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Article

J. Biol. Regul. Homeost. Agents. 2024; 38(3): 2673–2691
https://doi.org/10.23812/j.biol.regul.homeost.agents.20243803.211

Unveiling the Medicinal Power of *Citrus limon* Essential Oil: A Comprehensive Exploration of Antioxidant and Antimitotic Properties, Phytochemical Composition, and Molecular Inter-Actions

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Published: 1 March 2024

Background: The essential oil (EO) extracted from *Citrus limon* has gained attention for its potential bioactive properties due to its promising chemical composition. This study aimed to comprehensively evaluate the antioxidant and antimitotic activities of *Citrus limon* EO.

Methods: Volatile compounds were thoroughly analyzed using headspace and gas chromatography techniques. Molecular docking studies identified D-limonene and α-bergamotene as having the highest antioxidant potential. Assessment of antioxidant capacity employed 2,2-diphenyl-1-picrylhydrazyl (DPPH) and β-Carotene bleaching assays, alongside the Total Antioxidant Capacity (TAC) assay. Absorption, distribution, metabolism, excretion and toxicity (ADMET) tests were conducted to confirm the EO's suitability for pharmaceutical applications. The antimitotic activity was evaluated using the *Lepidium sativum* test.

Results: Results indicated a 1.99% essential oil yield, with D-limonene identified as the predominant constituent. In the DPPH assay, the half-maximal inhibitory concentration (IC_{50}) value was 1.27 $\pm$ 0.03 mg/mL, and the β-carotene bleaching assay exhibited an IC_{50} value of 6.74 $\pm$ 0.12 mg/mL. The TAC assay determined the EO's total antioxidant capacity as 212.2 $\pm$ 1.35 µg Ascorbic acid (AA) eq/mg EO. The essential oil demonstrated significant antimitotic activity, reaching a maximum of 83.33% at a concentration of 250 µg/mL.

Conclusions: These findings underscore the noteworthy antioxidant and antimitotic capabilities of *Citrus limon* essential oil, primarily attributed to D-limonene and other bioactive components.

Keywords: *Citrus limon*; essential oil; antioxidant activity; antimitotic activity; headspace

Introduction

Citrus limon also known as lemon is a tree from the rutaceae family, native to Asia [1]. Lemon tree is very known with its high nutritional value by the existence of multitude of natural compounds citric acid, ascorbic acid, minerals, and flavonoids. *C. limon* is the most significant fruit tree crop worldwide with an annual production of over 102 million tonnes. Despite the fact that vitamin C concentration has traditionally been linked to its health-related benefits, it has recently been shown that flavonoids also play a role in this [2]. Flavonoids may have a variety of biological effects, including anti-oxidative, anti-inflammatory, anti-allergic, antiviral, anti-proliferative, antimutagenic, and anticarcinogenic actions, according to some authors [3–5] . The main way to acquire *Citrus* essential oils, including lemon essential oil, is through the cold-pressing or steam distillation of the *Citrus* fruit peels. Lemon essential oil in particular is well-known for its energizing scent and has been utilized for its aromatic and flavorful capabilities in the food, fragrance, and cosmetic industries [6]. The use of aromatic molecules from essential oils in aromatherapy, a well-known technique for promoting relaxation, lowering stress, and influencing mood, has been shown to have an effect on human behavior. Some people have reported feeling better after inhaling specific essential oils, particularly lemon oil, which has been shown to improve mental and emotional health [7]. *Citrus* tree is very important in Morocco since it generates more than 432 million dollars [8]. 128,000 ha is dedicated to all type of *Citrus* growing with a total of 2.4 million tonnes of total production between 2015 and 2019, 2.5% of it is from *C. limon* [9]. *Citrus* peels can be a rich source of essential oils, but by re-

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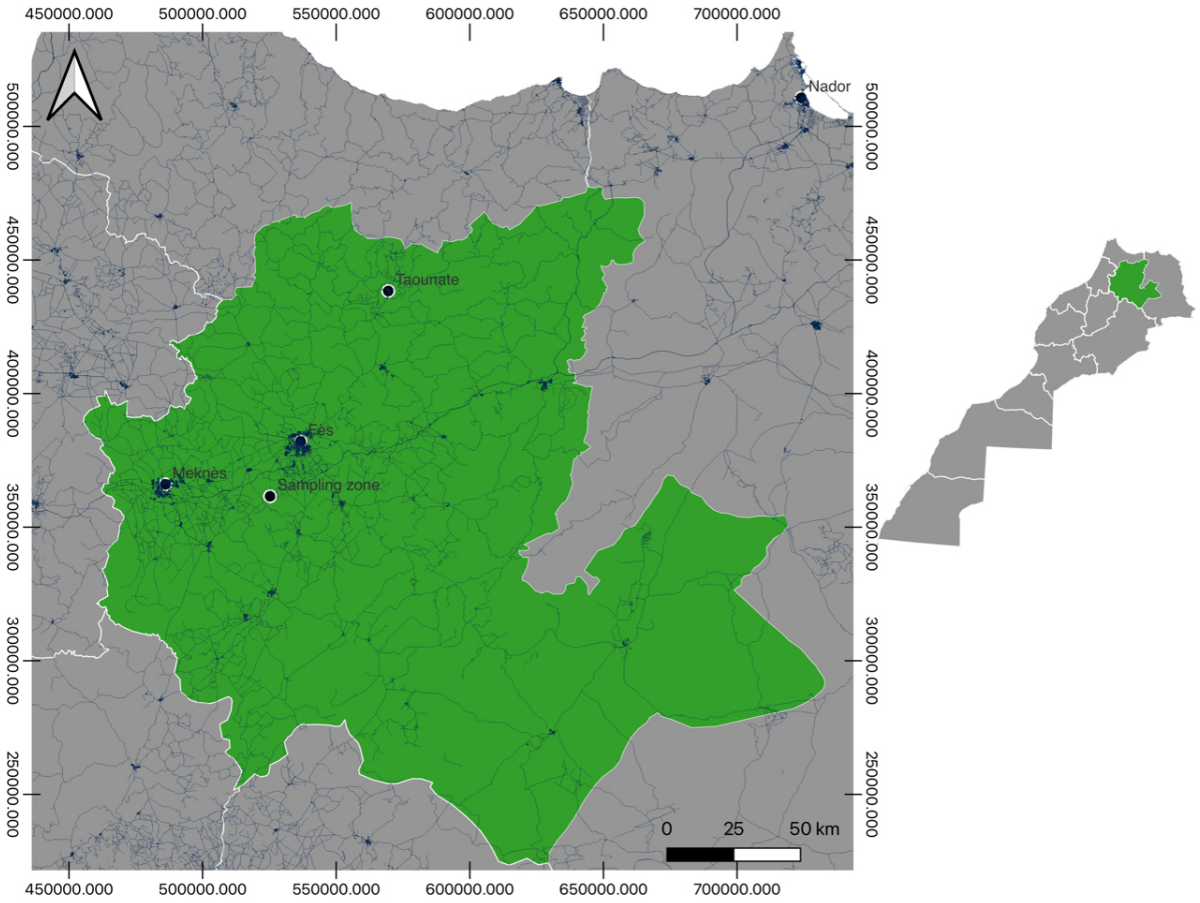

Fig. 1. Sampling zone in the Ain Taoujdate region, geographic coordinates 33°52'55.5"N 5°08'38.4"W.

Table 1. *Citrus limon* essential oil (EO) yield.

	Extraction 1	Extraction 2	Extraction 3	Mean ± Std
Quantity of EO (mg)	1942.12	1825.87	2213.34	1993.77 ± 198.33
Yield in %	1.94%	1.82%	2.21%	1.99 ± 0.19%

cycling trash from the agricultural economy, their use can also help reduce waste and advance sustainability. Furthermore, *C. limon* essential oil is known to have large spectrum of effects such antimicrobial, antioxidant and antiproliferative effects [10] due to the presence of several bioactive compounds. The present investigation aims to determine the phytochemical composition of *C. limon* essential oil using gas-chromatography and headspace technique coupled to mass spectroscopy. Followed by, an *in-silico* study for the abundant molecules identified and the analysis of their pharmacokinetic properties. Finally, the *in vitro* antioxidant and antimitotic activities of *C. limon* essential oil (EO) were assessed.

Materials and Methods

Study Area and Sampling

The investigation was conducted in the Fes Meknes region, more precisely in the proximity of the town of Taoujdate, (Fig. 1) on a modern farm, including several fruit crops and citrus fruits, including *Citrus limon*, which are distributed over an area of 50 hectares. The culture of *Citrus limon* is planted on a plot of 5 ha. Crop irrigation is provided by a modern irrigation system that respects the needs of each crop.

The fruits were harvested in February 2022 from various shrubs where a 10 Kg of lemons were taken from lemon trees (Fig. 2).

Extraction Method

Citrus limon essential oil was obtained using hydrodistillation technique, where 100 g of lemon zest was put

Fig. 2. Location and detailed plan of study area.

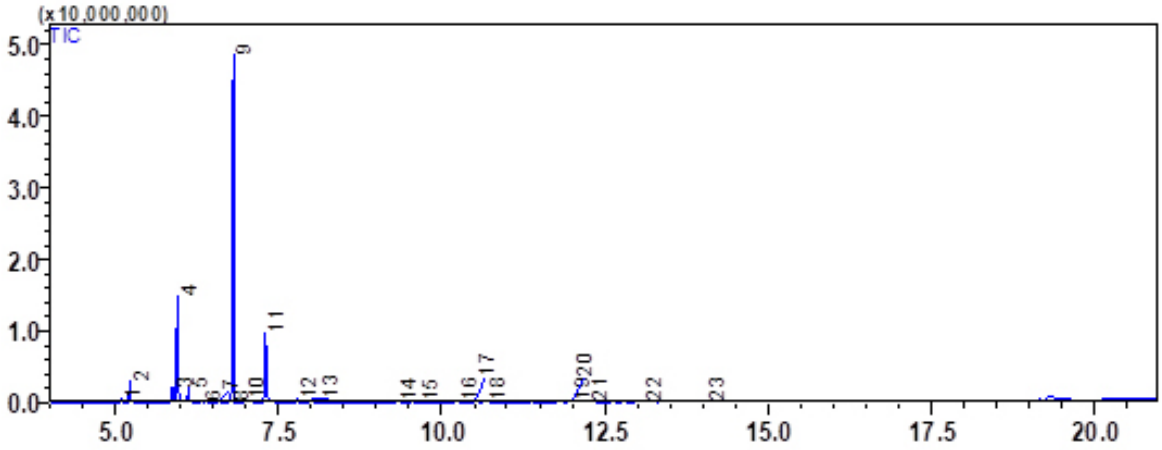

Fig. 3. Headspace chromatogram of *Citrus limon* EO compounds.

in a round bottom flask with 300 mL of distilled water were subject to hydrodistillation for about 2 hours until essential oil stabilization. After the extraction process, the produced oil was treated with anhydrous sodium sulfate (Na_2SO_4) to eliminate any residues of water. For later use, the produced essential oil was weighed and kept at 4 °C in a dark, sealed glass jar.

$$\text{EO yield } (\%) = \frac{\text{Weight of EO } (g)}{\text{Initiale weight of lemon peels } (g)} \times 100$$

Headspace (HS) and Solid-Phase Microextraction (SPME)

In a 20 mL transparent glass vial, 1 g of dried lemon zest was placed and was subjected to direct HS-SPME. The headspace glass vial was heated at 80 °C for 10 min in order to reach thermal equilibrium. The SPME fiber (DVB/CAR/PDMS, 2 cm–50/30 µm) was inserted into the headspace of the glass vial to absorb volatile organic compounds for 30 minutes. Following the sampling process, the SPME fiber was quickly removed from the zest sample,

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Table 2. *Citrus limon* EO composition with Headspace-Solid Phase Microextraction (HS-SMPE).

N°	Compounds name	Retention time	%
1	α-Thujene	5.093	0.90
2	α-Pinene	5.223	4.68
3	Sabinen	5.875	2.94
4	β-Pinene	5.950	20.19
5	β-Myrcene	6.118	4.60
6	1-Octanal	6.328	0.22
7	(+)-2-Carene	6.593	1.08
8	p-Cymene	6.744	0.49
9	D-Limonene	6.806	43.61
10	D-sylvestrene	6.996	1.12
11	γ-Terpinene	7.298	14.58
12	(+)-4-Carene	7.793	0.63
13	1-Undecanal	8.003	0.90
14	p-Menth-1-en-4-ol	9.328	0.33
15	α-Terpineol	9.553	0.23
16	2-Isopropenyl-5-methylhex-4-enal	10.233	0.43
17	2-Decenal, (E)-	10.515	0.48
18	trans-Citral	10.670	0.60
19	Nerol acetate	11.967	0.31
20	2-Dodecenal, (E)-	12.017	0.68
21	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate	12.237	0.37
22	α-Farnesene	13.075	0.29

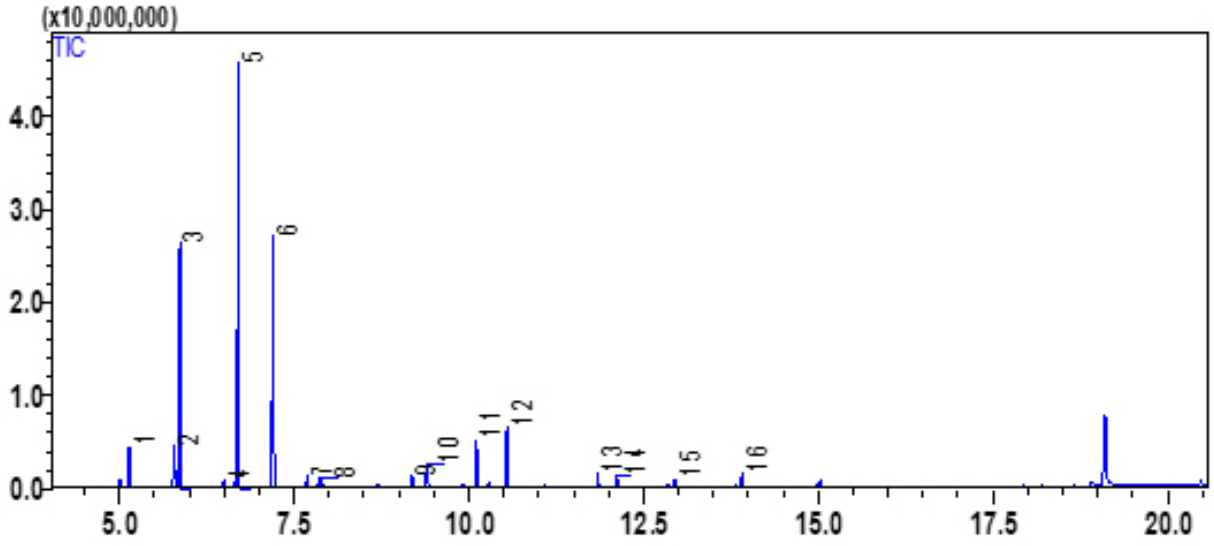

Fig. 4. Gas chromatography-mass spectrometry (GC-MS) chromatogram of *Citrus Limon* EO compounds.

then the analytes were subject to thermal desorption within the injector port of the Gas chromatography-mass spectrometry (GC-MS). Using the splitless injection method, this desorption took place for 5 minutes at a temperature of 250 °C.

Characterization of Aroma Compounds in C. lemon EO Using GC-MS

Shimadzu GC-2010 gas chromatograph (GC) equipped with a 30 m × 0.25 mm column, possessing a film thickness of 0.25 µm and coated with 5% phenyl methyl siloxane, was used for better detection analysis of essential oil obtained through hydrodistillation. The GC was coupled with a GC-MS-QP2010 mass detector, and the carrier gas used was helium maintained at a constant pressure of 100 KPa. The temperature program for the GC oven initiated at 50 °C for approximately one minute, followed by a linear temperature ramp of 10 °C per minute until reaching a final temperature of 250 °C. A 1 µL sample injection of the essential oil dissolved in

Table 3. *Citrus limon* EO composition with Gas chromatography-mass spectrometry (GC-MS).

N°	Compounds Name	Retention time	%
1	α-Pinene	5.141	3.37
2	Sabinen	5.787	4.07
3	β-Pinene	5.863	17.98
4	α-Terpinen	6.494	1.09
5	D-Limonene	6.706	27.70
6	γ-Terpinene	7.199	22.77
7	(+)-2-Carene	7.689	1.59
8	Linalol	7.853	1.08
9	4-Terpineol	9.189	1.66
10	α-Terpineol	9.402	2.42
11	2-Isopropenyl-5-methylhex-4-enal	10.110	4.63
12	trans-Citral	10.547	6.08
13	Nerol acetate	11.848	1.52
14	Geraniol acetate	12.115	1.15
15	α-Bergamotene	12.952	0.96
16	β-Bisabolene	13.911	1.93

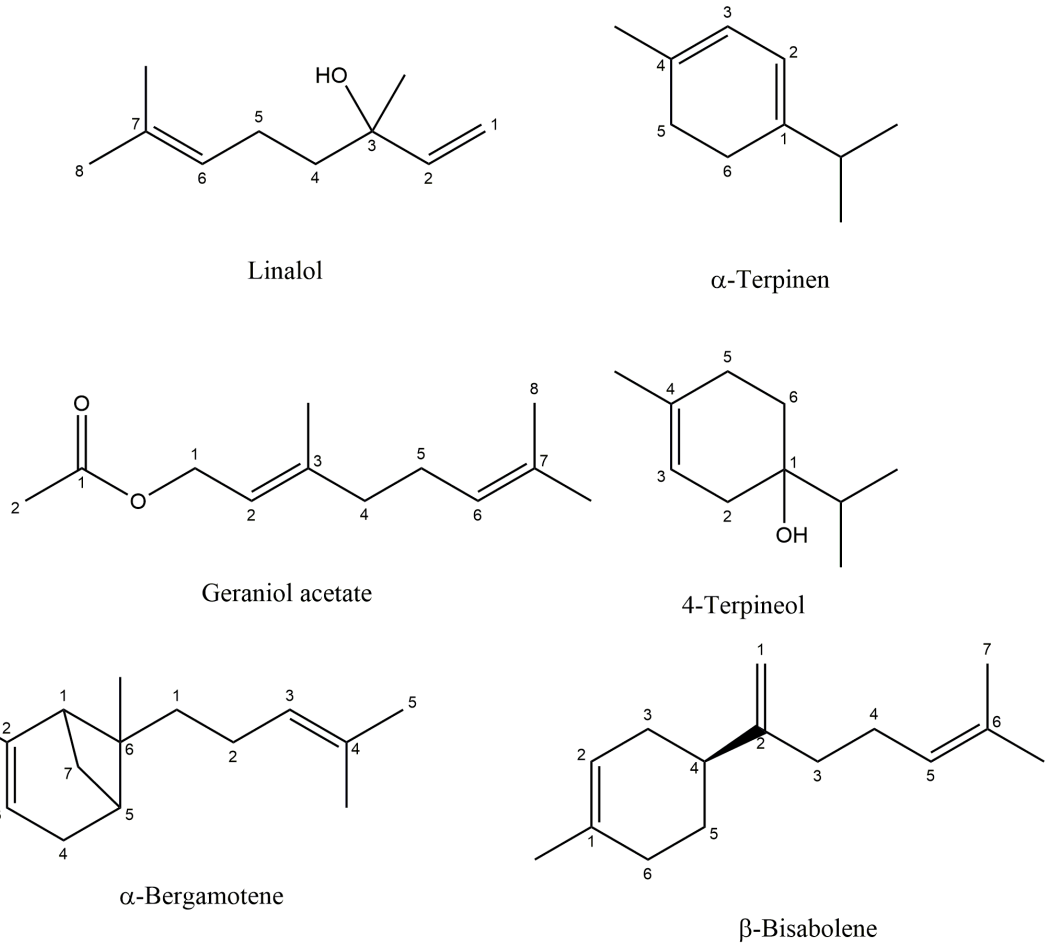

Fig. 5. Identified compounds in *Citrus limon* EO with GC-MS (GC-MS specific compounds Components).

n-hexane was employed. During the analysis, The GC-MS system was in scan mode when in use. By comparing the collected mass spectra with the reference information kept in the National Institute of Standards and Technology (NIST147) computer library, compounds were identified. The LabSolutions software (version 2.5) was used for data management and acquisition.

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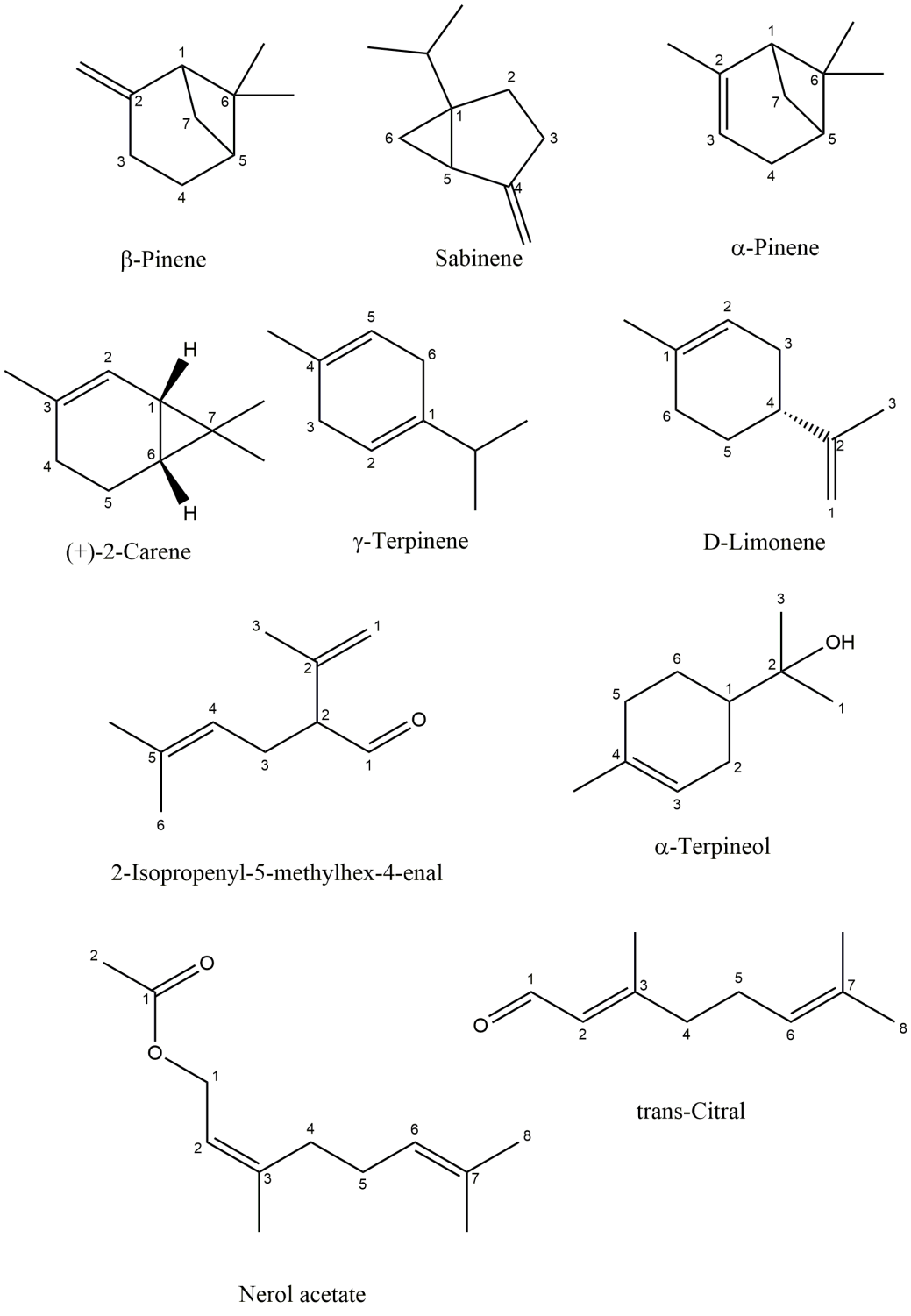

Fig. 6. Identified compounds in *Citrus limon* EO with headspace and GC-MS (Shared Components).

In Vitro Antioxidant Potential of C. lemon EO

Total Antioxidant Capacity (TAC)

The TAC assay was conducted using phosphor-molybdenum method as described by [11] where 0.1 mL of citrus zest EO was mixed with reagent solution composed of (28 mM sodium phosphate (Na_3PO_4), 0.6 M sulfuric acid (H_2SO_4) and 4 mM ammonium molybdate $(NH_4)6Mo_7O_{24}$). The reaction mixture was then incubated for 90 minutes at 50 °C. Following cooling at room temperature the mixture absorbance was measured at 695 [12]. Ascorbic acid was utilized as positive control. All measurements were carried out three times.

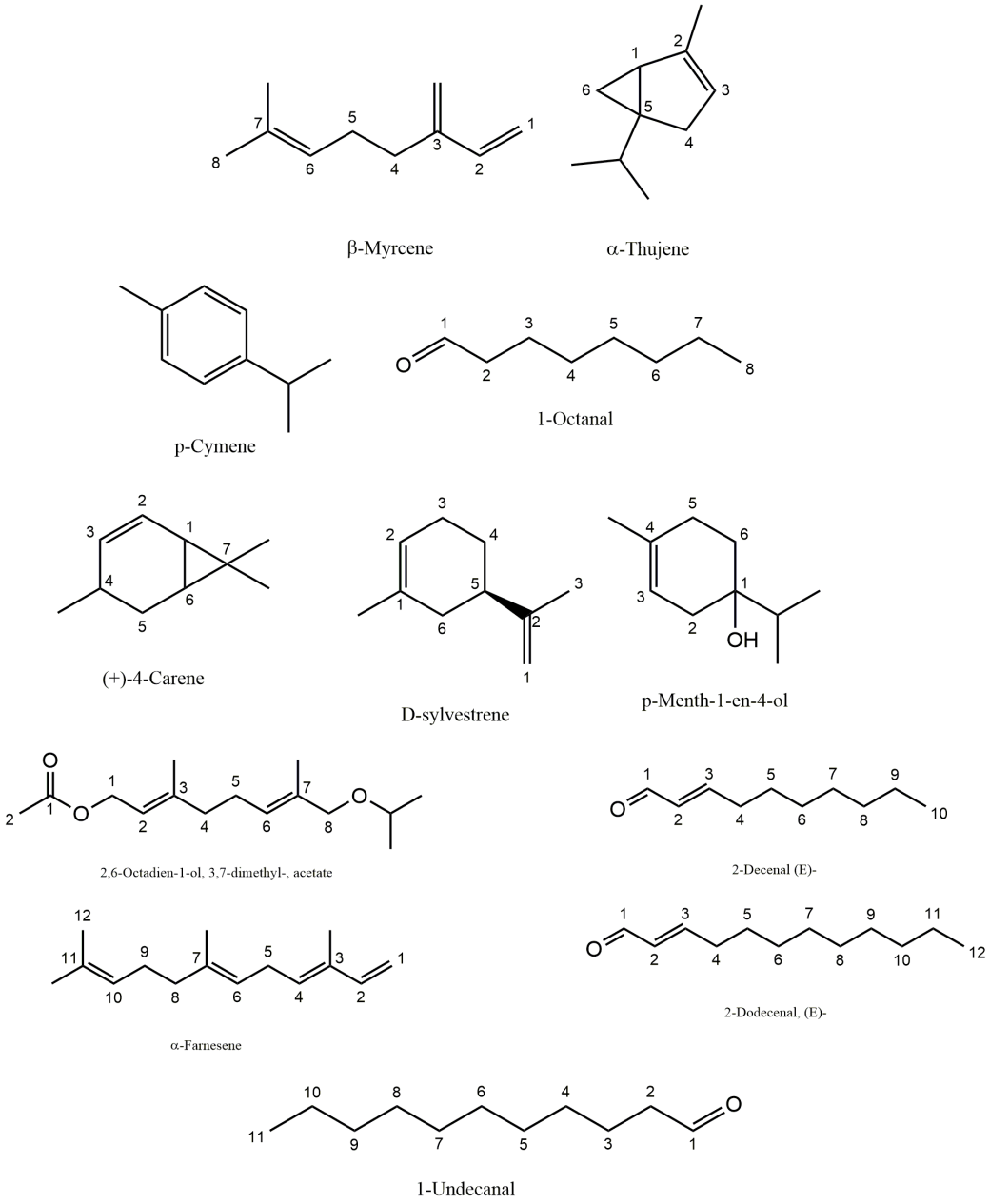

Fig. 7. Identified compounds in *Citrus limon* EO with headspace (HS-SMPE specific Components).

Free Radical Scavenging Activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH)

C. limon EO's capacity to scavenge the stable radical DPPH• was evaluated using the protocol described by [13] with slight modifications, where 1.8 mL of 4% DPPH• solution prepared in methanol was mixed with 0.2 mL Citrus EO prepared at different concentrations as mentioned respectively (0.5, 1, 1.5, 2, 2.5 and 3 mg/mL). The reaction mixture was then incubated for about 30 minutes in dark conditions at room temperature. At 517 nm, the mixture's absorbance was finally compared. A positive control was ascorbic acid. All measurements were carried out three times.

The radical scavenging percentage of *C. limon* EO was calculated according to the formula:

$$\mathrm{RSA}(\%) = \left[\left(\frac{A_B - A_S}{A_B}\right)\right] \times 100$$

A_S: Absorbance of the sample at 517 nm.

A_B: Absorbance of the Control.

The half-maximal inhibitory concentration (IC_{50}) value was derived through a calculation from the dose-response inhibition.

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Table 4. The antioxidant capacity of *Citrus limon* EO.

EO/Reference	Total Antioxidant Capacity (µg AA eq/mg EO)	DPPH Scavenging Capacity IC_{50} (mg/mL)	β-Carotene Bleaching Assay (mg/mL)
Citrus Limon EO	212.2 ± 1.35	1.27 ± 0.03	6.74 ± 0.12
Ascorbic acid (AA)	-	0.25 ± 0.05	-
Butylated hydroxyanisole (BHA)	-	-	0.084 ± 0.02

DPPH, 2,2-diphenyl-1-picrylhydrazyl; IC_{50}, half-maximal inhibitory concentration.

Table 5. The free binding energy's heat diagram (Kcal/mol) for some of *Citrus limon* EO compounds.

Ligand	Revealed by (GC-MS or Head Space or Both)	Proteins		
		1N8Q	1OG5	2CDU
		Binding affinity energy (Kcal/mol)		
Native ligand	–	−6.0	−6.6	−8.6
(+)-4-Carene	Head Space	−6.1	−5.8	−5.8
α-pinene	Both	−5.6	−5.6	−5.5
β-pinene	Both	−5.6	−5.6	−5.6
D-Limonene	Both	−6.0	−6.3	−5.6
γ-Terpinene	Both	−5.1	−6.1	−5.6
2-Isopropenyl-5-methylhex-4-enal	Both	−4.7	−5.1	−5.3
Sabinene	Both	−5.4	−6.2	−5.4
trans-Citral	Both	−4.5	−5.6	−5.1
α-Bergamotene	GC-MS	–9.0	–10.6	–8.9

1OG5: cytochrome P450; 1N8Q: lipoxygenase; 2CDU: NADPH oxidase.

β-Carotene Bleaching Assay

The potential of *C. limon* essential oil (EO) to mitigate β-carotene bleaching, primarily driven by free radicals, was investigated. The generation of free radicals was instigated by the natural oxidation of fatty acids. The assessment of this process was conducted utilizing the methodology detailed in the work of [14], with certain modifications. Firstly, 200 mg of Tween 80, 20 mg of linoleic acid, and 2 mg of beta-carotene were combined with 10 mL of chloroform in a flask with a round bottom. After, the chloroform was eliminated from the mixture using a rotary evaporator set at 40 °C. The reagent solution was then created by immediately adding 100 mL of distilled water. 0.2 mL of this emulsion was aliquoted into distinct test tubes, each containing the respective sample solution. Right after the introduction of the emulsion, the initial absorbance of the samples was recorded (t0). Subsequently, the tubes were placed within a water bath set at 50 °C for a duration of 2 hours, accompanied by consistent agitation. Finally, a second measurement at 470 nm after the lapse of 2 hours was conducted in comparison with the blank. Butylated hydroxyanisole (BHA) was used as a positive control in this experiment. Three measurements were made for each concentration.

The following formula was used to determine the residual color:

$$(\beta - \text{ carotene Bleaching } \%) = \left[\left(\frac{\text{OD}at_{to} - \text{OD}at_{t1}}{\text{OD}at_{t0}}\right)\right] \times 100$$

Antimitotic Assay

For the evaluation of the antimitotic activity of citrus zest EO, Lepidium sativum seeds were used for a preliminary evaluation of the possible cytotoxic effect of the EO. Thus, *Lepidium sativum* seeds were purshaced from local market and were germinated in petri dishes (8 dishes were cultered for each concentration) with filter paper that has been soaked in distilled water for twenty-four hours, then 1 mL of the essential oil to be evaluated was applied to each dish at various concentrations. The dishes are then incubated at 25 °C. The findings were read after 12 to 72 hours of incubation. As a positive control, colchicine was employed, and distilled water was used as a negative control. The dishes are incubated in the dark at a temperature of 25 °C at different time lapse. The biotest is based on measuring the length of a Lepidium sativum rootlet placed in a medium containing the essential oil to be tested [15].

The EO antimitotic activity was assessed by calculating measuring the root length which gives accurate results about cell growth inhibition.

The inhibition percentage was calculated as follow:

$$\text{Inhibition } \% = \frac{Lc - Lt}{Lc} \times 100$$

Lc: The length of control rootlets.

Lt: The length of rootlets treated with the citrus essential oil.

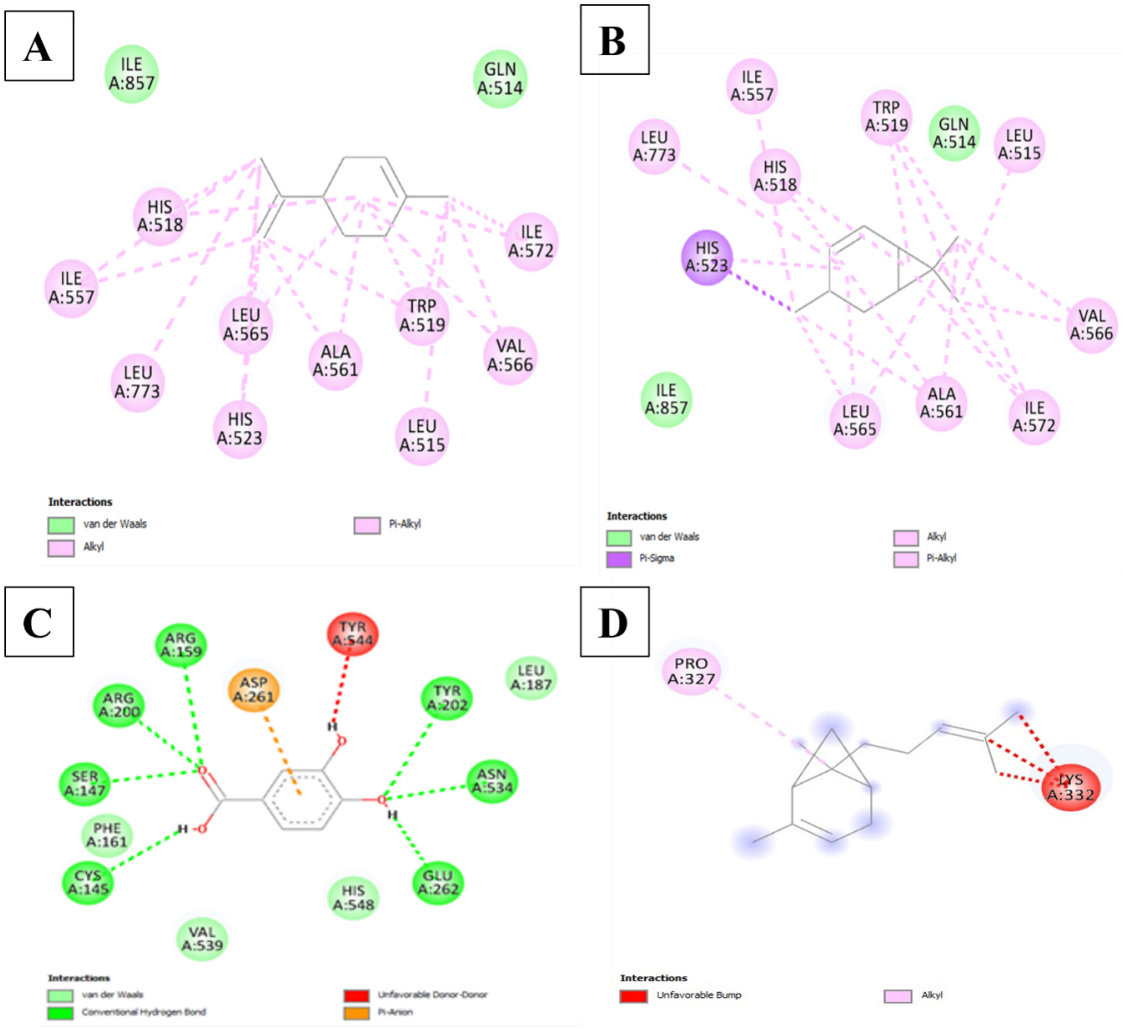

Fig. 8. 2D interaction diagram of the compounds D-limonene (A), (+)-4-carene (B), and protocatechuic acid (native ligand) (C), α-bergamotene (D), against the protein lipoxygenase (PDB ID: 1N8Q).

Molecular Docking in Silico Assay

Molecular docking techniques were used to anticipate the antioxidant activity of *C. limon* essential oil (EO), enabling an exploration of the potential therapeutic benefits of its main identified compounds. A well-established procedure, previously examined and documented, was adopted for the docking analysis. The three-dimensional (3D) structures of the main characterized bioactive compounds within *C. limon* EO originated from PubChem (https://pubchem.ncbi.nlm.nih.gov, accessed on July 10, 2023) for application in the docking analysis. PyMol software, a molecular visualization and analysis tool was used to convert the bioactive compounds downloaded from PubChem from "3D sdf" to "pdb". To study the interactions between the target proteins, crystallographic structures of the target proteins, identifiable by their distinctive PDB IDs, were retrieved from the Protein Data Bank website (https://www.rcsb.org/, accessed on July 10, 2023).

Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) Prediction and Boiled Egg Model

In order to evaluate the ADMET properties of *C. limon* EO main identified compounds, a computational method was adopted for better assessment of the drugs ability to permeate cell membranes, interact with transporters, their contribution to drug absorption and excretion, as well as their stability during metabolism. Hence, citrus EO bioactive compounds' physicochemical characteristics, drug similarity, and pharmacokinetic characteristics utilizing ADMET's two webservers: SwissADME (http://www.swissadme.ch/) who was employed for a comprehensive evaluation of the physicochemical properties, drug-likeness, and pharmacokinetic characteristics of the Citrus limon Essential Oil (EO) compounds, also to determine the boiled egg model and pkCSM (http://biosig.unimelb.edu.au/pkcsm/) who contributes to the ADMET analysis by providing additional insights into the pharmacokinetic properties of the identified EO compounds. It aids in assessing drug absorption, distribution, metabolism, and excretion [16–18].

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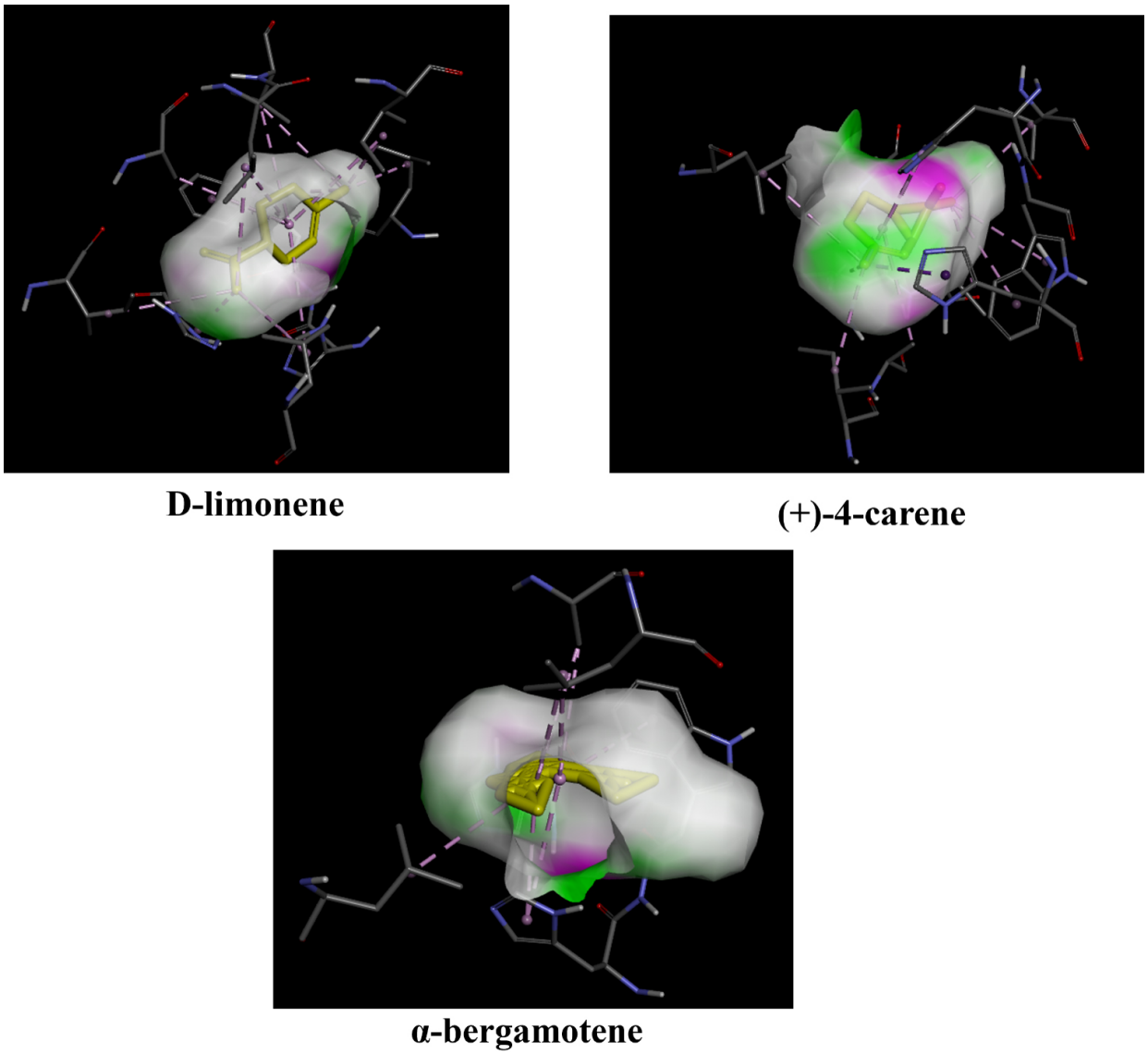

Fig. 9. 3D interaction diagram of the compounds D-limonene, (+)-4-carene and α-bergamotene against the protein lipoxygenase (PDB ID: 1N8Q).

Software

QGIC version 3.16 was used in drawing maps (Figs. 1,2).

Statistical Analysis

ANOVA was used to do statistical analysis on the data, which included multiple-group comparisons (One-way-analysis of variance).

Results

Essential Oil Extraction from C. limon

The essential oil yield is represented by the amount of oil (measured in milligrams) extracted per 100 grams of lemon peel. In our study, we conducted three repetitions, and the essential oil yield from our experiment is displayed in the Table 1. The hydrodistillation method was employed to extract the essential oil from the peels of *C. limon*; the resulting yield was 1.99 ± 0.19%.

Phytochemical Analysis Using GC-MS and Headspace

Two techniques, Headspace-Solid Phase Microextraction (HS-SMPE) and Gas Chromatography-Mass Spectrometry (GC-MS), were used to analyze the composition of *C. limon* essential oil (EO). The constituents of the oil were identified by comparing their mass spectrometry (MS) data with entries in the National Institute of Standards and Technology (NIST147) computer library. Table 2 and Fig. 3 show the chemical make-up and chromatograms of the investigated oils for the HS-SMPE analysis, and in Table 3 and Fig. 4 for the GC-MS analysis, respectively. The chem-

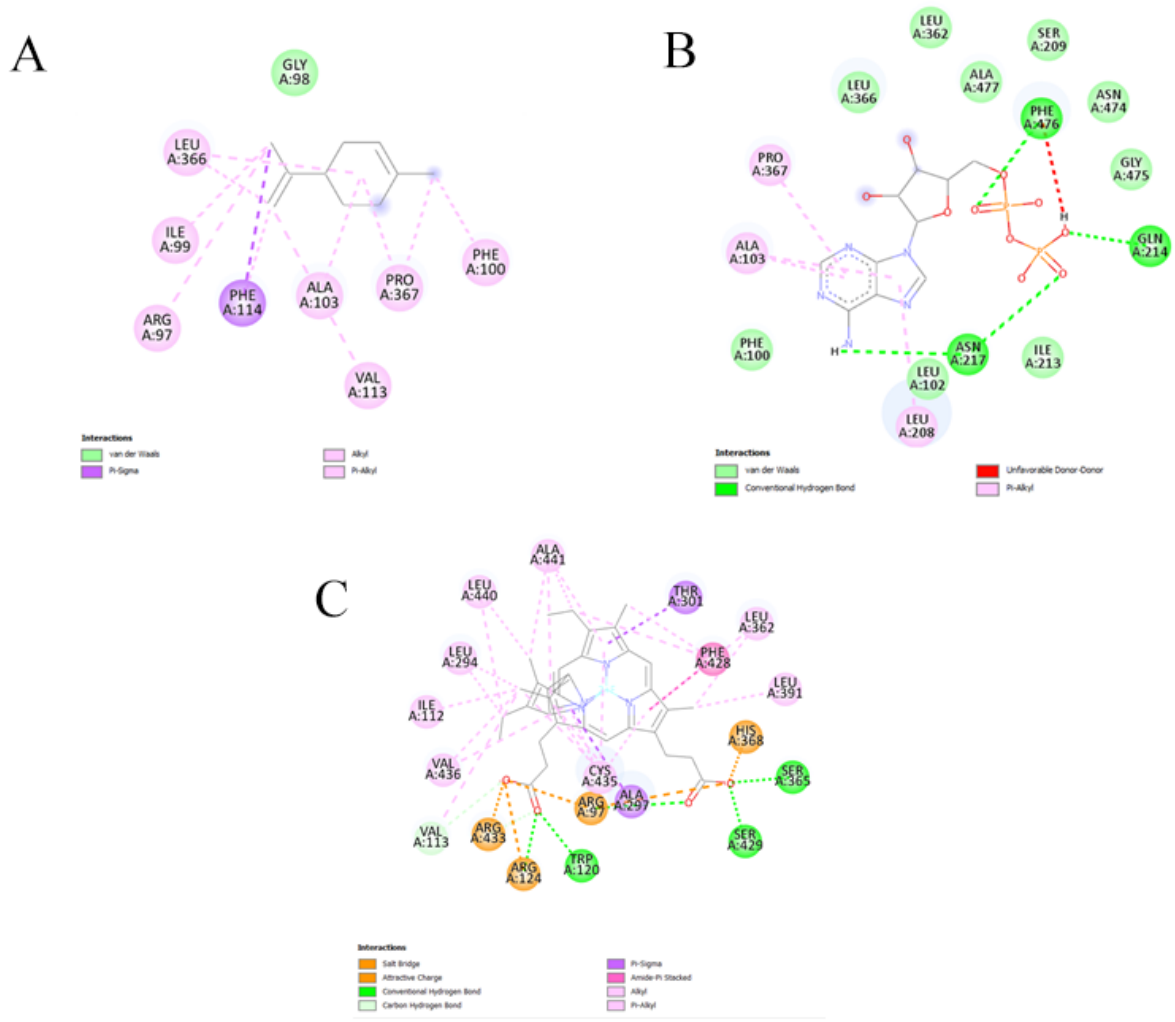

Fig. 10. 2D interaction diagram of the compounds D-limonene (A), and warfarin (native ligand) (B), α-bergamotene (C), against the CYP450 protein (PDB ID: 1OG5).

ical characterization using HS-SMPE and GC-MS analysis reported the presence of multitude of bioactive compounds in citrus peels EO.

Regarding HS-SMPE analysis, it was revealed the presence of 22 compounds. This analysis indicated that the EO was dominated by D-limonene with a percentage of 43.61% followed by β-pinene with 20.19%. γ-terpinene was found with a percentage of 14.58%, while α-pinene and β-myrcene were found with smaller percentages of 4.68 and 4.60% respectively.

On the other hand, the GC-MS has shown the presence of 16 compounds (Fig. 5) which is less than that detected by HS-SMPE. It was noted that D-limonene was the major compound identified in GC-MS analysis with a percentage of 27.70% which is significantly lower than that recorded in HS-SMPE. Following D-limonene, γ-terpinene (22.77%), and β-pinene (17.98%). Additionally, trans-citral and 2-isopropenyl-5-methylhex-4-enal were found to be present with percentages of 6.08% and 4.63% respectively.

Hence, between the two techniques used (HS-SMPE and GC-MS), a set of 10 compounds was identified as shared (Fig. 6), α-pinene, sabinene, β-pinene, D-limonene, γ-terpinene, (+)-2-carene, α-terpineol, 2-isopropenyl-5-methylhex-4-enal, trans-citral, and nerol acetate (as indicated in Fig. 4). These compounds have consistently been detected in *Citrus limon* EO using both GC-MS and HS-SMPE analysis, underscoring their presence. In the context of the HS-SMPE analysis, an additional 12 compounds (Fig. 7) were found to be specifically detected by this technique These compounds include α-thujene, β-myrcene, 1-octanal, (E)-2-decenal, p-cymene, 2,6-octadien-1-ol,3,7-dimethyl-, acetate, D-sylvestrene, (+)-4-carene, 1-undecanal, p-menth-1-en-4-ol, (E)-2-dodecenal and α-farnesene. On the other hand, GC-MS analysis revealed the presence of six (6) compounds that were not detected in the HS-SMPE analysis. These compounds are α-terpinen, linalol, 4-terpineol, geraniol acetate, α-bergamotene, and β-bisabolene. This difference under-

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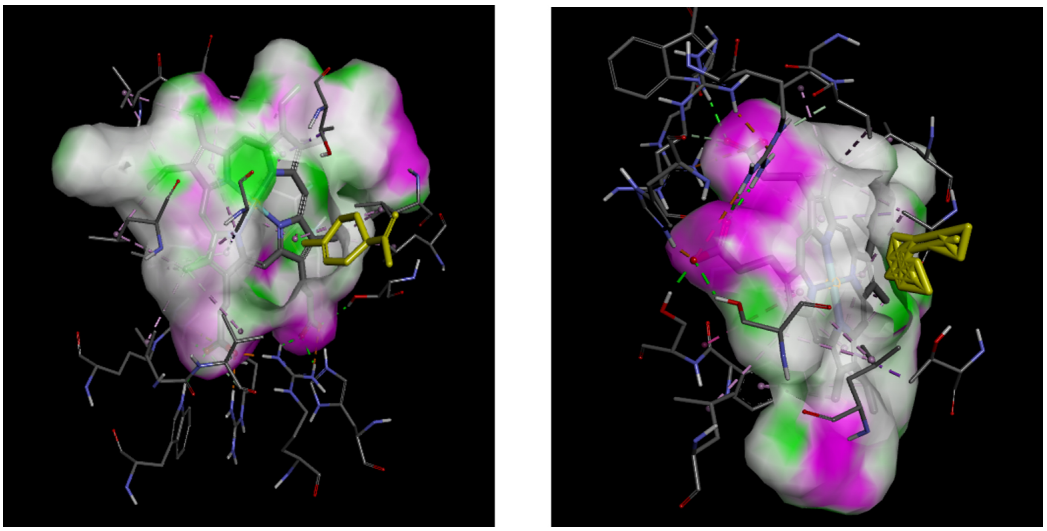

D-limonene **α-bergamotene**

Fig. 11. 3D interaction diagram of the compounds D-limonene and α-bergamotene against the CYP450 protein (PDB ID: 1OG5).

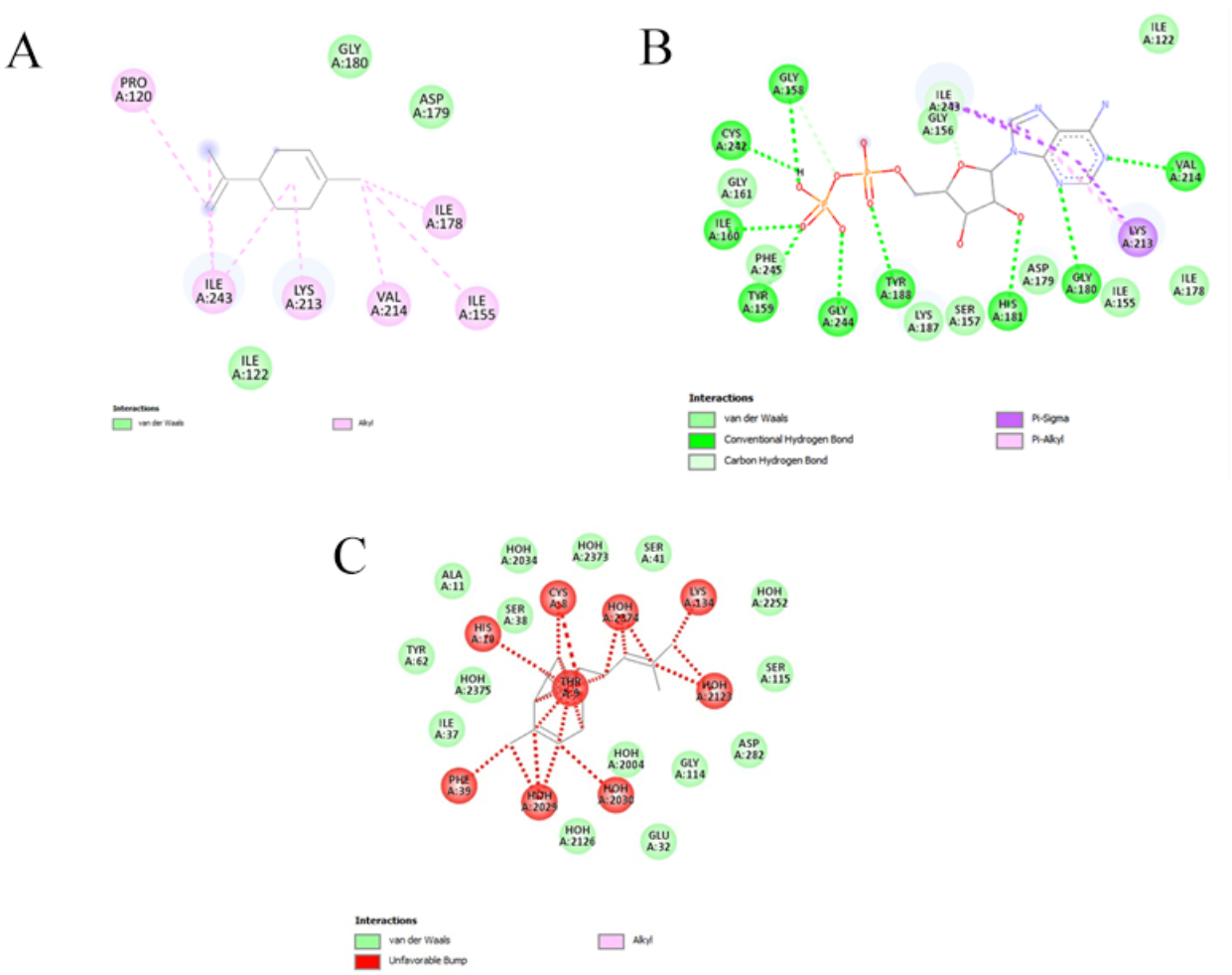

Fig. 12. 2D interaction diagram of the compounds D-limonene (A), and adenosine-5′-diphosphate (native ligand) (B), α-bergamotene (C), against NADPH oxidase (PDB ID: 2CDU).

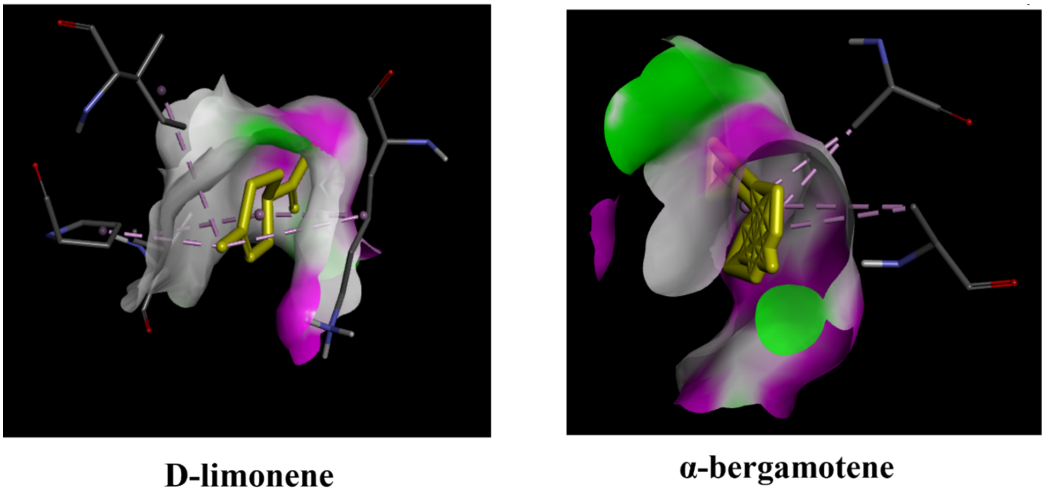

Fig. 13. 3D interaction diagram of the compounds D-limonene and α-bergamotene against NADPH oxidase (PDB ID: 2CDU).

Table 6. The inhibition rate of each concentrations of the of *C. limon* EO and colchicine at 250 µg/mL.

Tested conentration	12 hours	24 hours	48 hours	72 hours
50 µg/mL	14.63%	33.33%	21.05%	21.05%
100 µg/mL	39.02%	42.52%	31.57%	40%
150 µg/mL	46.34%	54.02%	48.24%	53.33%
200 µg/mL	65.85%	68.96%	65.78%	66.66%
250 µg/mL	75.6%	82.75%	81.57%	83.33%
Colchicine at 250 µg/mL	54%	60%	58%	62%

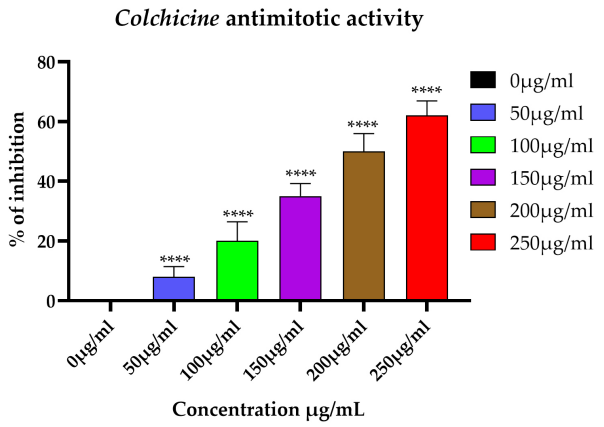

Fig. 14. Colchicine antimitotic activity. **** $p < 0.0001$.

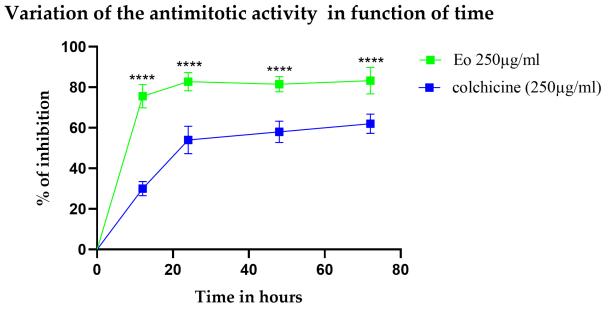

Fig. 15. The variation of the antimitotic activity of both colchicine and *Citrus limon* EO in function of time. **** $p < 0.0001$.

scores the complementary nature of the two analytical methods in capturing the diverse composition of *Citrus limon* essential oil.

In Vitro Antioxidant Potential of Citrus Peels EO

The antioxidant potential of citrus peels EO was evaluated using tree different techniques starting with total antioxidant activity followed by free radical scavenging activity and finally evaluating the anti-bleaching effect using β-carotene assay. Firstly, it was indicated that citrus peels EO are endowed with total antioxidant activity that was equal to 212.2 µg Ascorbic acid (AA) eq/mg EO. However, the EO showed moderate scavenging potential of DPPH radicals with an IC_{50} of 1.27 mg/mL while ascorbic acid used as control had high scavenging potential with an IC_{50} 0.25 mg/mL. finally, the EO showed moderate to weak potential to inhibit the bleaching effect of linoleic acid on β-carotene that was indicated by higher IC_{50} that was equal to 6.74 mg/mL which was higher compared to BHA used as positive control that showed a very strong anti-bleaching effect with an IC_{50} of 0.084 mg/mL (Table 4).

Journal of
BIOLOGICAL REGULATORS
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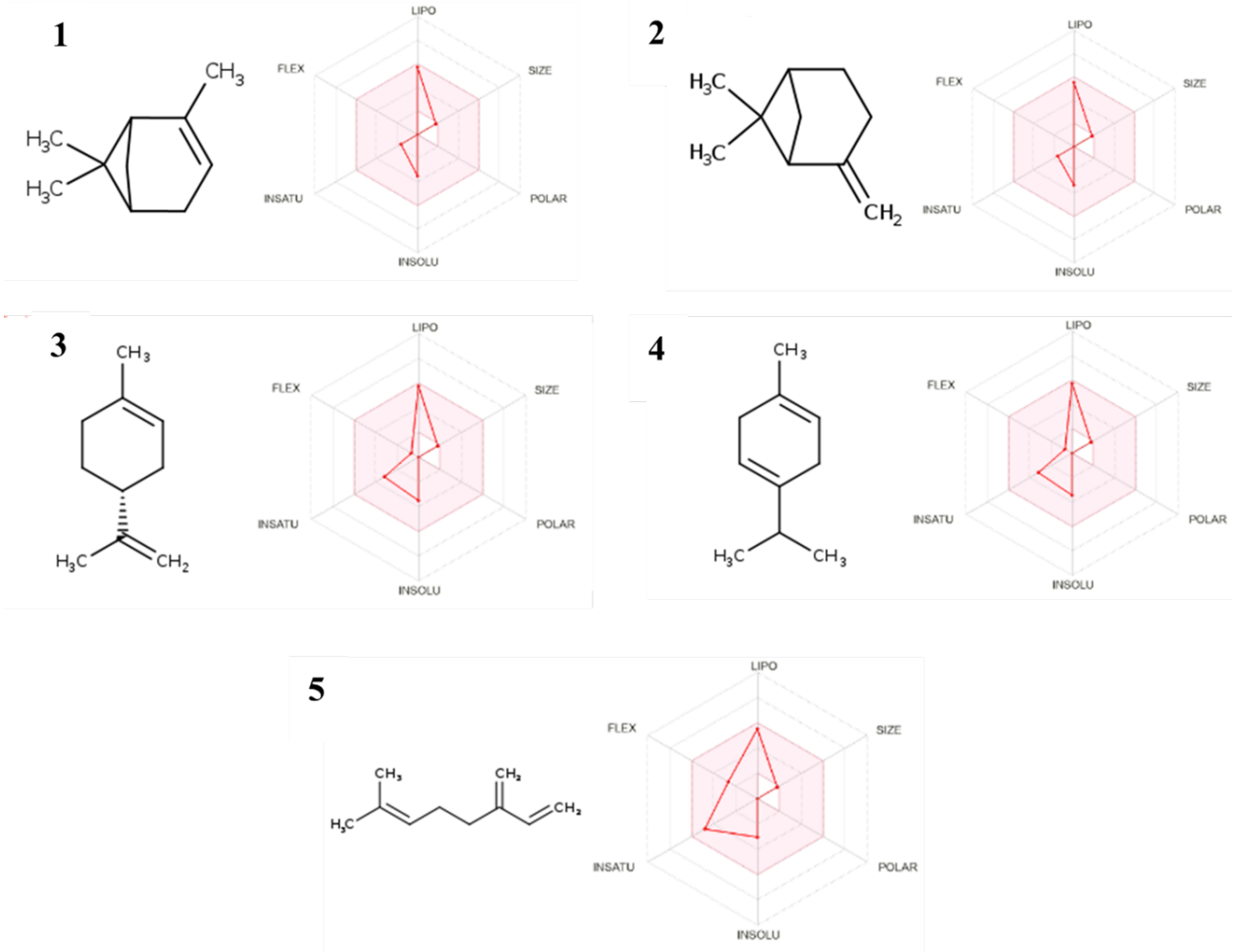

Fig. 16. Bioavailability radars for phytoconstituents considering six physicochemical properties (lipophilicity, size, polarity, solubility, flexibility and saturation) ideal for oral bioavailability. (1) α-Pinene, (2) β-Pinene, (3) D-Limonene, (4) γ-Terpinene, (5) β-Myrcene.

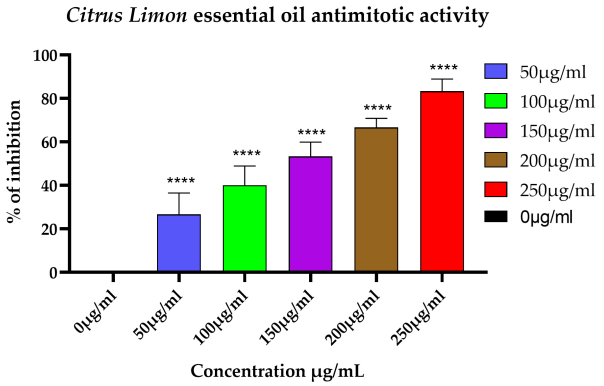

Fig. 17. *Citrus limon* essential oil antimitotic activity. **** $p < 0.0001$.

Molecular Doking

For better understanding of the antioxidant molecular mechanism of citrus peels EO, Molecular docking was used which allows the prediction of the possible molecular interactions between the identified molecules using HS-SMPE and GC-MS. A class of metal-containing enzymes known as lipoxygenases is in charge of accelerating the lipid peroxidation of free polyunsaturated fatty acids [19]. This process involves a redox mechanism wherein the conversion of the Fe^{2+} iron in the active site to Fe^{3+} triggers the formation of a fatty acid hydroperoxide radical centered on oxygen. This molecular transformation has been implicated in the development of various pathogenic diseases [20].

In this investigation, we concentrated on lipoxygenase (1N8Q) and cytochrome P450 (1OG5) two distinct lipoxygenases. Our research identified three compounds for the first targeted protein that had binding affinities that were either the same as or greater than those of the natural ligand. (+)-4-Carene, namely, D-limonene with a binding energy of –6.1 kcal/mol, D-limonene with –6.0 kcal/mol, and α-bergamotene with –9.0 kcal/mol (Figs. 8,9). In the context of the second protein (CYP450), four molecules were

Table 7. Absorption, distribution, metabolism, excretion and toxicity (ADMET) properties of the most abundant phytochemicals identified in *C. limon* essential oil.

Compound N.		1	2	3	4	5
Drug-Likeness	Lipinski's rule of five	Yes	Yes	Yes	Yes	Yes
	Bioavailability Score (%)	0.55	0.55	0.55	0.55	0.55
Absorption	Water Solubility	–2.74	–2.74	–3.50	–3.45	–4.49
	Caco2 Permeability	1.38	1.38	1.40	1.41	1.4
	Intestinal Absorption (Human) (%)	96.0	96.0	95.8	96.2	94.7
	Skin Permeability	–1.82	–1.82	–3.89	–3.94	–1.04
	P-glycoprotein Substrate	No	No	No	No	No
	P-glycoprotein I Inhibitor	No	No	No	No	No
	P-glycoprotein II Inhibitor	No	No	No	No	No
Distribution	VDss (human)	0.66	0.66	0.39	0.41	0.36
	permeability BBB	0.79	0.79	0.73	0.75	0.78
	Permeability CNS	–2.20	–2.20	–2.37	–2.04	–1.90
Metabolism	CYP2D6 Substrate	No	No	No	No	No
	CYP3A4 Substrate	No	No	No	No	No
	CYP2D6 Inhibitor	No	No	No	No	No
	CYP3A4 Inhibitor	No	No	No	No	No
Excretion	Total clearance	0.04	0.04	0.21	0.21	0.43
	Renal OCT2 Substrate	No	No	No	No	No
Toxicity	AMES Toxicité	No	No	No	No	No
	Hepatotoxicity	No	No	No	No	No
	hERG I Inhibitor	No	No	No	No	No
	Skin sensitivity	No	No	Oui	No	No

1, α-Pinene; 2, β-Pinene; 3, D-Limonene; 4, γ-Terpinene; 5, β-Myrcene.

identified as inhibitors due to their binding energies closely resembling that of the natural ligand (D-limonene at –6.3 kcal/mol, γ-terpinene at –6.1 kcal/mol, sabinene at –6.2 kcal/mol, and α-bergamotene at –10.6 kcal/mol, compared to warfarin at –6.6 kcal/mol, the native ligand of CYP2C9) (Figs. 10,11). Remarkably, our study showcased that only α-Bergamotene, with a binding free energy exceeding –8.6 kcal/mol (Table 5), stands out as the compound with the most robust interaction. This finding implies that α-Bergamotene, a component of our essential oil, holds significant promise as a potential antagonist against the NADPH oxidase protein (Figs. 12,13). In light of these findings, it can be reasonably inferred that our essential oil is a rich source of potent antioxidant compounds. Particularly noteworthy are D-Limonene and α-Bergamotene, which exhibit strong potential for eliciting powerful antioxidant activity. This findings should be confirmed with an in-vitro study for this two molecules.

Antimitotic Assay

This assay was used to evaluate the potential effect of *Citrus* peels EO to inhibit cell division. This assay involves observing the impact of EO on the mitotic process in *Lepidium sativum* root length. According to the collect experimental data it was observed that EO of citrus peels and colchicine (positive control) are endowed with antimitotic effect in a dose-dependent manner.

With increasing concentration, the antimitotic activity of *C. limon* essential oil demonstrates a gradual augmentation. Notably, at a concentration of 250 mg/mL, *C. limon* essential oil attains its peak antimitotic efficacy, reaching 83.33%. This trend underscores the concentration-dependent nature of *C. limon* essential oil's antimitotic attributes. In contrast, colchicine displays diminished antimitotic activity at each concentration level compared to *C. limon*. At a concentration of 250 g/mL, colchicine exhibits a maximal antimitotic effectiveness of 62% (Fig. 14). This discrepancy implies that, in relation to *C. limon* essential oil, colchicine exerts a less pronounced antimitotic influence. Finally, *C. limon* EO frequently showed superior antimitotic activity in comparison to colchicine. The assay revealed a notable concentration disparity for both EO and colchicine, a finding corroborated by the statistical analysis indicating a significant difference between the concentrations ($p < 0.0001$).

At a concentration of 50 µg/mL, the antimitotic activity of the *C. limon* essential oil is relatively low. The percentages (values) of inhibition are 14.63% at 12 hours, 33.33% at 24 hours, 21.05% at 48 hours, and 21.05% at 72 hours. Increasing the concentration to 100 µg/mL leads to a noticeable improvement in antimitotic activity. The inhibition percentages are 39.02% at 12 hours, 42.52% at 24 hours, 31.57% at 48 hours, and 40% at 72 hours. Further increasing the concentration to 150 µg/mL results in higher inhibition percentages across all time intervals. The val-

Journal of
BIOLOGICAL REGULATORS
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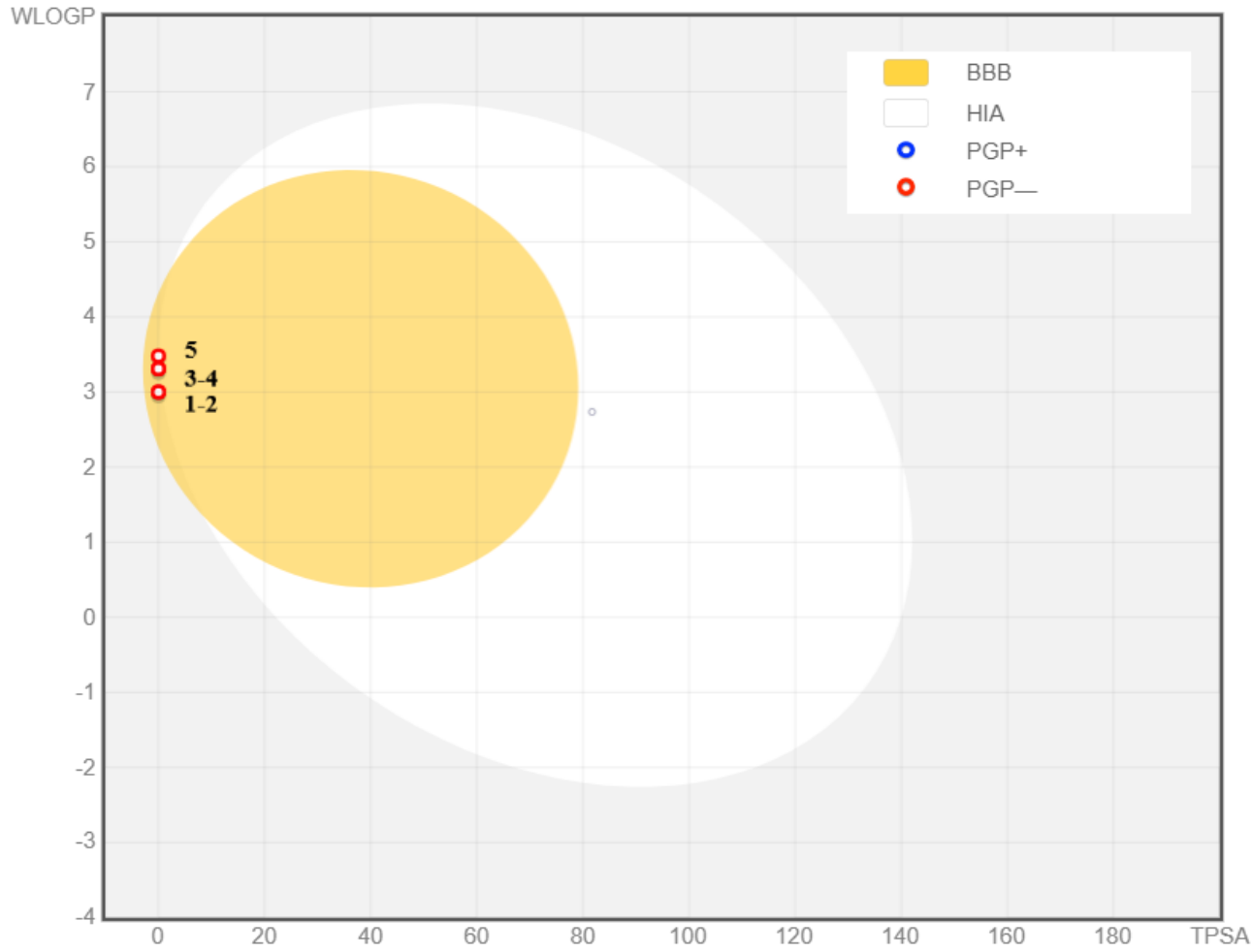

Fig. 18. Boiled egg model some of *Citrus Limon* EO compounds. (1) α-Pinène, (2) β-Pinène, (3) D-Limonène, (4) γ-Terpinène, (5) β-Myrcène.

ues are 46.34% at 12 hours, 54.02% at 24 hours, 48.24% at 48 hours, and 53.33% at 72 hours. At 200 µg/mL, the antimitotic activity continues to improve, with inhibition percentages of 65.85% at 12 hours, 68.96% at 24 hours, 65.78% at 48 hours, and 66.66% at 72 hours. The highest concentration tested, 250 µg/mL, shows the most substantial antimitotic activity. The inhibition percentages are 75.6% at 12 hours, 82.75% at 24 hours, 81.57% at 48 hours, and 83.33% at 72 hours (Fig. 15, Table 6). Comparing the antimitotic activity of lemon essential oil at different time intervals, we can observe some variations. Generally, the highest inhibition percentages tend to occur at 24 and 72 hours, while the values at 12 and 48 hours are somewhat lower. This indicates that the antimitotic effect of lemon essential oil becomes more prominent with longer exposure time. Colchicine (250 µg/mL) shows the following inhibition percentages: 30% at 12 hours, 42% at 24 hours, 58% at 48 hours, and 62% at 72 hours. Comparing it to the essential oil at the same concentration, lemon essential oil (250 µg/mL) demonstrates higher inhibition percentages than colchicine (250 µg/mL) at all time intervals. This suggests that lemon essential oil, at this concentration, has a stronger antimitotic activity compared to colchicine. At 24 and 72 hours, the difference in inhibition percentages between lemon essential oil and colchicine is particularly notable, with lemon essential oil showing significantly higher values. It's important to note that colchicine is a well-known natural compound derived from the autumn crocus (*Colchicum autumnale*) plant which is widely recognized for its antimitotic properties [21]. The fact that lemon essential oil, at the same concentration, demonstrates higher inhibition percentages indicates its potential as a promising alternative or complementary substance for antimitotic activity. Furthermore, there is a clear relationship between the concentration of lemon essential oil and its antimitotic activity. As the concentration increases, the inhibition percentages also increase, suggesting a dose-dependent response. This implies that higher concentrations of lemon essential oil are more effective at inhibiting cell division. In summary, the results demonstrate that lemon essential oil

exhibits antimitotic activity, and its effectiveness is influenced by both the concentration and duration of exposure. Higher concentrations and longer exposure times generally lead to greater inhibition of cell division. The essential oil of Citrus limon exhibited heightened activity across all concentrations, a observation substantiated by a statistical analysis with a p-value < 0.0001. This underscores the robust efficacy of Citrus limon essential oil in the context of the study.

ADMET Prediction Analysis

The restricted absorption, distribution, metabolism, excretion and toxicity (ADMET) characteristics of a drug can compromise its efficacy. In addition, pharmacokinetics is thought to be the biggest obstacle to drug development in clinical research, which make it incredibly expensive. Consequently, *C. limon* essential oil has been evaluated for its ADMET properties to determine whether it will likely be a candidate for medication development by using *in silico* approaches.

Numerous aspects, including physicochemical characteristics, absorption, distribution, metabolism, and toxicity, were considered in the present investigation. The ADMET prediction analysis concentrated on the five primary components isolated from *Citrus limon* essential oil (EO) (Table 7). These components, known as α-pinene, β-pinene, D-limonene, γ-terpinene and β-myrcene chosen due to their significant presence in the EO composition and their recognized potential in therapeutic applications. The objective was to gain a comprehensive understanding of the pharmacological behavior and potential implications of these key chemicals.The parameters mentioned above were used to evaluate compounds (Table 7). Lipinski's rule of five requires a set of physicochemical characteristics, including: $<$5 H-bond donors, $<$10 H-bond acceptors, N or O $\leq$10, MLOGP $\leq$4.15 and PM $<$500 DA [22]. Unexpectedly, all of the phytoconstituents listed above (numbered 1–5) satisfy Lipinski's rule of five. However, compounds 1 and 2 violate the rule by going above the lipophilicity limit (MLOGP $>$4.15).

Martin (2005) states that every substance that follows Lipinski's rule of five gets a bioavailability score of 0.55. [23]. The bioavailability score is 0.55 as a result (Fig. 16).

The logS scale states that substances having a water solubility between –4 and 0 (expressed in logS (log mol/L)) possess an excellent solubility [16]. Therefore, phytochemical compounds in this situation are regarded as good water-soluble chemicals with the exception of β-Myrcene. –4.49. All of the aforementioned phytochemicals have an elevated Caco2 permeability (expressed as log P in 10–6 cm/s) and consequently a high absorption rate in the human intestine.

None of the substances were either a substrate for P-glycoprotein or an inhibitor of glycoproteins I or II, according to absorption results. The steady-state volume of distribution in humans is represented by VDss (given in Log L/Kg), compounds are reported to be well distributed in plasma. With the largest logBB and the best blood-brain barrier penetration, chemicals 1–5 are all deemed to have reasonable central nervous system (CNS) permeability.

Indicating whether a molecule is an inhibitor or substrate of the two main isoenzymes CYP2D6 and CYP3A4, which are crucial for drug metabolism, in order to characterize the metabolic interactions among substances and cytochrome P450 [17]. None of the substances mentioned previously is a CYP2D6 or CYP3A4 substrate or inhibitor. Although components 1 through 5 were discovered to be non-substrates for renal OCT2 (Organic Cation Transporter 2), β-Myrcene had the highest overall clearance (mL/min/kg) at 0.43 log mL/min/kg. Molecules (1–5) have no AMES toxicity, and none of the substances in the table seem to affect how the human liver works.

Inhibiting the *hERG* gene by stopping potassium channels can result a ventricular arrhythmia [24], but none of the compounds inhibit this gene. All the above phytochemicals, with the exception of D-limonene, cannot induce allergic contact dermatitis.

Bioavailability Radars

The bioavailability radars of the compounds that were identified are shown in Fig. 17, with the pink area denoting the oral bioavailability space, into which the molecular graph must completely fit in order for the compound to be classified as a medication. In the current research, all compounds adhere to the appropriate space for oral bioavailability as indicated above.

BOILED-Egg Model

The BOILED-Egg model offers a preliminary explanation of how to measure intestinal absorption (IA) and blood-brain barrier (BBB) permeability as a function of lipophilicity (WLOGP) and polarity (TPSA). [25] While chemicals found inside the egg yolk (the yellow area) represent compounds with high BBB permeability, the white area represents molecules with high intestine absorption. If a molecule is a P-glycoprotein substrate (blue) or a P-glycoprotein non-substrate (red), it is indicated by the color of the dots.

The phytocompounds in this instance are found to be P-glycoprotein no-substrates, they are well absorbed and perfectly penetrate in the blood-brain-barrier (Fig. 18).

Discussion

Our investigation into the extraction of *C. limon* essential oil (EO) has yielded noteworthy results, revealing a yield of 1.99% $\pm$ 0.19 mg. This finding significantly surpasses the work of Manabi Paw, who reported a yield of 0.41% for *C. limon* EO [26]. Our study employed both Head-Space Solid-Phase Microextraction (HS-SMPE) and Gas chromatography-mass spectrometry (GC-

Journal of BIOLOGICAL REGULATORS and Homeostatic Agents

MS) techniques, consistently identifying D-limonene as the primary constituent in *C. limon* EO. This aligns with the research conducted by Raad A. Kaskoos, who also identified D-limonene as the major component, with a content of 29.52% using GC-MS [27]. Importantly, our findings extend beyond the identification of constituents. We conducted an investigation into the antioxidant properties of *C. limon* EO using the DPPH assay, resulting in an IC_{50} value of 1.17 mg/mL, in line with Monica Rosa Loizzo's study [28]. Our findings support this by demonstrating a similar IC_{50} value. Additionally, Ben Hsouna A's research [29] on *C. limon* EO showed remarkable β-carotene bleaching inhibition with an IC_{50} of 40.147 µg/mL, which differs from our result of $IC_{50} = 6.74 \pm 0.12$ mg/mL. This variation can be attributed to the presence of diverse antioxidant molecules like D-limonene and α-bergamotene. Particularly noteworthy is α-bergamotene, which exhibits significant antioxidant effects by binding strongly to critical proteins involved in free radical production, such as 2CDU and NADPH oxidase. This enzyme catalyzes the generation of superoxide via the one-electron reduction of oxygen using NADPH as the electron donor [30]. Recent research has established the neuroprotective potential of inhibiting NADPH oxidase enzymes, showcasing the significance of α-bergamotene in our EO [31]. Furthermore, considering the potential development of new drugs, the antimitotic properties of *C. limon* EO are noteworthy. Morita *et al*. [32] highlighted the significance of antimitotic agents in drug development.

Considering the diverse therapeutic functions demonstrated by *C. limon* EO, including its antioxidant and antimitotic activities, we acknowledge the importance of exploring the Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) properties of its major constituents. Our study yielded positive results for all phytochemicals tested, offering promising prospects for the incorporation of *C. limon* EO into the pharmaceutical sector.

The enhanced therapeutic potential of *C. limon* EO, as revealed by its robust antioxidant and antimitotic activities, along with favorable ADMET properties, signifies its promising role in drug development and the pharmaceutical industry. Our study not only corroborates previous findings but also extends the understanding of the specific mechanisms through which key constituents exert their beneficial effects. However, it's important to acknowledge the limitations of our study, and further research is warranted to elucidate these mechanisms more precisely.

Conclusions

In conclusion, this study aimed to identify the chemical composition of a citrus peel essential oil using GC-MS and HS-SMPE, revealing D-limonene as the predominant compound. The research further explored the antioxidant potential through various methods, with D-limonene and α-bergamotene demonstrating significant antioxidant capabilities according to molecular docking analysis. Additionally, the essential oil exhibited noteworthy antimitotic activity compared to colchicine on *Lepidium sativum* root length. Importantly, ADMET analysis found no violations, suggesting the essential oil's safety and potential utility. Overall, these findings contribute valuable insights into the chemical, antioxidant, and biological properties of the citrus peel essential oil.

Availability of Data and Materials

Data involved in the present work are available from corresponding authors upon request.

Author Contributions

Conceptualization: MR, MD; methodology: MR, SA and MD; software: SA; validation: MR, MD, SA, MH, SH, NG and RM; formal analysis: MR, MD and SA; data curation: MR, MD, SA, RM, MH, SH and NG; writing—original draft preparation: MR, MD, SA, RM, MH, SH and NG; writing—review and editing: MR, MD, SA, RM, MH, SH and NG; visualization: MR and SA; interpretation: MR, MD, SA, MH, SH, NG and RM; supervision: NG; funding acquisition: RM. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

The authors extend their appreciation to Researchers Supporting [Project number (RSP2024R119)], King Saud University, Riyadh, Saudi Arabia for funding this work.

Funding

This work was supported by Researchers Supporting Program [Project number (RSP2024R119)], King Saud University, Riyadh, Saudi Arabia.

Conflict of Interest

The authors declare no conflict of interest.

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