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Research Article

Pharmacological and Phytochemical Screening of the Essential Oil from peels of *Citrus limetta* Risso

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Abstract

The current study investigated the anti-microbial, enzymatic inhibition potential, anti-oxidative, and anti-depressive properties of the essential oil (EO) of peels of *Citrus limetta* Risso (Clp) plant. GC-MS analysis was performed to screen the Clp-EO active constituents. Limonene was found to be the main constituent of Clp-EO through NMR spectroscopic data. The Clp-EO was found to possess great anti-fungal inhibition of *C. albicans* (20 mm) as effective as the standard agent (Amphotericin B). On the other hand, its anti-bacterial activity was moderate at inhibiting S. aureus (8 mm) when compared to the standard (Ciprofloxacin). The antioxidant activity test was performed using DPPH radicals and the Clp-EO flourished strong antioxidant (90%) activity. Clp-EO exhibited substantial inhibition (IC50 = 28.13 ± 0.5 µg/mL) against prolyl endopeptidase, while notable inhibition (IC50 value of 62.52 ± 1.49 µg/mL) was observed in case of tyrosinase. Similarly, Clp-EO exhibited a strong inhibition (IC50, 38.58 ± 1.26 µg/mL) against α-glucosidase enzyme, while, in case of CA-II inhibitory assay, Clp-EO exhibited a notable inhibition (39.64%). Moreover, reductions in behavioral patterns *in vivo* indicated anxiolytic activity. These findings imply that Clp-EO could be a natural preservative in food or as an effective treatment against a variety of ailments.

Keywords: *Citrus limetta* Peels; Antioxidant; Antidiabetic; GC-MS; Enzyme Inhibition

Introduction

Diabetes mellitus (DM), a well-known metabolic disorder, has become an alarming global problem in recent years [1,2]. One satisfying approach to cure postprandial hyperglycemia is to reduce the cleavage of glucose from disaccharide and starch by inhibition of α-glucosidase enzyme in the humans [1,3]. α-Glucosidase (EC 3.2.1.20) is an enzyme that breaks down large molecules (starch and disaccharides) into glucose. The use of α-glucosidase inhibitors (AGIs) is an effective strategy in the treatment of diabetes [4]. Several AGIs from natural sources (NS) can efficiently control blood glucose levels but are linked with serious gastrointestinal side effects [5]. In recent years, AGIs from NS have received tremendous attention because of the highly abundant compounds in nature and their promising biological activities [6]. Therefore, it is important to search for alternatives remedies that can display α-glucosidase inhibitory activity without any side effects.

Prolyl endopeptidase (EC 3.4.21.26; PEP). first reported as a cytosolic enzyme from the human uterus [7], and is associated with learning and memory. PEP degrades several neuropeptides like TRH, substance P, and AVP substrates [8]. Inhibition of PEP is proven to be correlated with increased cognitive function and protection from memory weakening linked with cerebral aging [9].

Tyrosinase (EC 1.14.18.1) as a targeted enzyme used in food and cosmetic industries [10,11]. In the case of food industry, tyrosinase is mostly involved in the oxidation of phenolic like compounds into highly reactive quinines, preceding to the blackening of the products in processing or storage [10]. On the other hand, tyrosinase also affected the formation of melanin, which plays a crucial role for protecting the skin organs from UV radiation in animals. However, excess amount of melanin in the skin can result in abnormal pigmentation syndromes [12]. Furthermore, melanogenesis can be controlled by inhibiting the activity of tyrosinase enzyme [13]. Most skin-lightening agents, such as hydroquinone, kojic acid, arbutin, and azelaic acid are effective tyrosinase inhibitors with undesirable side effects such as cytotoxicity, vitiligo, irritation, skin peeling, and redness [14,15]. Therefore, natural sources and compounds with tyrosinase inhibitory activity have the possibility to be employed in both the food and cosmetic industries.

Citrus limetta Risso (Clp), (Omani name "shamom", locally named as "mosambi" or "lomi) is native to South and Southeast Asia, India, Sri Lanka, Pakistan, North Africa, and Arabian peninsula especially Oman [16,17]. Traditionally, it is used in the treatment of malaria, jaundice, and flu like symptoms such as fever, headaches, and nausea [18]. The essential oil of *Citrus limetta Risso* contains monoterpene hydrocarbons such as α-pinene, β-pinene, myrcene, camphene, linalool as well as limonene as the major constitute [18,19]. Several studies have reported that limonene, abundant component of Clp-EO, exhibits significant antimicrobial, antidepressant, anticonvulsant, analgesic, anti-inflammation, oxidative stress, and antioxidant activity [20-24]. Recently, Narayanankutty and his co-workers reported anti-bacterial, antioxidant, and anti-cancer potential of Clp-EO [25]. Furthermore, EOs of *Citrus* include monoterpenes and its derivatives, esters, ketones, alcohols, aldehydes, β-pinene, terpinenes, and limonene as a major component [26]. Limonene is usually considered harmless and safe, rarely causing skin irritations but having no harmful effects [27]. It is commonly applied as the flavorings for various foods, cosmetics, and medicines as well as a fragrance in the perfume industry [27,28]. However, the peels of the fruit, which account for 50% of the fruit's weight, are usually discarded as waste material.

The significance of this study lies in its unique approach to discover the overlooked possibilities of *Citrus limetta Risso* peel extract (Clp-EO) as a source of bioactive compounds with therapeutic properties. By employing phytochemical screening methods to precisely identify the constituents of Clp-EO and conducting a series of pharmacological experiments, this research aims to not only confirm the effectiveness of Clp-EO's bioactive compounds against disease-causing agents, but also unlock new and cost-effective avenues for drug development. The findings of this study hold the promise of contributing to the creation of novel medications derived from readily available and underutilized resources, ultimately benefiting both the pharmaceutical industry and public health.

Materials and Methods

Chemicals/Instruments

Analytical grade methanol (MeOH), dimethyl sulfoxide (DMSO), phosphate buffer, 2,2 –diphenyl-1-picrylhydrazyl (DPPH) bought from Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany. α-Glucosidase enzyme, and P-nitrophenyl-α-D-glucopyranoside were purchased from Sigma Aldrich, Switzerland. Prolyl endopeptidase, carbonic anhydrase (CA-II), and tyrosinase enzymes were obtained from Sigma-Aldrich, St. Louis, MO, USA. Citalopram was obtained from Al Rusail, Muscat. Clevenger apparatus was used for extraction of oil, while GC-MS analysis was performed through Perkin Elmer Clarus 600 GC device. Nuclear Magnetic Resonance (NMR) spectra were obtained using a 600 MHz-operating NMR spectrometer from Bruker in Zürich, Switzerland. Enzymatic assay required a 96-well plate reader (SpectraMax-384, Molecular Devices, CA, USA). The incubator (Gen Lab, Mino/75F, and Serial No. Y5K041, Germany) was used for incubation. The absorbance was taken through a UV spectrophotometer (Great Britain, Model Biomate-8832) purchased from Thermo Spectronic Shifting Company, Europe. The rotatory evaporator (Model RE801) was from Yamato Rotary Evaporator, Japan, used for the evaporation of organic solvents.

Plant material

The plant was collected from the botanical garden of the University of Nizwa in which the plant was grown and nurtured. The collection was obtained during the early morning hours and transferred to the lab for extraction. The peels were carefully removed from the fruit material.

Extraction of the essential oil

The peels were cut into small pieces to increase the surface area for extraction. The Clevenger apparatus was set up for extraction of the essential oil from the peels of the fruit. The distillation continued for 3 to 4 h, until no more quantity of EO left. The separated oil was dried after by adding anhydrous sodium sulphate then filtered and dichloromethane was removed obtaining dried essential oil. The isolated EO was transferred to a vial and kept in the refrigerator for further processing [28].

Gas chromatography-mass spectrometry (GC-MS)

GC-MS analysis was performed in Sultan Qaboos University, College of Agricultural and Marine Sciences, Central laboratories, on a Perkin Elmer Clarus, fitted with a SP-2560 Supelco capillary column (100 m × 0.250 mm i.d. × 0.2 µm film thickness) coupled to Clarus 600C MS. Ultra-high purity helium (99.9999%) from air products was used as carrier gas at a constant flow of 1.0 ml/min. The injection, transfer line, and ion source temperatures were 250, 240 and 240 ºC, respectively. The ionizing energy was 70 eV. Electron multiplier (EM) voltage was obtained from autotune. All data were obtained by collecting the full-scan mass spectra within the scan range 35-500 amu. The injected sample volume was 1 µL with a split ratio of 50:1. The oven temperature program was 60 ºC (holds for 1 min.) and accelerated at a rate of 8 ºC/min–280 ºC hold for 25 minutes. The unknown compounds were identified by comparing the spectra obtained with mass spectrum libraries (NIST 2011 v.2.3 and Wiley, 9th edition) and further confirmed with C_7-C_{30} saturated Alkanes standards (cat. # 49451-U). The Kovats Index (KI) was calculated for each compound, serving as a retention index used in gas chromatography to characterize and identify compounds based on their retention times.

Nuclear magnetic resonance (NMR)

The oil was prepared for analysis by dissolution in deuterated chloroform ($CDCl_3$). The NMR instrument was calibrated and configured for the specific NMR technique, with the magnetic field strength and operating parameters properly set. The prepared EO sample was carefully loaded into a specialized NMR tube, ensuring there were no air bubbles or contamination. The NMR tube, containing the oil sample, was inserted into the NMR spectrometer. Data acquisition was initiated, subjecting the sample to a strong magnetic field and radiofrequency pulses. Raw NMR data was processed to eliminate baseline distortions, correct phase, and amplitude, and apply necessary transformations.

Antimicrobial and antifungal testing

Bacterial and fungal cultures

The microorganisms used in this study were obtained from the NMSRC microbiology laboratory at the University of Nizwa in the Sultanate of Oman. This experiment used isolates of the following bacteria and fungi: *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 25922), *Candida albicans* (ATCC 14053), and *Candida kruzei* (ATCC 6258).

Preparation of bacterial strains

S. aureus and *E. coli* strains were sub-cultured on nutrient agar (Liofilchem, Teramo, Italy). The subculture was prepared by inoculating direct colony from master plate (original) into fresh nutrient agar (24 g in 1L Distilled water (D.H_2O) and autoclaved 121 °C for 15 min) to get fresh bacteria and the inoculation was done by taking single pure colony using a sterilized loop. After incubation of 24 h at 28 °C, the strains were ready for antimicrobial sensitivity testing [29].

Preparation of fungal strain

C. albicans and *C. kruzei* strains were sub-cultured on potato dextrose agar (PDA) (Liofilchem, Italy). The subculture was prepared by inoculating direct colony from master plate (original) into fresh PDA (41.0 g in 1L D. H_2O and autoclaved 121 °C for 15 min) to get fresh fungi with same morphology and inoculation was done by taking 1uL using disposable loop. After incubation of 24 h at 28 °C, strains were ready for antimicrobial sensitivity test.

Antioxidant activity

The assessment of antioxidant activity in the oil was conducted following the used method [25]. Various concentrations of the oil sample were prepared using MeOH, specifically at levels of 40, 60, 80, 100, and 200 µg/mL. From each concentration, 3 mL sample was combined with 1 mL of MeOH solution containing 0.3 mM DPPH (2,2-diphenyl-1-1-picrylhydrazyl). The resulting mixture was vigorously agitated and left in darkness at room temperature for 30 min. Subsequently, the absorbance was measured at 516 nm and contrasted against a blank sample composed of 3 mL of the oil and 1 mL of MeOH. The control sample was prepared in the same manner but lacked the addition of oil. The assessment of the radical scavenging activity at the tested concentrations was determined as a percentage inhibition using the subsequent formula:

$$\% \text{ Radical Scavenging Activity} = \frac{(A° - As)}{A°} \times 100$$

A° represents the absorbance of the control sample.

As represents the absorbance of the test sample.

In-vitro CA-II inhibitory assay

In the present investigation, a bioassay involving carbonic anhydrase II (CA-II) was conducted to identify potential compounds suitable for novel medications. The experiment was carried out in 96-well plates, where freshly prepared aqueous solutions containing 20 mg/mL of bovine erythrocytes CA-II, 20 µL of 4-nitrophenyl acetate (4-NPA) at a concentration of 0.7 mM in ethanol, and 140

µL of buffer solution (HEPES-tris-HCl) at a pH of 7.4 were utilized. The tested Clp-EO and the enzyme were pre-incubated for 15 min in a 96-well plate before the reaction was initiated by the addition of the substrate. The reaction was initiated by adding 20 µL of 4-NPA, and the subsequent product production rate was continuously monitored for 30 min with 1min intervals at 25 °C using a microplate reader (SpectraMax-384, Molecular Devices, CA, USA). To determine the % inhibition, the following equation was employed [30].

α-Glucosidase inhibitory assay

In this research, DMSO was utilized as the solvent system for dissolving all the tested samples. For this assay, different concentrations of Clp-EO's were added to each well of 96-well plate along with an enzyme solution (2 U/2 mL, 20 µL/well), and a phosphate buffer (50 mM, pH 6.8, 135 µL/well). Subsequently, the plate was incubated at 37 °C for 15 min, and a blank absorbance reading was recorded without the substrate. Afterward, changes in absorbance were continuously monitored for 30 min at 400 nm following the addition of the substrate (4-nitrophenyl α-D-glucopyranoside, 0.7 mM, 25 µL) [31].

Prolyl endopeptidase inhibitory activity

A spectrophotometric assay to measure the inhibitory activity of prolyl endopeptidase was conducted according to the method outlined [32] with minor adjustments. In a 200 µL reaction volume, 140 µL of sodium phosphate buffer (50 mM, pH 7.0), 20 µL of the Clp-EO's (dissolved in MeOH at a concentration of 0.5 mM), and 20 µL of prolyl endopeptidase solution (0.02 U/well) were combined in a 96-well plate. For the control, 20 µL of MeOH was used instead of the Clp-EO's, while bacitracin (0.5 mM) served as the positive control. The reaction mixture was then incubated at 30 °C for 10 minutes, and a pre-reading was taken at 410 nm. Subsequently, 20 µL of a substrate solution, Z-gly-pro-4-nitroanilide (dissolved in 1,4-dioxane at a concentration of 0.4 mM), was added, and the change in absorbance was continuously recorded at 410 nm over a 30-min period using a 96-well plate reader. The final concentration of MeOH and 1,4-dioxane in the reaction mixture was 10% v/v. All reactions were conducted in triplicate in 96-well microplates.

Tyrosinase inhibitory assay

The tyrosinase inhibition assay was carried out in a 96-well plate using a spectrophotometer microplate reader, following a method adapted from [33]. Initially, 10 µL of the Clp-EO was placed into a 96-well microplate, alongside the positive control, "kojic acid." The solution was then mixed with 60 µL of phosphate buffer and 10 µL of mushroom tyrosinase (30 U/mL in phosphate buffer) to form the reaction mixture. After an incubation period, 20 µL of L-DOPA in phosphate buffer was introduced, and the reaction mixture was spectrophotometrically measured at 480 nm. All reactions were performed in triplicate, and the results were reported as the mean. The *in vitro* testing results, including % inhibition and IC_{50} values, were analyzed using EZ-Fit, which is a curve-fitting enzyme kinetics program provided by Perrella Scientific Inc. in Amherst, USA.

Anti-depressant activity

To perform the anti-depressant activity, a tail-suspension test was used [34]. Four test groups of 3 mice were established. The protocol is as follows: The weight of each mouse was measured on a scale; the drug or the control was administered using insulin syringe, followed by a 30-min wait to allow the drug or the sample to take effect. The mice were videotaped for 6 min but the first two min were neglected, as the mice must adjust to the change in position. The 4 min of monitoring were reported by calculating the ratio of the periods of movement and physical activity to the periods of quietness and stillness in the mice. The analysis of the videotape was done by measuring the immobility time by three different raters individually to promote precision and accuracy in the reported outcomes. The first group received 200 mg/kg of Clp-EO dissolved in DMSO (10% DMSO with buffer) and phosphate buffer (1:9 ratio) for IP injection. Group 2 received 100 mg/kg of Clp-EO in the same solvent. The control group received only solvents used for Clp-EO. The standard group was injected with citalopram (10 mg/kg), as appositive control, using the same solvent that was used to dissolve the EO.

Statistic evaluation

Notably, all experiments were performed in triplicate to reduce the chances of mistakes, and the differences in the results are reported as Standard Error Mean (SEM). Excel and the SoftMax Pro package were used to examine the results for biological activity. The following formula was used to determine the % inhibition.

IC_{50} values of all tested substances were calculated using EZ-FIT (Perrella Scientific, Inc., USA). Notably, all experiments were performed in triplicate to reduce the chances of mistakes, and the differences in the results are reported as Standard Error Mean (SEM).

Results and Discussion

Chemical composition of Clp-EO

The high yield of essential oil (6 g) was extracted corresponds to 0.8% of the total peels (740 g). This yield was considered high

and sufficient to perform the experiments needed for the study. In another study, the amount extracted using the same method was 0.313% of extraction yield of the same oil [18].

Limonene (96.72%), as a major component in essential oil, was determined using GC-MS analysis, followed by β-myrcene (2.11%) (Table 1). Other compounds like α-pinene, sabinene, and linalool is present in smaller amounts. The Retention Time (RT) and Retention (RI) Index are crucial for compounds identification, ensuring the results are consistent with known standards. In comparison to another study published by Hasan., *et al*. (2022) on the same essential oil of *Citrus limetta*, notable differences in both chemical profiles emerge [35]. In this study, β-myrcene appears as a component, contributing 2.11% to the total amount which is absent in another study. Limonene is the predominant compound in both studies, representing 96.72% and 95.98% of the total. While both studies share some common compounds, such as α-pinene and α-terpinene, the variations in identified components and their respective percentages indicate discrepancies in the chemical makeup of the Clp-EO. These differences may stem from various factors, including geographical variations in plant material and extraction methods [36,37].

Name	RT_{min}	$RI_{(cal)}$	$RI_{(NIST)}$	% Clp-EO
α-Pinene	8.171	932	922	0.62
Sabinene	9.494	972	964	0.13
β-Myrcene	10.118	991	981	2.11
Limonene	11.43	1029	1018	96.72
Linalool	13.937	1100	1082	0.25
Total				99.83

Table 1: GC-MS analysis of essential oils from peels of *C. limetta.*

$RI_{(calc)}$ = Retention index calculated.

[a]Elution order on Rtx-5MS capillary column.

$RI_{(NIST)}$ = Retention index obtained from database (NIST, 2011).

RT_{min} = Retention time (min).

Total =99.83% (peels).

NMR analysis of limonene

As the oil contains > 96% limonene, it was subjected to NMR analysis to confirm its structure. The NMR data showed perfect resemblance to the structure of limonene (Figure 1). The ^{1}H-NMR represented singlet absorption due to the two allylic methyl groups at δ_H 1.71 (3H, s, H-7), and 1.79 (3H, s, H-10) (Ferreira Farias., *et al.* 2019). In ^{1}H NMR, resonances at δ_H 5.38 (1H, 120.6, C-2), and 4.69 (2H, 108.4, C-9) determined the presence of one olefinic proton and one exocyclic methylene, respectively. Three NMR resonances appeared at δ_H 2.28 (δc 30.6), 2.08 (δc 41.1), and 1.62 (δc 27.6) methylenes represented the presence of three methylene in the ring [38].

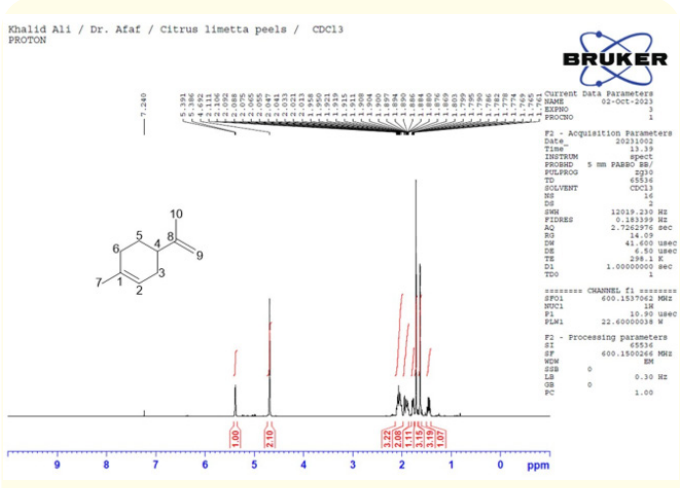

Figure 1: ^{1}H-NMR and structure of limonene

Antimicrobial potential

The Clp-EO exhibits an 8 mm inhibition zone against *S. aureus* (Gram (+) bacteria), which is smaller than the inhibition zone of the standard (40 mm). This suggested that the sample has weaker antimicrobial activity against *S. aureus* compared to the standard. Gram (-) bacteria (*E. coli*) was not susceptible to Clp-EO at all doses (Table 2; Figure 2). Furthermore, the oil inhibited the growth of *C. albicans,* at levels like that of standard (20 mm). The sample showed no activity against *C. kruzei.* (Table 2). The antibacterial activity observed in this study is in complete agreement to that of reported by Hasan., *et al.* however, an inhibition of 20 mm was observed against *S. aureus* [35]. Similarly, B'chir and his Co-workers reported 10 mm ZOI for *E. coli* [36]. The disparity between these findings may be attributed to multiple factors, including variations in the concentration of antimicrobial compounds within the samples, differences in the extraction methodology employed, or variances in the sensitivity of the test strains. However, the findings for *C. albicans*, notably, align with the results reported [36], where the same ZOI was observed against *C. albicans* (Figure 3). It is imperative to acknowledge that the antimicrobial activity of Clp-EO oil may exhibit variability based on numerous factors, including the source of the fruit, the extraction methods employed, and the composition of the oil. Consequently, direct comparisons between different studies can be challenging. Nevertheless, the results obtained in this study indicated a noteworthy antimicrobial activity of Clp-EO against a range of pathogens, encompassing *S. aureus, E. coli,* and *C. albicans* [35,39].

Compound	*S. aureus* (Gram positive bacteria)	*E. coli* (Gram negative bacteria)	*C. albicans C. kruzei* (Fungi)	
Clp-EO	8 mm	NA	20 mm	NA
Standard	40 mm	30 mm	20 mm	20 mm

Table 2: Antimicrobial activity of EO from peels of *C. limitta.*

NA = No activity.

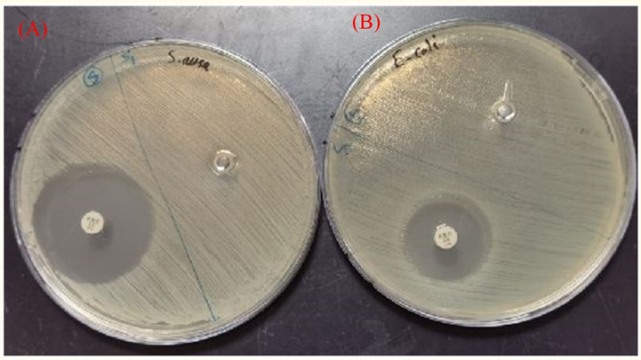

Figure 2: Zone of Inhibition (ZOI) of EO from *C. Limetta* oil against A) *S. aureus*, B) *E. coli*.

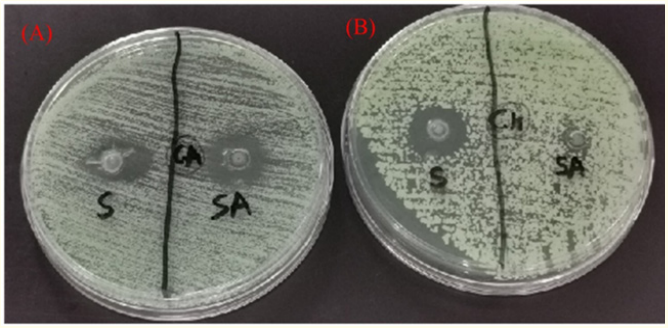

Figure 3: Zone of Inhibition (ZOI) of EO from *C. Limetta* oil against A) *C. albicans*, B) *C. kruzei*.

Antioxidant activity

The % RSA values represent the ability of Clp-EO to scavenge DPPH radicals and reflect its antioxidant activity. At the highest concentration of 500 µg/mL, Clp-EO exhibited 92.88% Radical scavenging activity (RSA), indicating its capacity to neutralize approximately 93% of the DPPH radicals. This demonstrated robust antioxidant activity. Notably, as the concentration of Clp-EO decreased, the % RSA values remained relatively high, indicating that Clp-EO maintained its antioxidant activity even at lower concentrations. Throughout the tested concentration range, % RSA values consistently exceeded 90%, signifying the sample's consistent and notable ability to scavenge DPPH radicals (Fig. 4). Our results are in complete agreement with the reported values of the different species belonging to the same genus [18,28,39].

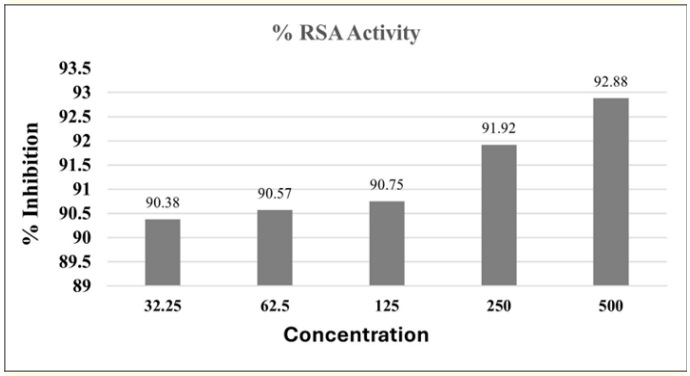

Figure 4: The radical scavenging activity of the peels EO from *C. limetta*.

Enzymatic assays

Enzyme assays have a wide range of applications in drug discovery, from plants to natural materials. The ability of the EO from *Citrus limetta* peels to inhibit four key enzymes associated with human pathologies. Therefore, CA II (diuretic, antiglaucoma, antitumor and antiepileptic agent), α-glucosidase (diabetes), endoprolyl endopeptidase (neurodegenerative diseases), and tyrosinase (hyperpigmentation) enzymes were examined in the current study. The Clp-EO exhibited less than 50% (39.64%) inhibition against CA-II (Table 3) in comparison to standard (acetazolamide) [40]. It is important to note that Clp-EO is a complex mixture with multiple components, making it challenging to determine a specific IC_{50} value, which is typically feasible for pure substances.

On the other side, Clp-EO exhibited a noteworthy activity with IC_{50} values of 38.58 ± 1.26 µg/mL, indicating strong inhibition against the target enzyme compared to the standard (Acarbose, 377.26 ± 1.20 µg/mL, Table 3) at the concentration of 0.5 mg/mL. These results indicated that Clp-EO may be used as an as an effective enzyme inhibitor in food supplements. The limonene could be responsible for the inhibition of α-glucosidase enzyme, as it is present at high levels in the active samples (96.72%). Although, Clp-EO has demonstrated a significant inhibition suggesting that this monoterpene could contribute to the α- glucosidase inhibitory activity. Hamden and Co-Workers reported that administration of terpenes to diabetic exerts blood glucose lowering effect in alloxan-induced diabetic rat [41]. Similarly. Padilla-Camberos., *et al.* reported 28.2% inhibition (conc. 20 mg/mL) by the aqueous ex-

tract of Clp peels against α-glucosidase enzyme [42]. Furthermore, *C. limetta* fruit peel also displayed a notable anti-hyperglycemic effect in streptozotocin induced diabetic rats. *In vitro* α–amylase and *in vivo* hypoglycemic effect of methanol extract of *C. macroptera*. fruit was also performed [43]. Thus, the antidiabetic activity of Clp-EO suggests the EO of *C. Limetta* peel as potential therapeutics for diabetic conditions.

Prolyl endopeptidase (PEP) is an enzyme which plays a role in the metabolism of proline-containing neuropeptides, e.g., vasopressin, substance P and thyrotropin-releasing hormone (TRH), which have been suggested to be involved in learning and memory processes [8]. The citrus oil determined notable activity with IC_{50} of 28.13 ± 0.5 µg/mL in comparison to Z-prolyl-prolinal (standard, Table 3). This low IC_{50} value indicates Clp-EO's high potency as an inhibitor of PEP. These results collectively suggested that Clp-EO holds great promise as an inhibitor for PEP.

Tyrosinase is a multifunctional, copper-containing enzyme that is widely distributed in nature and is involved in melanogenesis. It catalyzes both the hydroxylation of L-tyrosine (monophenolase activity) and the oxidation of L-DOPA (diphenolase activity) to *o*-quinone, which induces the production of melanin pigments [12]. In this study, the inhibitory potential of Clp-EO on tyrosinase enzyme activity was investigated, with kojic acid serving as the control reference. Clp-EO exhibited remarkable tyrosinase inhibition, with a percent inhibition of 80% (IC_{50} = 62.52 ± 1.49 µg/mL) (Table 3). Recently, Imen and his Co-workers (2023) recorded the IC_{50} value between 12.66 ± 0.66 mg/mL and 120.65 ± 3.43 mg/mL in the EOs extracted from orange peel waste dried in the oven at different temperatures [44]. This result underscores the efficacy of Clp-EO as a tyrosinase inhibitor. The high % inhibition demonstrates the ability of Clp-EO to substantially reduce the enzymatic activity of tyrosinase. The results suggested that Clp-EO might effectively reduce the activity of the enzyme, making it potentially valuable in formulations targeting pigmentation disorders and related dermatological conditions.

Enzyme	Sample	% Inhibition	IC_{50} ± µg/ml (S.E.M)
CA-II	"Clp-EO"	39.64	N/A
Standard	Acetazolamide	80.00	8.64 ± 0.27
α-Glucosidase	"Clp-EO"	83.17	38.58 **± 1.26**
Standard	Acarbose	59.37	377.26 ± 1.20
Prolyl endopeptidase	Clp-EO	87	28.13 ± 0.5
Standard	Z-prolyl-prolinal	95	0.001 ± 0.0004
Tyrosinase	Clp-EO	80	62.52 ± 1.49
Standard	Kojic acid	95	7.49 ± 0.21

Table 3: Enzyme inhibition studies of the essential oil extracted from the peels of *C. limetta.*

Concentration= 0.5 mg/mL; N/A = Not applicable; S.E.M = Standard Error Mean.

Anti-depressant activity

The tail suspension test (TST) was used in this study to assess the impact of *Citrus limetta* peel's EO on depression in mice. The mice in the negative control group exhibited relatively low levels of immobilization (75.87 sec., Table 4). This duration of immobilization suggested that the control solution, 10% DMSO in phosphate buffer, did not significantly affect the mice activity levels. The control group's behavior can be considered as a baseline for comparison. The mice in the positive control group (10 mg/kg of citalopram) displayed a moderate level of immobilization, with a mean duration of 62 sec. Mice with tested Clp-EO at a dose of 100 mg/kg group determined an observable increase in immobilization.

These findings suggested a reduction in behavioral activity compared to the control group. The observed decrease in the behavioral activity within this group could potentially be attributed to anxiolytic activity rather than antidepressant effects, as previously reported with essential oil from peels of different citrus fruits [45]. Limonene, a naturally occurring monoterpene found in citrus peels, has demonstrated anxiolytic properties in various studies. Several preclinical studies have shown that limonene can reduce anxiety-like behaviors in animal models, such as the elevated plus maze and open field tests. These effects are attributed to limonene's interaction with the GABAergic and serotonergic neurotransmission systems. Lima., *et al.* 2013 investigated the anxiolytic effects of limonene in the elevated plus maze test and found that limonene administration significantly increased the time spent in the open arms of the maze, indicating reduced anxiety-like behavior [46]. Available studies also indicated that there are anxiolytic effects of limonene in the marble-burying test and found that limonene administration significantly reduced the number of marbles buried, suggesting anxiolytic activity [47-49]. Anxiolytics are known to reduce anxiety and may lead to lower activity levels in animal models. Given the varying levels of movement within this group, it is possible that the essential oil administered to these mice having

anxiolytic properties, which might contribute to reduced activity levels. Further investigations into the specific anxiolytic mechanisms and components of the essential oil are warranted to confirm this hypothesis.

Group	Weight (g)	Dose injected (mL)	Immobility (seconds)			
			Rater 1	Rater 2	Rater 3	Mean
10% DMSO in phosphate buffer (Negative control)	35.00	0.1167	100	60	77	79
	34.26	0.1142	85	76	71	77
	32.25	0.1075	80	68	67	71.6
Mean = 75.87 sec						
Citalopram (Positive control)	33.50	0.1117	86	45	75	69
	32.21	0.1073	58	52	55	55
	34.72	0.1150	68	58	60	62
Mean = 62.0 sec						
Group 1 (200 mg/kg)	30.3	0.1010	105	60	90	85
	24.61	0.0987	171	165	171	169
	31.4	0.1046	159	133	162	151
Mean = 135.0 sec						
Group 2 (100 mg/kg)	31.05	0.1035	95	79	90	88
	28.12	0.0937	130	100	120	117
	27.75	0.0925	209	183	214	202
Mean = 135.67 sec						

Table 4: Anti-depressant behavior of EO from peels of *Citrus limetta*.

Conclusion

In conclusion, this research comprehensively explored the EO extracted from *Citrus limetta Risso* peels, with a focus on its active components and pharmacological properties. Limonene was identified as the primary constituent through GC-MS and NMR spectroscopy. The results demonstrated promising antimicrobial and antifungal activity against *S. aureus* and *C. albicans*. Furthermore, the oil exhibited strong antioxidant (90%) activity using DPPH assay. Clp-EO exhibited substantial inhibition against prolyl endopeptidase (IC_{50}, 28.13 ± 0.5 µg/mL), tyrosinase (IC_{50}, 62.52 ± 1.49 µg/mL), α-Glucosidase (IC_{50}, 38.58 ± 1.26 µg/mL). This present research will open new avenues for pharmaceutical exploration by utilizing the abundant resources offered by *Citrus limetta Risso* peels. As a viable source of bioactive compounds with therapeutic properties, Clp-EO will contribute to the nutraceutical field readily available option for future drug development endeavors.

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Disclosure Statement

The authors declare no conflict of interest.

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