-Eo.

	PC-Eo	BHT	TEAC	Asc Ac
DPPH IC_{50}(mg/mL)	0.52 ± 0.0007	0.024 ± 0.00	–	–
ABTS IC_{50}(mg/mL)	0.47 ± 0.008	–	0.02 ± 0.00	–
BCBT IC_{50}(mg/mL)	2.09 ± 0.017	–	–	0.032 ± 0.00
RP EC_{50}(μg/mL)	1.2 ± 0.00	–	–	10.24 ± 0.07
TAC (mg/mL)	257.26 ± 0.21	–	–	–

ABTS: 2,2′-Azinobis-(3-Ethylbenzthiazolin-6-Sulfonic Acid, Asc Ac: Ascorbic acid, BCBT: β-carotene bleaching test, BHT: 2,6-di-*tert*-butyl-4-methylphenol, DPPH: 2,2-diphenyl 1-picrylhydrazyl, RP: Reducing Power, TAC: Total Antioxidant Capacity, TEAC: Trolox equivalent antioxidant capacity.

Table 3
Antibacterial effect of PC-Eo using Disc diffusion method.

Bacteria[a]	Diameter of Inhibition zone (mm ± SD)[b]	
	PC-Eo	Fusidic acid (10 μg/disc)
***Bacillus subtilis* ATCC 6633**	22.43 ± 1.34[a]	14.63 ± 2.22[b]
Salmonella enterica serotype Typhi	27.43 ± 0.56[a]	10.63 ± 1.65[b]
***Escherichia coli* ATCC 27853**	11.07 ± 2.25[c]	15.70 ± 0.65[a]
***Staphylococcus aureus* ATCC 29213**	17.69 ± 1.45[b]	14.06 ± 0.61[b]
Fungic[a]	**PC-Eo**	**Fluconazole**
Candida albicans	14 ± 0.57	12 ± 1.05

Fusidic acid was used as positive control. Results are expressed as means ± SD, of three independent measurements; [a] Final microbial density was around 10^6 CFU/mL for bacteria and 10^4 CFU/mL for *Candida* species, [b] Diameter of inhibition zone including disc diameter of 6 mm (10 μL of oil/disc). Data with the same letter in the same test present non-significant difference by Tukey's multiple range test (ANOVA, $p < 0.05$).

DPPH test, *Petroselinum crispum* essential oil demonstrated a strong antioxidant activity, exhibiting 81 % inhibition compared to 95 % for the BHT standard (Fig. 3a and b). This is further elucidated by an IC_{50} value of 0.52 ± 0.0007 mg/mL, whereas the positive control (BHT) had an IC_{50} of 0.024 ± 0.00 mg (Table 2).

The ABTS test revealed even higher antiradical capacities, with an IC_{50} value of 0.47 ± 0.008 mg/mL, signifying a concentration required to inhibit 60 % of radicals. These values, although lower than those reported by H. Zhang et al. and Irene Marín et al., where IC_{50} values were 80.21 mg/mL and 12.91 g/L for DPPH, are substantial and indicative of strong antioxidant potential [10,44]. The highest antioxidant activity ranged from 69.82 ± 3.07 to 50.06 ± 2.47 mmTrolox® eq./10 g extract for ABTS [45].

According to β-carotene bleaching test, it was determined to be 2.09 ± 0.017 mg/mL. Considering the previous study, our results were determined strongly when compared with Okan et al., with a RAA of 78.21 % [46].

The results from the RP assay, depicted in Table 2, revealed a relatively high EC_{50} value of 1.2 ± 0.00 μg/mL compared to the ascorbic acid standard (10.24 ± 0.07 μg/mL). These findings surpass those reported by Irene Marín et al., for Spanish parsley, where the FRAP value oscillated between 0.40 ± 0.00 and 0.93 ± 0.07 mmol/L Trolox [10].

The results presented in Table 2 indicate that our essential oil possesses a remarkable total antioxidant capacity value of 233.72 ± 0.12 mg AAE/mL of Eo, in comparison to 257.26 ± 0.21 mg EAA/g for Quercetin and 269.2 ± 0.10 mg EAA/g for BHT as standards. These results significantly exceed those reported by El-Sayed et al., for Egypt *Petroselinom crispum,* which had an EC_{50} of 166.83 ± 1.96 acid eq./g extract [47].

However, limited studies have been conducted on various species within the Apiaceae family. Recent research on the antioxidant potential of metabolites synthesized by *Foeniculum vulgare* has been documented. According to Khammassie et al., both essential oils and non-volatile metabolites represented by crude extracts were found to possess significant antioxidant potential, sharing several capacities with those observed in *Petroselinum crispum* Eo [35].

In their study, Khammassie et al., highlighted that ethanolic extracts of wild fennel seeds exhibited notable antioxidant activity, as evidenced by scavenging free radicals DPPH and ABTS, as well as in reducing power assays and total antioxidant activity [6].

In a subsequent investigation by Khammassie et al., Tunisian essential oils from Zaghouan demonstrated the highest antioxidant activity (8.5 ± 0.05 mg GAE/g DW). Additionally, essential oils from Oued Zarga exhibited the most potent DPPH radical scavenging activity (IC_{50} = 7.7 ± 0.43 mg/mL), while those from the In Gulf and Baladar regions displayed the highest ABTS radical scavenging activity (IC_{50} = 86.86 ± 0.23 mg/mL and 87.33 ± 0.17 mg/mL, respectively). Furthermore, the most significant reducing power assay was observed in Ain Ghlel and Charchara essential oils (EC_{50} of 70.46 ± 0.17 mg/mL and 71.31 ± 0.61 mg/mL, respectively) [16].

The robust antioxidant capacities observed in parsley extracts are often associated with the dominant essential oil constituents, particularly phenylpropanoids like apiol, myristicin, and 1-allyl-2,3,4,5-tetramethoxybenzene, which were detected in the highest concentrations (Table 1). The antioxidant potency of the essential oil is a result of the synergistic interactions among its complex chemical components. Given this substantial antioxidant capacity of PC-Eo, we conducted further antimicrobial tests to validate this activity.

Table 4
Antibacterial effect of PC- Eo using broth microdilution method.

Bacteria[a]	PC-Eo (% v/v)			Fusidic acid (μg/mL)		
	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
***B. subtilis* ATCC 6633**	0.5	0.5	1.0	32	32	1.0
***S. enterica* serotype Typhi**	0.5	1.0	2.0	64	32	2.0
***E. coli* ATCC 27853**	2.0	2.0	1.0	356	356	1.0
***S. aureus* ATCC 29213**	0.25	1.0	4.0	64	64	1.0
Fungi[a]	**PC-Eo (% v/v)**			**Fluconazole (μg/mL)**		
	MIC	**MFC**	**MFC/MIC**	**MIC**	**MFC**	**MFC/MIC**
Candida albicans	1.25	2.5	2.0	32	64	2.0

[a] Final microbial density was around 10^6 for bacteria and 10^4, for fungi; MIC: Minimum inhibitory concentration in % (v/v), MBC: Minimum Bactericidal concentration in % (v/v). Fusidic acid and fluconazole were used as standard drugs.

Table 5
Prediction of Lipinski physico-chemical features for nine molecules extracted from PC essential oil.

Compounds number	Physico-chemical properties					Lipinski's five rules
	MW	MR index	Log P	HBA	HBD	(No/Yes)
Rule	≤500(g/mol)	130≥ MR index ≥40	<5	≤10	<5	
C 1	136.23	45.22	2.63	0	0	Yes
C 2	136.23	45.22	2.59	0	0	Yes
C 3	136.23	47.12	2.65	0	0	Yes
C 4	150.22	45.42	2.08	1	0	Yes
C 5	192.21	53.10	2.67	3	0	Yes
C 6	208.25	60.02	2.89	3	0	Yes
C 7	238.28	66.52	3.23	4	0	Yes
C 8	222.24	59.59	2.85	4	0	Yes
C 9	222.24	59.60	2.82	4	0	Yes

MW: molecular weight (g/mol); MR: molar refractivity index; HBA: Hydrogen bond acceptors; HBD: Hydrogen bond donors; Log P: partition coefficient (indicating lipophilicity in octanol/water solvent).

3.3. Antimicrobial activity of PC-Eo

The disc diffusion method was employed to assess the inhibitory effects of Moroccan organic EOs against *Bacillus subtilis* ATCC 6633, *Salmonella enterica serotype Typhi*, *Escherichia coli* ATCC 27853, *Staphylococcus aureus* ATCC 29213 and *Candida albicans*. Table 3 outlines the inhibitory halos and antibacterial/antifungal properties of the PC-tested oil. Eo activities were categorized as low (<10 mm), moderate (>10–15 mm), and high (>15 mm) based on the inhibition zone diameter [20]. The PC-Eo exhibited the highest antibacterial activity against *Salmonella enterica serotype* Typhi (27.43 ± 0.56 mm), followed by *Bacillus subtilis* (22.43 ± 1.34 mm), and *Staphylococcus aureus* (17.69 ± 1.45 mm), while moderate activity was observed against *Escherichia coli* (11.07 ± 2.25 mm).

In comparison to reference antibiotics, gram-positive bacteria were more susceptible, and these findings were statistically significant (ANOVA, $p < 0.05$). In terms of antifungal potential, disc diffusion demonstrated moderate activity against *Candida albicans* (14 ± 0.57 mm); the outcomes were noteworthy and similar to the antifungal medication that was used as a reference (Table 3). The broth microdilution method determined MIC, MBC, and MFC values (Table 4). The lowest MIC and MBC values were recorded with Gram-positive bacteria (*S. aureus*, *B. subtilis*, *S. enterica*), ranging from 0.25 to 0.5 % v/v for MIC and 0.5–1.0 % v/v for MBC. For Gram-negative bacteria (*E. coli*), MIC and MBC values were 2.0 % v/v. These results supported the disc-diffusion method findings (Table 3). For the *Candida albicans* fungal strain, MIC = 1.25 % v/v, and MFC = 2.5 %, confirming the notable antifungal efficacy of PC-Eo. MBC/MIC and MFC/MIC ratios indicated that PC-Eo has a bactericidal and fungicidal mechanism.

This conclusion is based on the observation that antimicrobial agents fall into the bactericidal or fungicidal category if the ratio of MBC/MIC to MFC/MIC is 4.0 or lower. According to this ratio, it should be possible to obtain tested agent concentrations that are capable of eliminating 99.9 % of the treated microorganisms. Ratios higher than 4.0 could suggest difficulties in administering dosages high enough to kill microorganisms at the same level, designating the substance as bacteriostatic [20].

Previous studies have indicated that essential oils exhibit potent inhibitory effects on the mycelium growth of plant pathogenic fungi [48]. In a study by Viuda et al., parsley essential oil demonstrated inhibition zones of 11.50 mm only against *L. innocua* and did not exhibit any inhibitory effect on the other two tested bacteria (*S. marcescens* and *P. fluorescens*) at all added doses (2 μL, 5 μL, and 10 μL) [49]. Numerous earlier reports on parsley seed oil support the evidence of inhibitory effects of seed oil on the growth and development of fungi [50].

Within the same family (Apiaceae), essential oils extracted from the leaves of *12 Foeniculum vulgare* Mill populations, growing wild in Tunisia, were subjected to evaluation against eight pathogenic bacteria and fungi, showcasing potent inhibitory effects akin to those of our plant, *Petroselinum crispum*.

The results elucidated the sensitivity of the tested strains to fennel essential oils, particularly pronounced against gram-positive

Table 6
Prediction of ADME-Tox features for nine molecules extracted from PC-Eo.

Compounds Number	A	D		M							E	T		
	human intestinal Absorption	Blood-brain barrier permeability	Central nervous system permeability	Substrate Cytochromes		Inhibitor					Total Clearance	AMES test of toxicity	Hepatotoxicity	Skin Sensitization
				2D-6	3A-4	1A-2	2C-19	2C-9	2D-6	3A-4				
	(% Absorbed)	(Log BB)	(Log PS)	(No/Yes)							Numeric (Log ml/min/kg)	(No/Yes)		
C 1	96.041	0.791	−2.201	No	No	No	No	No	No	No	0.043	No	No	No
C 2	95.984	0.829	−1.867	No	No	No	No	No	No	No	0.03	No	No	No
C 3	96.045	0.747	−2.023	No	No	No	No	No	No	No	0.196	No	No	**Yes**
C 4	96.41	0.807	−2.328	No	No	No	No	No	No	No	−0.004	No	No	**Yes**
C 5	97.684	0.293	−2.149	No	No	**Yes**	No	No	No	No	0.199	No	No	No
C 6	96.861	0.44	−2.101	No	No	**Yes**	No	No	No	**Yes**	0.366	**Yes**	No	**Yes**
C 7	98.641	0.35	−2.376	No	No	No	No	No	No	No	0.491	No	No	No
C 8	98.965	0.468	−2.356	No	No	**Yes**	No	No	No	**Yes**	0.269	No	No	No
C 9	98.269	0.464	−2.27	No	No	**Yes**	No	No	No	**Yes**	0.252	No	No	**Yes**

A: Absorption; D: Distribution; M: Metabolism; E: Excretion; T: Toxicity.

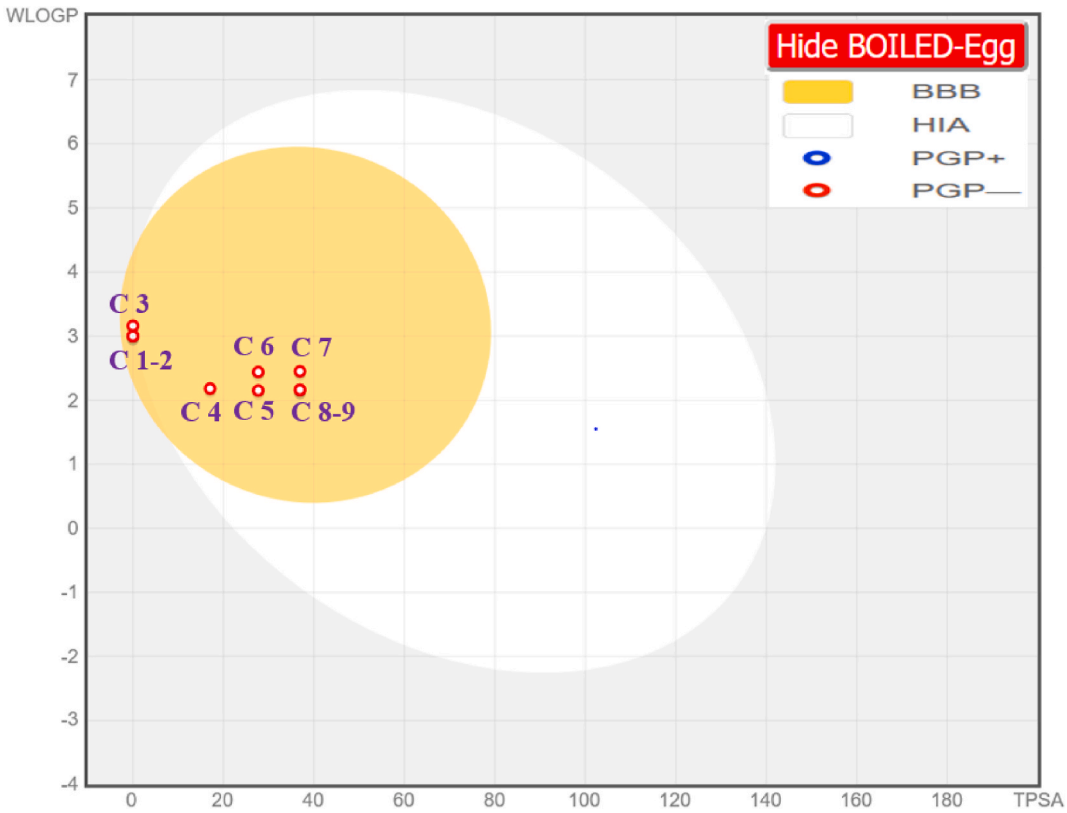

Fig. 4. The predictive model of Egan Boiled egg for nine extracted molecules.

strains. Notably, essential oils derived from the Zaghouan population exhibited the highest efficacy against *M. coli*, *L. coli*, and *Staphylococcus aureus*. These findings significantly contribute to our comprehension of the chemical diversity present in wild fennel across varied geographical origins and pedoclimatic conditions, underscoring the existence of distinct chemotypes of essential oils within Tunisian natural populations of *F.* vulgare [16].

Microorganisms are affected by various antimicrobial mechanisms by EOs, including attacking the phospholipid bilayer on their cell membranes, disrupting enzyme systems, compromising their genetic material, and forming fatty acid hydroperoxidase [51], through oxygenation of unsaturated fatty acids. Proteins and lipids may be harmed by the EOs' ability to coagulate in the cytoplasm. Their mode of action would resemble that of other phenolics, meaning it would involve the disruption of electron flow, active transport, the proton motive force (PMF), and the coagulation of cell contents. On the other hand, it is known that lipid molecules surround and reside in the cytoplasmic membrane of enzymes like ATPases [10]. The hydrophobic properties of EOs and their constituents allow them to partition into the lipids of the bacterial cell membrane and mitochondria, causing structural distortion and increasing susceptibility to antimicrobial action, ultimately resulting in cell content leakage [52]. The specific mechanism of action and antibacterial activity of each constituent essential oil are therefore influenced by its molecular makeup. Furthermore, the toxicity of the EOs appears to be related to the hydrophobicity optimum range [53].

The efficacy of EOs is also influenced by the type of bacteria. Gram-negative bacteria, such as *Bacillus subtilis* and *Salmonella enterica*, were generally less sensitive than gram-positive bacteria, as exemplified by *Bacillus subtilis* [3]. Different bacteria exhibit varying susceptibility to an Eo, likely due to differences in the structure of their cell membranes. Gram-negative bacteria possess more complex cell envelopes compared to Gram-positive bacteria. Gram-negative bacteria consist of two layers that provide rigidity and protection to the cell [54]. In contrast, gram-positive bacteria lack the outer membrane, potentially making them more susceptible to the action of phenolic components present in EOs [52]. In fact, the lipophilic ends of the lipoteichoic acids in the cell membrane of Gram-positive bacteria may aid in the penetration of hydrophobic compounds, whereas the external membrane of Gram-negative bacteria makes their surfaces extremely hydrophobic [48].

3.4. Drug-likeness of PC-Eo components

Based on *in vitro* investigations and once the chemical composition of *Petroselinum crispum* essential oil had been identified by Gas chromatography, all nine active molecules were examined using *in-silico* ADME-Tox predictions based on Lipinski rules of five, bioavailability radars, and the predictive model of Egan boiled-egg. As shown in Table 5, the nine extracted molecules of PC- Eo meet all five rules of Lipinski physicochemical properties (Molecular weight less than 500 g/mol, molar refractivity index comprised in [40,

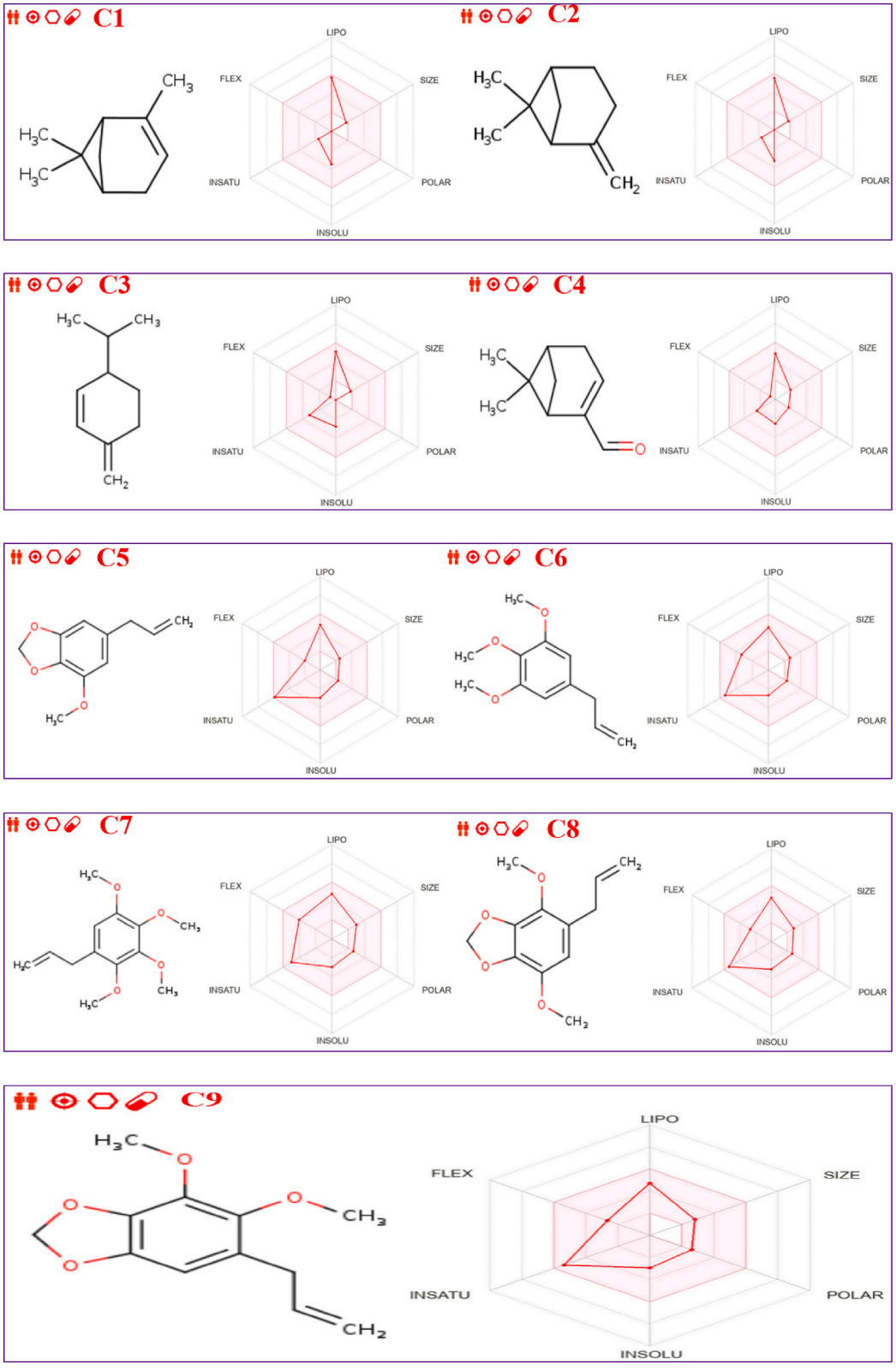

Fig. 5. Oral bioavailability prediction of extracted molecules from PC-Eo. **C1**: α -Pinene, **C2**: β -Pinene, **C3**: β -Phellandrene, **C4**: Myrtenal, **C5**: Myristicin, **C6**: Elemicin, **C7**: 1-allyl-2,3,4,5-tetramethoxybenzene, **C8**: Apiol, **C9**: Dillapiole.

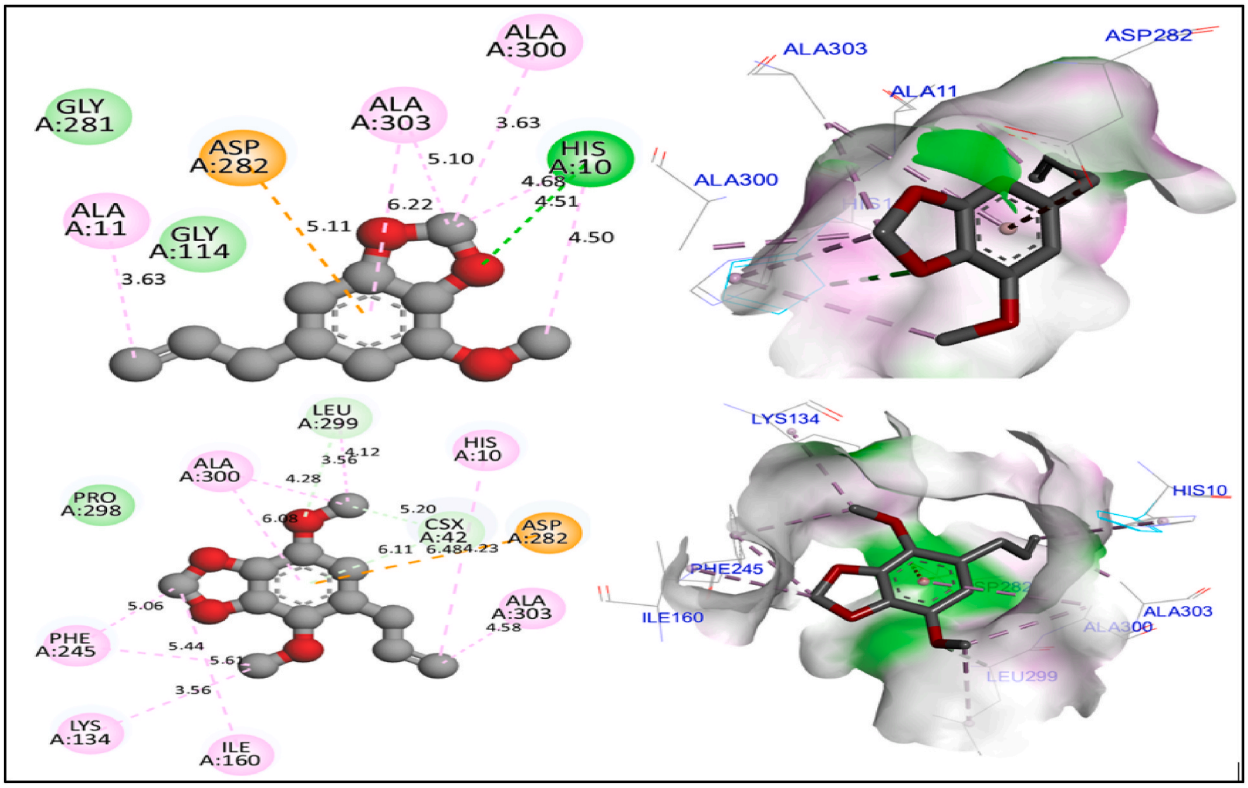

Fig. 6. 2D and 3D views of intermolecular interactions produced between NADPH oxidase protein (2CDU.pdb) and the major compounds of PC-Eo (C5 and C8, respectively).

130] rang, lipophilicity in octanol/water solvent defined by a Log P value inferior to 5, numbers of donors and acceptors of Hydrogen bonds do not exceed 5 and 10, respectively [55]. So, they were predicted with a large resemblance to candidates-drugs [26]. Furthermore, the extracted compounds were predicted with good ADME-Tox pharmacokinetic profiles, justified by the good levels of human intestinal absorption (HIA exceeding 95 %), good permeabilities to the central nervous system (CNS) and the blood-brain barrier (BBB), explained by the ranges included in [0,1] Log BB, and [−1, −3] Log PS, respectively. In addition to their safety, they do not cause any undesirable side effects of hepatotoxicity and skin allergy, except for the compounds labeled C3, C4, C6, and C9 which have been predicted by their positive effects to generate skin allergy. Furthermore, the metabolism of studied molecules demonstrates that C5, C6, C8, and C9 compounds were defined as potent inhibitors of 1A2 cytochrome, while C6, C8, and C9 compounds were equally predicted as potent agents of 3A4 cytochrome.

Most importantly, the major compounds of this essential oil, namely (C5) and (C8) were predicted to be completely non-toxic, without causing the undesirable effects of hepatotoxicity and skin allergy, well-absorbed by the human organism, well-permeable to BBB and CNS, and predicted as potent inhibitors of 1A2 cytochrome. So, they were discovered with very good ADMET profiles, as resulted in Table 6.

Moreover, Egan's boiled egg predictive model, widely applied in the drug discovery field, detects potent CNS inhibitors as shown in Fig. 4, in which we noticed that all extracted compounds from PC-Eo are part of yellow Egan egg, so they were predicted to traverse the blood-brain barrier and therefore considered potent CNS inhibitors (Egan et al., 2000) [25]. The oral bioavailability of the extracted molecules was also examined using the bioavailability radars test, based on six physicochemical features of lipophilicity (LIPO), flexibility (FLEX), unsaturation (INSATU), solubility (INSOLU), polarity (POLAR), and size (SIZE), which displays the highest levels of bioavailability due to the localization of all nine molecules into the pink area as an ideal zone of oral bioavailability [56], as presented in Fig. 5.

3.5. Molecular docking

In the present study, two major compounds of PC-Eo were docked to three targeted proteins, to explore the inhibition mechanisms of antioxidant, antifungal, and antibacterial activities against *Lactobacillus Sanfranciscensis, Candida Albicans*, and *Escherichia-Coli* pathogenic strains.

The results obtained in Fig. 6 show that the main compounds labeled C5 and C8 were docked into the active sites of NADPH oxidase protein target encoded by 2CDU.pdb, with binding energies of −5.67 and −5.44 kcal/mol respectively, producing a variety of chemical bonds in A chain of the targeted protein, which include one Hydrogen bond created with His10 amino acid residue (AAR), one Pi-Anion

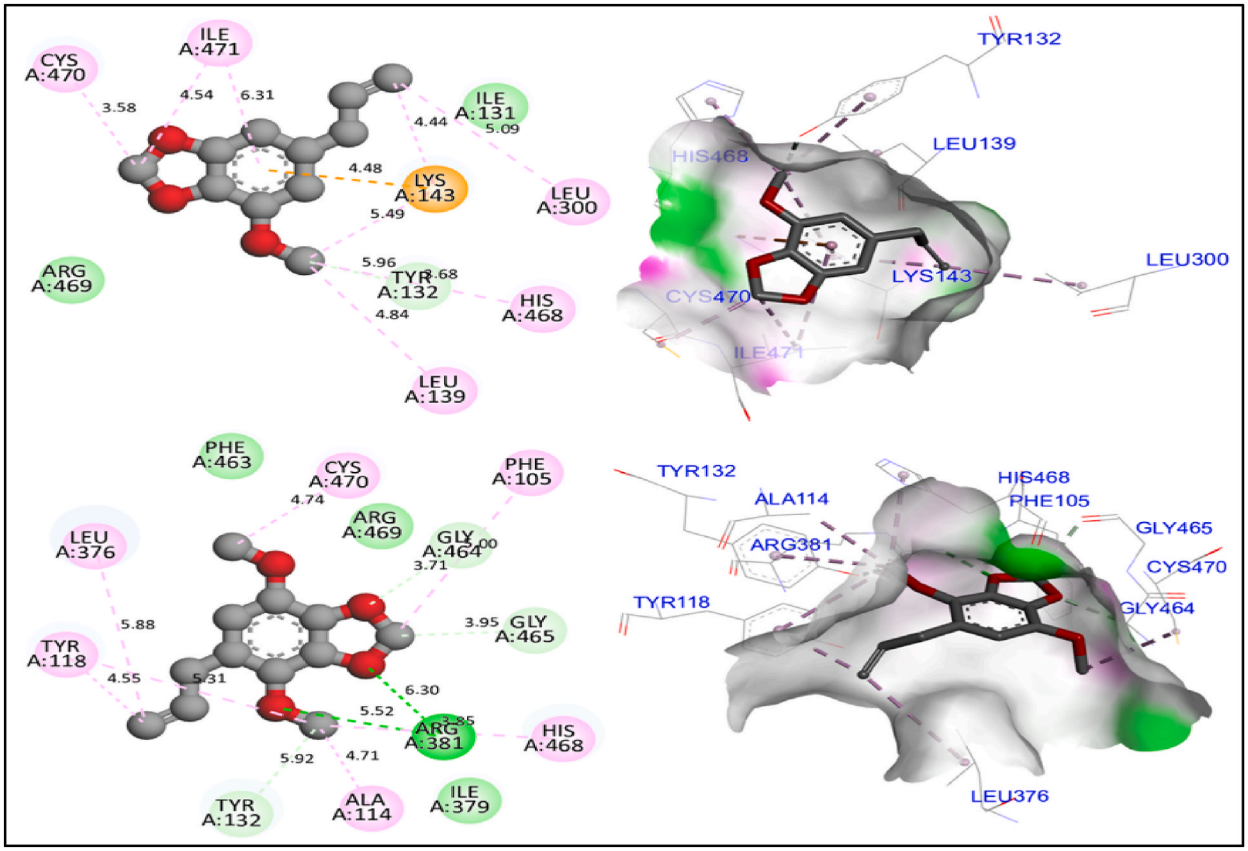

Fig. 7. 2D and 3D views of intermolecular interactions produced between Sterol 14-alpha demethylase (CYP51) protein (5TZ1.pdb) and the major compounds of PC-Eo (C5 and C8, respectively).

bond detected with Asp282 AAR, more than Alkyl and Pi-Alkyl bonds detected with Ala11, Ala300, and Ala303 AARs for the compound C5. Almost the same intermolecular interactions were obtained in (C8-NADPH oxidase protein) complex, more than additional Alkyl bonds which were produced towards Phe245, Lys134, Ile160, and two Carbon–Hydrogen bonds resulted towards Csx40 and Leu299 AARs.

Regarding the molecular docking results exposed in Fig. 7, the major compounds of PC essential oil were equally docked to the active sites of antifungal protein from Candid Albicans, encoded in the protein data bank by 5TZ1.pdb, in which the compound C5 was complexed to targeted protein with a binding energy of −4.63 kcal/mol, forming one Pi-anion bond with Lys143 AAR, more than Alkyl, and Pi-Alkyl bonds created with His468, Leu300, Leu139, Cys470, and Ile471 AARs, while the compound C8 was complexed with the same receptor protein with a binding energy of −4.30 kcal/mol, creating one Hydrogen bond with Arg381, two Carbon–Hydrogen bonds with Gly464, Gly465, and Tyr132 AARs, more than six Alkyl bonds detected towards His468, Cys470, Phe105, Tyr118, Leu376, and Ala114 AARs.

For the next time, C5 and C8 were also docked to the active sites of DNA Gyrase-B from *Escherichia-Coli* with binding energies of −4.48 and −4.32 kcal/mol, as resulted in Fig. 8. Based on the recently published work of Caixin Yuan, and Xiuqiao Hao [29], DNA Gyrase-B encoded in PDB basis by 6F86, was considered as the targeted protein of antibacterial activity against pathogenic strains, in which the main compounds of PC-Eo created similar intermolecular interactions as two Pi-anion bonds detected with Gly50, and Arg76 AARs, more than chemical bond produced with Gly77 AAR, and another Alkyl bond with Pro77 AAR.

To conclude the inhibition mechanisms against the pathogenic strains, the results of six molecular docking simulations are well summarized in Table 7, in which we concluded that antioxidant effect of major compounds extracted from PC-Eo is justified by the presence of common intermolecular interactions which were produced towards Ala11, Ala300, Ala303, and Asp282 AARs of NADPH oxidase protein, while the antifungal activity against *C. albicans* is also explained by the presence of common interactions detected towards His468 and Cys470 AARs of CYP51 protein. So that antibacterial effect is clearly demonstrated by the production of similar interactions created between the same examined compounds and Glu50, Arg76, Gly77, and Pro79 AAR of DNA Gyrase-B protein.

4. Conclusion

Parsley essential oils (EOs), derived from Moroccan seeds, have demonstrated significant antioxidant activity across various

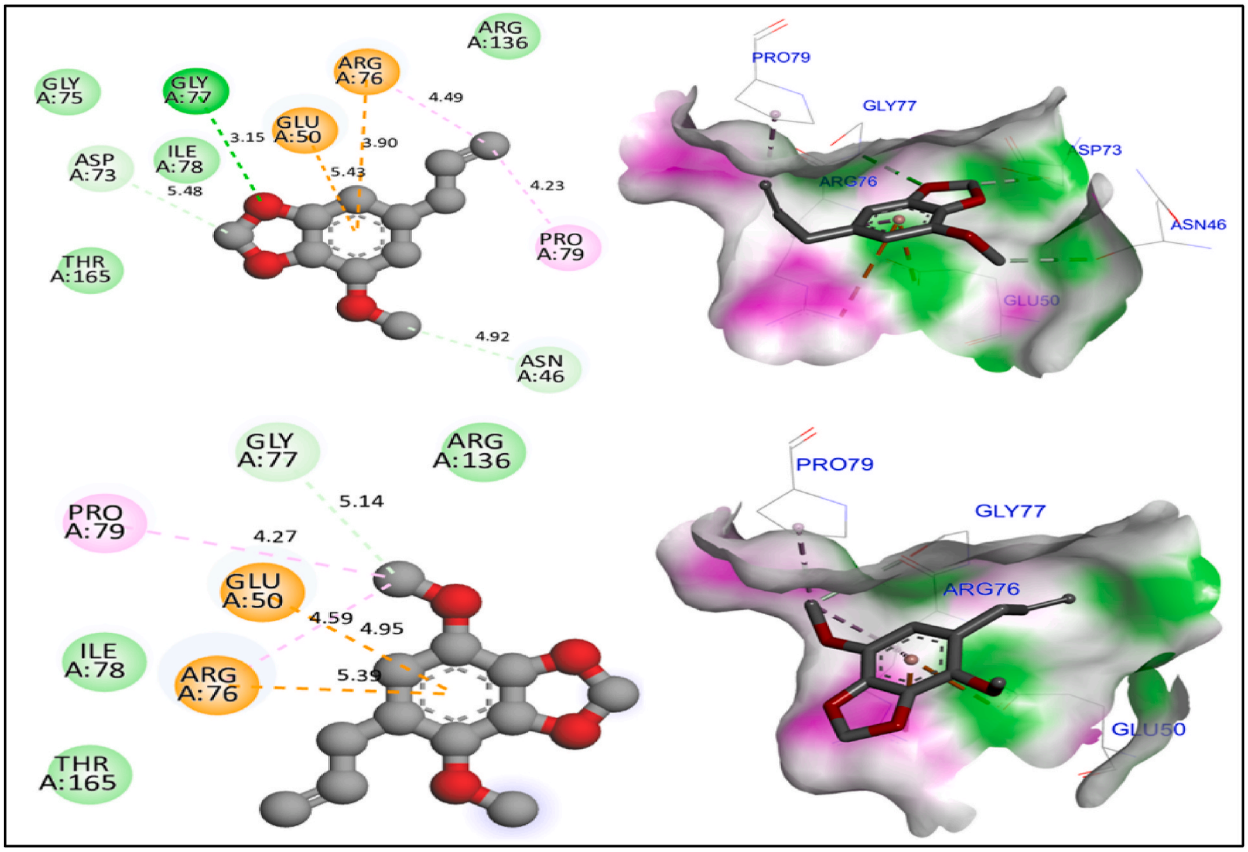

Fig. 8. 2D and 3D views of intermolecular interactions produced between DNA Gyrase -B-protein (6F86.pdb) and the major compounds of PC-Eo (C5 and C8, respectively).

Table 7
Intermolecular interactions produced between the main compounds of PC-Eo and three targeted proteins.

Major chemical compounds	Types of intermolecular interactions			
	Hydrogen bond	Alkyl, Pi-Alkyl bonds	Pi-Anion bond	C–H bond
NADPH oxidase protein (2CDU.pdb)				
Myristicin (C5)	His10	**Ala11-Ala300-Ala303**	**Asp282**	–
Apiol (C8)	–	**Ala11-Ala300-Ala303**-Phe245-Lys134-Ile160	**Asp282**	Leu292-Csx40
CYP51 protein (5TZ1.pdb)				
Myristicin (C5)		**His468-Cys470**-Leu139-Leu300- Ile471	Lys143	–
Apiol (C8)	Arg381	**His468-Cys470**-Phe105-Leu376-Tyr118-Ala114-	–	Gly464-Gly465-Tyr132
DNA Gyrase-B protein (6F86.pdb)				
Myristicin (C5)	**Gly77**	**Pro79**	**Glu50-Arg76**	Asp73
Apiol (C8)	–	**Pro79**	**Glu50-Arg76**	**Gly77**

methods, likely attributed to bioactive compounds identified through GC-MS analysis in the volatile portion of *P. crispum*. Notably, primary compounds within PC-EO exhibit favorable pharmacokinetic characteristics according to ADMET simulation.

Amidst a growing preference for natural additives over artificial preservatives, essential oils present promising prospects in the food, cosmetic, and pharmaceutical industries. With their environmentally friendly and biodegradable qualities, PC-EOs could serve as effective biopesticides in agriculture. Additionally, their antimicrobial effects further enhance their potential applications.

Moreover, the medical and pharmaceutical sectors stand to benefit from PC-EOs' robust biomedical applications, including their potential utilization in nanodelivery systems. However, validating the pharmacological effects of this plant necessitates more in-vivo and clinical research. Furthermore, comprehensive toxicity evaluations are essential to ensure safety across various applications.

In conclusion, parsley essential oils hold immense promise in various industries, driven by their antioxidant, antimicrobial, and pharmacokinetic properties. Continued research and development efforts are imperative to fully exploit their potential and ensure

their safe and effective utilization in diverse applications.

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CRediT authorship contribution statement

Ghizlane Nouioura: Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mohamed El fadili:** Writing – review & editing, Software, Data curation, Conceptualization. **Naoufal El Hachlafi:** Writing – review & editing, Data curation, Conceptualization. **Hatem A. Abuelizz:** Funding acquisition. **Ahmed Elfallaki Elidrissi:** Methodology, Investigation. **Mohamed Ferioun:** Data curation. **Najoua Soulo:** Formal analysis. **Sara Er-rahmani:** Funding acquisition. **Badiaa Lyoussi:** Writing – review & editing, Supervision. **Elhoussine Derwich:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interestsHatem A. Abuelizz reports financial support was provided by King Saud University, Riyadh, Saudi Arabia. Hatem A. Abuelizz reports a relationship with King Saud University, Riyadh, Saudi Arabia. that includes: board membership and funding grants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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