Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2023**; 28(9): 229
https://doi.org/10.31083/j.fbl2809229

Original Research

Expediting Multiple Biological Properties of Limonene and α-Pinene: Main Bioactive Compounds of *Pistacia lentiscus* L., Essential Oils

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Academic Editor: Marcello Iriti
Submitted: 8 April 2023 Revised: 23 April 2023 Accepted: 2 August 2023 Published: 27 September 2023

Abstract

Background: Screening new natural molecules with pharmacological and/or cosmetic properties remains a highly sought-after area of research. Moreover, essential oils and volatile compounds have recently garnered significant interest as natural substance candidates. In this study, the volatile components of *Pistacia lentiscus* L. essential oils (PLEOs) isolated from the fruit and its main compounds, alpha-pinene, and limonene, are investigated for antioxidant, antidiabetic, and dermatoprotective activities. **Methods**: *In vitro* antioxidant activity was investigated using 2,2′-diphenyl-1-picrylhydrazyl (DPPH), fluorescence recovery after photobleaching (FRAP), and 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) methods. The antidiabetic and dermatoprotective effects were studied using enzyme inhibitory activities. **Results**: Antioxidant tests showed that PLEO has the best activity (ranging from 29.64 $\pm$ 3.04 to 73.80 $\pm$ 3.96 µg/mL) compared to its main selected molecules (ranging from 74 $\pm$ 3.72 to 107.23 $\pm$ 5.03 µg/mL). The α-glucosidase and α-amylase assays demonstrated that the elements tested have a promising antidiabetic potential with IC_{50}values ranging from 78.03 $\pm$ 2.31 to 116.03 $\pm$ 7.42 µg/mL and 74.39 $\pm$ 3.08 to 112.35 $\pm$ 4.92 µg/mL for the α-glucosidase and α-amylase assays, respectively, compared to the standard drug. For the tyrosinase test, we found that the EOs (IC_{50} = 57.72 $\pm$ 2.86 µg/mL) followed by limonene (IC_{50} = 74.24 $\pm$ 2.06 µg/mL) and α-pinene (IC_{50} = 97.45 $\pm$ 5.22 µg/mL) all exhibited greater inhibitory effects than quercetin (IC_{50} = 246.90 $\pm$ 2.54 µg/mL). **Conclusions**: Our results suggest that the biological activities of PLEO, as well as its main compounds, make them promising candidates for the development of new strategies aimed at improving dermatoprotection and treating diseases associated with diabetes mellitus and oxidative stress.

Keywords: *Pistacia lentiscus*; α-pinene; limonene; enzyme inhibitory; antidiabetic; dermatoprotective.

1. Introduction

The inhibition of the enzymatic activity involved in the development of human pathologies is an interesting treatment for diseases and constitutes a guideline for the discovery of new drugs [1]. This therapeutic approach is based on the development of inhibitors acting on the regulation and/or mode of action of enzymatic activity [2–4]. In this context, several studies have suggested that: (i) the simultaneous inhibition of α-glucosidase and α-amylase enzymes represents an optimal approach to improve the management of diabetes, (ii) elastase inhibition constitutes an effective strategy to evaluate the dermatoprotective effect, and (iii) these inhibitors can play a crucial role in the prevention and/or treatment of pathologies by reducing the levels of oxidative stress [5–16]. Despite the availability of synthetic inhibitors, their side effects have prompted the search for new effective and protective inhibitors based on natural products [17,18]. Moreover, these plants contain a wide range of secondary metabolites, which have shown important biological activities and are suggested as promising drug candidates in the treatment of many human pathologies [19–26]. Among medicinal herbs, lentisk (*Pistacia lentiscus*), belonging to the Anacardiaceae family, has attracted much attention over the years because of its culinary, cosmetic, and therapeutic applications. This plant is found throughout the Mediterranean region, including Morocco. It is widely employed in Moroccan traditional medicine to treat pathologies such as digestive [27], cardiovascular, and diabetic disorders [28]. Phytochemical analyzes have shown the composition of different parts of this plant with varying levels of phenols, phenolic acids, condensed tannins, and flavonoids [29–31]. Bioassay results from several studies highlight the anti-inflammatory, antioxidant, antiparasitic, anticancer, and antimicrobial properties of *P. lentiscus* extracts [32–34]. In addition, our pre-

vious research has focused on *P. lentiscus* essential oils (PLEOs) [23]. The *in vitro* results of our studies demonstrated that the functional compounds present in PLEO possess antioxidant, anticancer, and antibacterial capacities. Furthermore, the EOs from the fruit are rich in α-pinene and limonene, compounds that have been widely studied for use in the food and cosmetics industry, and for their therapeutic potential. Several studies have examined the toxicity of α-pinene and limonene, both *in vivo* and *in vitro*, and have demonstrated the safety of these compounds for human use. Shahriari *et al*. [35] evaluated the oral toxicity of four substances, including α-pinene, on *Tribolium castaneum* Herbst Larvae. They determined LC_{50} values, representing the lethal concentration at which 50% of organisms die after a specific exposure time. After 24 hours of exposure, α-pinene was the least toxic substance (LC_{50} = 12.85 µL/mL). The acute and subacute toxicity of limonene was evaluated using mice and rats (male and female) [36]. In rats, the oral LD_{50} was 4.4 and 5.1 g/kg b.w. for males and females, respectively. In mice, the LD50 was 5.6 and 6.6 g/kg b.w. for males and females, respectively. In addition, no abnormalities were observed in the mucosa of the duodenum, bile ducts, or gallbladder 24 hours after infusion of 20 mL/day of limonene into the gallbladder [37]. Similarly, no major abnormalities were recorded in dogs after a daily infusion of $_{D}$-limonene (10 mL) for seven days [38]. Based on these studies, routine use of α-pinene and limonene appears safe. However, it is important to note that there is a possible risk of allergic reactions associated with these compounds, as with any product.

To our knowledge, the data on the biological effects of *P. lentiscus* fruit EO (PLFEO) and its main compounds, limonene, and α-pinene, are poor. This study aims to report for the first time a comparison between the biological effects of PLFEO and its main compounds, namely the antioxidant, antidiabetic, and dermatoprotective effects.

2. Materials and Methods

2.1 Chemicals and Reagents

α-pinene and limonene were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). p-Nitrophenyl-α-D-$_{D}$-glucopyranoside (p-NPG), 3,4-dihydroxy phenylalanine (L-DOPA), acarbose, 2,2′-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 6-hydroxy- 2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and ascorbic acid, α-glucosidase (from *Saccharomyces cerevisiae*) and α-amylase (from *Bacillus licheniformis*) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). All other reagents were obtained from commercial sources.

2.2 Plant Collection and Essential Oil Extraction

The fruit of *P. lentiscus* were collected from their natural habitat in the province of Ouezzane (north-west of Morocco: 34°47′50″ N and 5°34′56″ W) in October 2016 and authenticated at the scientific institute by Rabat. The voucher specimen has been stored in the Herbarium of the Botany Department at the Scientific Institute of Rabat/Morocco under the voucher specimen code RAB30. The samples were air-dried at room temperature in the shade. EOs were extracted by hydrodistillation using a Clevenger-type apparatus. The oils obtained were dried with anhydrous sodium sulphate, weighed, and then stored at 4 °C until their use.

2.3 Chemical Composition Analysis

In agreement with our earlier research [39], we proceeded to analyze the chemical composition of PLEO using the GC-MS method, following the instructions of Talbaoui *et al*. [40]. Analysis was performed on a TRACE GC ULTRA equipped with a non-polar VB5 capillary column (5% phenyl, 95% methylpolysiloxane) with a length of 30 m and an internal diameter of 0.25 mm, with a film thickness of 0.25 µm. This device was coupled to a Polaris Q mass spectrometer (EI 70 eV). The injector temperature was maintained at 250 °C, while the detector temperature was set at 300 °C. The oven temperature program was set to increase from 40 to 180 °C at a rate of 4 °C/min, then from 180 to 300 °C at 20 °C/min. Helium was used as the carrier gas at a flow rate of 1 mL/min. A 0.5 µL sample was injected in splitless mode. The individual components of the EOs were identified by comparing their relative retention times (RTT) with those of authentic samples or by comparing the relative retention indices (RRI) of the GC peaks with those of a homologous series of n-alkanes (series of C-9 to C-24 n-alkanes) reported in the literature. Each compound was confirmed by comparing its mass spectra with those of the NIST02 library data of the GC/MS system and the spectra of the Adams libraries (NIST/EPA/NIH, 2002; Adams, 2007) [41]. To determine the percentage of each individual component, the GC peak areas of each compound were normalized without any correction factors.

2.4 Antioxidant Activity Assays

To assess the antioxidant activity of PLEO, as well as α-pinene and limonene, three complementary antioxidant tests were conducted using the DPPH, FRAP, and ABTS assays. The testing procedures were performed according to our previous studies [42,43].

2.4.1 DPPH Free Radical-Scavenging Assay

The stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was used for the determination of free radical-scavenging activity of PLEO, α-pinene, and limonene. Aliquots (0.2 mL) of various concentrations of the EO, α-pinene, and limonene samples dissolved in methanol were added to 1.8 mL of a 0.004% methanolic solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm using a UV spectrophotometer. The percentage (%) to scavenge DPPH radicals were calculated using the following formula:

DPPH scavenging activity (AA in %) = $[(A_c - A_t)/A_c] \times 100$. 0where A_c is the absorbance of the control (without oil), and A_t is the absorbance of the test (with oil).

Trolox and ascorbic acid were used as a positive control, and EO concentrations providing 50% inhibition (IC_{50}) were calculated by plotting the inhibition percentages against the concentrations of the sample. The test was carried out in triplicate, and the IC_{50} values were reported as means ± SD.

2.4.2 Reducing Ferric Power Assay

Tested products and control were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [K3Fe (CN) 6] (2.5 mL, 1%). The mixture was then incubated at 50 °C for 20 min. A portion (2.5 mL) of trichloro acetic acid (10%) was added to the mixture, which was then centrifuged for 10 min at 3000 rpm. Finally, the upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and FeCl3 (0.5 mL, 0.1%), and the absorbance was measured at 700 nm using a spectrophotometer. The sample concentration providing 0.5 absorbance (IC_{50}) was calculated by plotting absorbance at 700 nm against the corresponding sample concentration. Trolox and ascorbic acid were used as positive controls. The test was carried out in triplicate, and IC_{50} values were reported as means ± SD.

2.4.3 ABTS Radical Scavenging Activity

The ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation was produced by the reaction between 5 mL of ABTS stock solution and 5 mL of 2.45 mM potassium persulfate ($K_2S_2O_8$) solution, stored in the dark at room temperature for 16 h. Before use, this solution was diluted with water to produce an absorbance of 0.700 ± 0.015 at 734 nm and equilibrated at 30 °C. PLEO, α-pinene, and limonene at various concentrations were diluted with dimethyl sulfoxide (DMSO) to produce a sample solution. A total of 2.5 mL of sample solution was homogenized with 97.5 mL of ABTS solution, the mixture was incubated at room temperature for 6 min, and its absorbance was recorded at 734 nm. Blanks were run in each assay. The inhibition percentage was calculated using the following formula:

ABTS scavenging activity (%) = $(A_0 - A_1)/A_0 \times 100$.

Where: A_0 is the absorbance of the control, and A_1 is the absorbance of the sample.

Trolox and ascorbic acid were used as positive controls. The test was carried out in triplicate, and ABTS scavenging ability was expressed as IC_{50} (µg/mL).

2.5 In Vitro Antidiabetic Activity

2.5.1 α-Amylase Inhibitory Assay

The inhibitory effects of PLEO, α-pinene, and limonene on α-amylase were examined using different concentrations of samples, reacting them with the enzyme α-amylase and a starch solution [42,43]. For this, a mixture of 250 µL of the samples and 250 µL of 0.02M sodium phosphate buffer (pH = 6.9) containing α-amylase (240 U/mL) was incubated at 37 °C for 20 minutes. Then, 250 µL of a 1% starch solution in 0.02 M sodium phosphate buffer (pH = 6.9) was added to the reaction mixture, which was then incubated at 37 °C for 15 minutes. Subsequently, 1 mL of dinitrosalicylic acid (DNS) was added, and the reaction mixture was incubated in boiling water for 10 minutes. After this step, the reaction mixture was diluted with 2 mL of distilled water, and absorbance was measured at 540 nm using a UV-Vis spectrophotometer. Acarbose was used as a positive control.

The percentage of inhibition was calculated using the following formula:

% of inhibition = (1 – (Abs enz+sub – Abs sub) – (Abs sample – Abs control)(Abs enz+sub – Abs sub)) × 100% of inhibition = 1 – Abs enz+sub – Abs sub – Abs sample – Abs controlAbs enz+sub – Abs sub × 100

The IC_{50} value was determined by the concentration of α-glucosidase inhibitor necessary to inhibit 50% of activity under assay conditions.

2.5.2 α-Glucosidase Inhibitory Assay

To assess the α-glucosidase inhibitory activity of PLEO, α-pinene, and limonene at three phenological stages, the pNPG substrate was used according to our previously published method with some modifications [42,43]. First, a mixture was prepared using 200 µL samples (at different EO concentrations with DMSO at a rate of less than 1%) and 100 µL of 0.1M sodium phosphate buffer (pH = 6.7) containing α-glucosidase enzyme at a concentration of 0.1 U/mL, then the mixture was incubated at 37 °C for 10 minutes. After preincubation, 200 µL of a 1 mM pNPG solution in 0.1 M sodium phosphate buffer (pH = 6.7) was added. Enzyme reactions were then incubated at 37 °C for 30 minutes. The α-glucosidase activity was determined at 405 nm after adding 1 mL of Na_2CO_3 (0.1 M) to the reaction mixture. The inhibitory activity of PLEO, α-pinene, and limonene was expressed as percent inhibition, and IC_{50} values were determined. Acarbose was used as a positive control.

2.6 Dermatoprotective Activity

2.6.1 Tyrosinase Inhibitory Assay

To evaluate the dermatoprotective effect, the tyrosinase inhibitory activity of PLEO, α-pinene, and limonene was determined according to the method previously described by Bouyahya *et al.* [42,43]. Briefly, 25 µL (at different concentrations of PLEO, α-pinene, and limonene prepared with DMSO at a rate <1%) of the sample was added to 100 µL of tyrosinase solution (333 U/mL, 50 mM phosphate buffer, pH 6.5) and incubated at 37 °C for 10 min. Subsequently, 300 µL of L-DOPA (5 mM) was added to the reaction mixture and incubated at 37 °C for 30 min. The

absorbance was measured at 510 nm using a spectrophotometer. The degree of tyrosinase inhibition was calculated at concentrations of 40, 60, 120, and 160 µg/mL of EO, the IC_{50} values were determined, and quercetin was used as a positive control.

2.6.2 Elastase Inhibitory Assay

The inhibitory effect of PLEO, α-pinene, and limonene on elastase activity was assessed using a method based on that described by Bouyahya [42,43], with some modifications. PLEO, α-pinene, and limonene samples were dissolved in methanol at varying concentrations (0.5, 1, 2, and 3 mg/mL). Then, 50 µL of each sample (prepared with DMSO at a rate of less than 1%) was mixed with 200 µL of an elastase solution in Tris-HCl buffer (0.2 M, pH 8.0). The reaction mixtures were incubated at 25 °C for 15 min, then 200 µL of a solution of N-succinyl-Ala-Ala-Ala-p-nitroanilide was added. The reaction was continued for 20 min at 25 °C, and absorbance was measured at 410 nm to determine percent elastase inhibition. In addition, the concentration of extract required to achieve 50% enzyme inhibition (IC_{50}) was calculated. Quercetin was used as a positive control in this experiment.

2.7 Statistical Analysis

Data analysis was performed using SPSS 21 (IBM-SPSS Statistics, Chicago, IL, USA). All experiments were conducted in triplicate, and the results were reported as the mean ± standard error (SD) based on three measurements. A one-way analysis of variance (ANOVA) was carried out, followed by a Tukey test, in order to compare the means between the different groups. Statistical significance was established at a level of $p < 0.05$.

3. Results and Discussion

3.1 Chemical Composition of PLEO

The identification of volatile compounds of PLFEO was performed in our previous study [39]. The results of GC-MS analysis revealed that the main compounds are α-pinene (20.46%) and limonene (18.26%) (Table 1, Ref. [39]). Due to these results, both compounds were individually tested for their biological effects in the present study. In addition, other minor components were identified in PLFEO, present at different percentages [39].

Table 1. Chemical composition of PLFEO [39].

Peak	RT (min)	Compounds	PLFEO (%)
1	4.241	Tricyclene	1.13
3	7.926	α-pinene	20.46
4	8.69	Camphene	1.06
5	10.064	Myrcene	8.95
6	10.087	Sabinene	2.14
7	10.126	β-pinene	1.87
8	10.45	β-Phellandrene	5.37
9	10.631	α-Phellandrene	4.82
11	11.407	Germacrene	1.27
12	11.479	Limonene	18.26
13	12.099	Cis-β-Ocimene	1.83
15	12.569	γ-Terpinene	5.35
16	13.493	Terpinolene	4.37
17	16.622	Borneol	1.86
18	17.134	γ-Terpineol	2.54
19	19.629	α-Terpineol	5.83
21	23.418	γ-Cadinene	4.38
22	25.051	α-Caryophyllene	3.72
Total			95.21

Compounds that represent less than 1% of EOs are not indicated. RT, Retention time; PLFEO, *P. lentiscus* fruit EO.

3.2 Antioxidant Activity of PLEO, α-Pinene, and Limonene

Plants and their different parts, such as fruit, often contain antioxidants that have the ability to neutralize free radicals. These are unstable molecules naturally produced by the body during normal metabolic processes and can cause cell damage. In order to assess the antioxidant activity of these natural substances, various *in vitro* methods can be used. In our study, we adopted an approach using three distinct methods to assess the antioxidant activity of PLEO, limonene, and α-pinene (Table 2). This approach allows us to obtain more reliable and complete results while taking into account experimental variations, thus facilitating comparison with other similar substances or studies.

Table 2. *In vitro* antioxidant activity of PLEO, α-pinene, and limonene.

	DPPH assay	FRAP assay	ABTS assay
PLEO	29.64 ± 3.04^{a}	38.57 ± 4.22^{b}	73.80 ± 3.96^{d}
α-pinene	74.00 ± 3.72^{c}	107.23 ± 5.03^{c}	74.18 ± 3.72^{d}
Limonene	85.34 ± 2.28^{c}	88.82 ± 4.63^{c}	100.43 ± 5.61^{c}
Trolox	34.12 ± 2.13^{b}	55.25 ± 4.19^{c}	54.74 ± 3.85^{c}
Ascorbic acid	22.61 ± 1.08^{a}	31.63 ± 1.42^{b}	44.37 ± 2.32^{c}

Antioxidant activity of PLEO, α-pinene, and limonene (IC_{50} as µg/mL).
Trolox and ascorbic acid: Used drugs (standards) for antioxidant activity.
Results were expressed as the mean of triplicates ± SD.
Different superscript letters (a, b, c, and d) in the same column indicate a significant difference ($p < 0.05$).
DPPH, 2,2′-diphenyl-1-picrylhydrazyl; FRAP, fluorescence recovery after photobleaching; ABTS, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid.

Our findings demonstrated that regardless of the method used, PLEO exhibits optimal activity (ranging from 29.64 ± 3.04 to 73.80 ± 3.96 µg/mL) compared to both controls (ranging from 74 ± 3.72 to 107.23 ± 5.03 µg/mL). However, these results were lower than those obtained with

the reference molecules, Trolox and ascorbic acid, with the exception of the DPPH and FRAP methods, where the PLEO (29.64 ± 3.04 and 38.57 ± 4.22 µg/mL, respectively) was found to be significantly more active ($p < 0.05$) than Trolox (34.12 ± 2.13 and 55.25 ± 4.19 µg/mL, respectively).

Several factors may explain the higher antioxidant activity of PLEO compared to its major compounds. It is important to note that a plant's EO contains several different elements, some of which may act synergistically to enhance the overall activity. These compounds can neutralize free radicals more effectively than a single compound by acting in tandem. Moreover, it is possible that limonene and α-pinene are not solely responsible for the antioxidant effect of *P. lentiscus*. Other less abundant compounds (camphene, myrcene, *p*-cymene, etc. [23]) but present in significant quantities in the EO could also contribute to the overall effect of the plant [44–46]. Therefore, comprehending the EO components are crucial to understanding its antioxidant capacity. Additionally, the EO extraction method can play a role in enhancing this ability. In fact, the extraction can preserve some volatile compounds that could be lost during the extraction. This diversity has been noted in several investigations evaluating the antioxidant potential of PLEO and its two main compounds. Regarding PLEO, Barra *et al*. [47] found that its anti-DPPH activity varies according to the harvest period indicating significant variability. Interestingly, this variation cannot be solely attributed to the chemical composition of the EO. Additionally, Mezni *et al*. [48] tested the impact of the pressing method on the antioxidant potential of PLFEO harvested from two different Tunisian regions. Therefore, this method improved the percentage inhibition of DPPH radical. As a promising source of omega-6 and -9, this oil can be used for nutritional purposes in addition to its therapeutic benefits.

In the same context, another study evaluated these properties in *P. lentiscus* (fruit and leaves) with an origin identical to ours (Moroccan), harvested from two regions in the east of the country [49]. A diversity of efficacy was observed between the two regions as well as between the two parts used. Using the DPPH test, an Italian research team has shown that EOs extracted from 21 *P. lentiscus* plants from southern Italy showed variable free-radical scavenging potential, spanning approximately 21% to 35% [50]. In Oran, Algeria, Abdelkader *et al*. [51] recorded the same anti-DPPH activity with *P. lentiscus* leaf EOs, showing a linear correlation between the reduction of DPPH radicals and the EO concentration of the plant. Likewise, EOs from the aerial parts of another Algerian variety exhibited significant iron-reducing activity, thus confirming the high antioxidant potential of Algerian *P. lentiscus* [52]. Two years later, fatty fruit oil from another Algerian plant exerted a very weak capacity to scavenge DPPH radicals (EC_{50} = 20.619 ± 0.312 mg/mL) compared to the synthetic antioxidant, BHA (EC_{50} = 0.012 ± 0.0001 mg/mL) [53]. These data support previously reported findings and shed light on other factors influencing variability in antioxidant activity, such as harvest time, geographic location, environmental and growing conditions, etc.

Considering the high antioxidant potential of PLEO from different origins, the elucidation of the mechanism(s) of action involved has become obvious. To this end, Mohamed *et al*. [54] evaluated the preventive effect of the application of this EO on the oxidative damage induced by nickel oxide (NiO) nanoparticles. They recorded several positive effects, including enhancement of the endogenous antioxidant system, inhibition of ROS production, and the improvement of cell survival. This suggests that PLEO could be a promising solution to protect and prevent cells from the harmful effects of NiO nanoparticles.

We have previously carried out a study identical to this experiment, indicating a promising antioxidant activity of PLFEO using the same *in vitro* methods (DPPH, FRAP, and ABTS) [23].

In order to optimize the antioxidant potential of EOs, a recent study was performed to determine the ideal harvest period for *P. lentiscus* seeds at three stages of maturation [55]. Effectively, the results confirmed the maturity stage's significant impact on the seed oil's antioxidant effect, especially the earliest stage, which presented stable and better-quality oils. This study suggested that the stage of maturity of *P. lentiscus* seeds can constitute a colossal factor to be considered in the preparation of highly active EOs and add to the other determining factors.

To better understand the mechanisms involved in the potential of PLEO, several studies have evaluated the effect of its main compounds, α-pinene, and limonene, following various preclinical methods.

The antioxidant effect of limonene was evaluated *in vitro* analyzing the activity of specific cellular antioxidant enzymes, namely superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), as well as on the relationship between normal lymphocytes and the modulation of H_2O_2 levels [56]. This molecule protected cells from oxidative stress induced by exogenous H_2O_2 by decreasing its levels through an increase in the activity of antioxidant enzymes.

A subsequent study evaluated the antioxidant effect of limonene (0.5 mL) in rats fed with an atherogenic suspension for one month. The antioxidant enzyme activity of arylesterase, an enzyme found in blood plasma and plays a crucial role in protecting against oxidative stress, was measured [57]. As a result, the activity of this enzyme was significantly increased in treated animals, suggesting another mechanism of action for this molecule. Another *in vivo* study assessed the impact of $_D$-limonene on antioxidant defense systems and lipid peroxidation in diabetic rats [58]. After 45 days of treatment, the administration of $_D$-limonene resulted in increased antioxidant enzyme activities and reduced levels of lipid peroxidation by-products in the plasma of rats.

Furthermore, the antioxidant potential of an organic compound obtained from limonene called (+)-limonene epoxide (LE) was evaluated both *in vivo* and *in vitro* [59]. *In vitro*, LE was able to inhibit the formation of ROS and reactive nitrogen species (RNS) while it decreased the levels of nitrite content and lipid peroxidation in mice. Interestingly, it enhanced the activity of CAT and SOD, which reinforces the idea that LE has beneficial antioxidant effects in brain. This corroborates the study conducted by Piccialli *et al*. [60], who demonstrated that limonene (10 µg/mL) inhibits the production of ROS released by Aβ1-42 oligomers.

In 2018, Shah and coworkers recorded results of the antioxidant activity of $_{D}$-limonene similar to that of Trolox in six different *in vitro* tests. However, it should be noted that these tests do not entirely reflect the antioxidant activity of this substance in the human body because its absorption, distribution, and metabolism can influence this activity, hence the need for further *in vivo* studies [61].

More recent studies have begun to improve this potential. Its low water solubility, further increases its degradation and limits its integration in specific applications. Therefore, Sarjono *et al*. [62] encapsulated limonene in chitosan microparticles, which exerted promising antioxidant activity (IC_{50} = 116 ppm), with limonene-chitosan coating enhancing the free radical inhibition effect. Following this approach, one year later, Souto *et al*. [63] developed a nanoparticle formulation based on LE and glycerol monostearate that showed similar improvement in reducing lipid peroxidation.

From these data, limonene could be used in the prevention of diseases associated with oxidative stress, especially cancer, justifying the aim of our study.

Furthermore, the other terpene, α-pinene, has also been investigated in multiple studies. The choice of treatment dose is a key factor when using a drug. Aydin *et al*. [64] verified this by evaluating the antioxidant activity of α-pinene on total oxidative stress (TOS) and total antioxidant capacity (TAC). In primary rat neurons, at doses of 10 and 25 mg/L, this bicyclic monoterpene significantly increased TAC levels without altering them in N2a cells. However, at higher doses, elevated levels of TOS were observed in both cell types. The same biochemical parameters (TAC and TOS) were examined in another study carried out in the same year using human lymphocytes, which supported the previous results. At 25 and 50 mg/L doses, α-pinene increased TAC levels, whereas at 200 mg/L it decreased TOS levels [65]. This suggests that α-pinene could potentially have beneficial antioxidant effects at appropriate doses, but at high doses, it may cause oxidative stress, prompting further research to determine effective and safe doses for possible clinical use.

Moreover, the antioxidant potential of α-pinene isolated from the EO of *Salvia lavandulifolia*, a plant belonging to the Lamiaceae family, was tested using the ORAC test to measure its efficacy against H_2O_2-induced oxidative stress [66]. Consequently, pre-treatment of U373-MG cells with this compound inhibited lipid peroxidation and ROS production, reduced cell viability loss, and enhanced the endogenous antioxidant system. These effects strongly protected the cells against oxidative damage caused by H_2O_2. The same authors conducted another study the following year using the same method on PC12 cells [66]. Pre-treatment with this monoterpene presented results consistent with their previous study, thus confirming the possible involvement of nuclear factor Nrf2 induction and ROS scavenging in the antioxidant mechanism of action of α-pinene.

Another antioxidant system adopted by Shahriari *et al*. [35] for treating larvae, *Ephestia kuehniella* Zeller, with α-pinene, showed significantly elevated activities of three antioxidant enzymes: CAT, SOD, and POD. This corroborates the results obtained from Khoshnazar *et al*. [67], who recorded a restoration of the function of these enzymes and a decrease in lipid peroxidation after *in vivo* treatment with α-pinene (100 mg/kg). However, in 2021, Xanthis and colleagues found that this molecule did not exert a significant direct effect (DPPH and ABTS). However, increased mRNA levels of antioxidant response genes were observed, indicating an indirect antioxidant effect [46].

Recently, rats with 3-nitropropionic acid-induced Huntington's disease showed low antioxidant enzyme activities and increased levels of oxidative markers [68]. The administration of α-pinene significantly normalized these parameters supporting its preventive potential against diseases related to oxidative stress.

3.3 Antidiabetic Activity of PLEO, α-Pinene, and Limonene

The transition from oxidative stress to type 2 diabetes (T2D) may result from damage to pancreatic insulin-producing β-cells or from increased resistance to this hormone. Diabetes, also known as diabetes mellitus (DM), is a chronic disease characterized by chronic hyperglycemia. Traditionally, many natural substances have been used in the treatment of this condition. In addition, several studies have shown that these substances can increase insulin sensitivity, improve glucose tolerance, and reduce blood glucose, via several mechanisms, often involving the improvement of insulin signaling, the stimulation of insulin production by pancreatic β-cells, inhibition of intestinal glucose uptake, and regulation of the expression of genes involved in glucose metabolism.

The evaluation of the antidiabetic activity of these substances, as well as the identification of the underlying mechanisms of action, can be carried out according to several preclinical approaches. Our study adopted the enzymatic assay to test this potential in PLEO and its two main molecules, α-pinene, and limonene. This method consists of measuring the inhibitory capacity of these substances on

the enzymes involved in carbohydrate metabolism, namely α-glucosidase and α-amylase (Table 3).

Table 3. Antidiabetic activity of PLEO, α-pinene, and limonene.

	α-amylase	α-glucosidase
PLEO	112.35 ± 4.92	116.03 ± 7.42
α-pinene	82.12 ± 4.52	95.62 ± 4.11
Limonene	74.39 ± 3.08	78.03 ± 2.31
Acarbose	396.42 ± 4.83	199.53 ± 3.26

Enzymes inhibitory activity of PLEO, α-pinene, and limonene (IC_{50} as µg/mL).
Acarbose: Used drug (standard) for antidiabetic activity against α-amylase and α-glucosidase.

We observed that these three elements exhibit promising antidiabetic potential with IC_{50} values ranging from 78.03 ± 2.31 to 116.03 ± 7.42 µg/mL and from 74.39 ± 3.08 to 112.35 ± 4.92 µg/mL for α-glucosidase and α-amylase assays, respectively, compared to the standard, acarbose (199.53 ± 3.26 and 396.42 ± 4.83 µg/mL, respectively). This indicates that PLEO and its major compounds have the ability to reduce the potential of these two enzymes involved in the breakdown of carbohydrates, thus contributing to the maintenance of a more stable blood glucose level in diabetics.

To the best of our knowledge, this is the first report on the antidiabetic potential of PLEO. However, several studies have been carried out using the extracts of this plant. Foddai *et al*. [69] also evaluated this potential *in vitro* using aqueous extracts of *P. lentiscus* fruit and leaves by targeting the inhibition of metabolic enzymes (α-glucosidase, α-amylase, and pancreatic lipase), the regulation of their activity is of major interest as pharmacological targets in the management of diabetes. These extracts potently and effectively inhibited the activity of these enzymes, positioning them as promising candidates in the prevention and management of DM. *In vivo*, crude *P. lentiscus* (100 mg/kg) showed anti-hyperglycemic activity and improved glucose tolerance in alloxan-induced diabetic rats [70].

Recently, Sehaki *et al*. [30] provided additional confirmation of this inhibitory capacity of digestive enzymes, α-glucosidase. Indeed, various extracts of *P. lentiscus* from fruit, stem, bark, and leaves harvested on Tizi-Ouzou in Algeria (littoral and mountains) have shown varied antidiabetic properties depending on the part of the plant and its origin, which are related to the variation of the chemical composition of PLEO.

α-pinene, the primary compound of this oil with 20.46% [23], exhibited remarkable antidiabetic properties. It showed promising hypoglycemic activity when administered orally at different doses in diabetic mice [71] and rats [72] compared to control and also in combination with antidiabetic drugs [73].

Furthermore, it is interesting to note that in our study, limonene was found to be the most active compound, with IC_{50} values of 74.39 ± 3.08 and 78.03 ± 2.31 µg/mL for α-amylase and α-glucosidase, respectively. This suggests that limonene could represent a promising target for the development of antidiabetic drugs. This explains the number of *in vivo* studies evaluating the antidiabetic potential of this monoterpene.

For optimal management of diabetes using limonene, More *et al*. [74] combined it with linalool in the oral treatment of STZ-induced diabetic rats using diaphragm tissue glucose uptake and OGTT assays. The results showed that limonene alone (100 µM) inhibited protein glycation by 85.61% and reduced HbA1c and blood glucose levels, whereas the limonene/linalool combination reinforced this potential by significantly improving glucose levels. The therapeutic approach combining limonene with other antidiabetics could represent a promising treatment option. Likewise, limonene monotherapy reduced blood glucose content in diabetic mice [75].

Moreover, Shakeel and Tabassum [76] administered a daily dose of D-limonene (300 mg/kg) to rats with T2D for four weeks to assess glycaemic parameters. At the end of the experiment, a significant improvement in HbA1c, serum insulin, and glycemia levels were recorded in diabetic animals, thus confirming the important positive impact of this monoterpene on the regulation of glycaemic parameters. However, the results of these studies, including ours, were not in agreement with those obtained by Sever Yılmaz & Özbek [77], who observed no hypoglycemic effect in alloxan-induced diabetic mice. Several reasons may explain this discrepancy between the results, namely the differences in the experimental protocols (dose administered, treatment duration, administration frequency, methods of measuring glycaemic parameters, animal model, etc.), biological variability (different reactions of animals according to their general state of health, sex, age, genetic composition, etc.), the quality of the molecule used, and certain experimental biases (inappropriate selection of animals, inappropriate measurement methods, etc.).

3.4 Dermatoprotective Activity

Skin is the largest organ the body and plays an essential role in protecting against external aggressions (pollution, UV rays, infections, etc.). Today, dermatoprotective products are becoming more and more popular. However, some may contain synthetic ingredients that may cause adverse effects. In contrast, natural substances, considered safer, are increasingly studied for their dermatoprotective potential.

Numerous preclinical studies have deciphered the mechanisms of action responsible for the beneficial effects of certain natural substances on the skin, such as protection against UV rays, improvement of the skin barrier, and reduced inflammation.

Therefore, we evaluated the dermatoprotective activity of PLEO as well as its major molecules by examining their impact on two key enzymes involved in the physiological processes of skin; elastase and tyrosinase (Table 4).

Table 4. Dermatoprotective activity of PLEO, α-pinene, and limonene.

	Tyrosinase	Elastase
PLEO	57.72 ± 2.86	72.37 ± 3.37
α-pinene	97.45 ± 5.22	64.18 ± 2.80
Limonene	74.24 ± 2.06	91.25 ± 3.06
Quercetin	246.90 ± 2.54	9.08 ± 0.21

Enzymes inhibitory activity of PLEO, α-pinene, and limonene (IC_{50} as µg/mL).
Quercetin: Used drug (standard) for dermatoprotective activity against tyrosinase and elastase.

Consequently, for the tyrosinase test, we found that PLEOs (IC_{50} = 57.72 ± 2.86 µg/mL) followed by limonene (IC_{50} = 74.24 ± 2.06 µg/mL) and α-pinene (IC_{50} = 97.45 ± 5.22 µg/mL), all exhibit more marked inhibitory effects than quercetin (IC_{50} = 246.90 ± 2.54 µg/mL). Although α-pinene presented the best enzymatic activity (IC_{50} = 64.18 ± 2.80 µg/mL), it remains lower than that of quercetin (IC_{50} = 9.08 ± 0.21 µg/mL). Our results suggest that PLEO, limonene, and α-pinene can be used to inhibit tyrosinase activity, thereby reducing melanin production in the skin. However, quercetin remains the most effective agent in inhibiting tyrosinase activity among the elements tested, thus underscoring the importance of conducting further preclinical trials. To the best of our knowledge, no study has investigated the dermatoprotective effect of the EO of this plant by measuring the activity of these enzymes, which makes our results innovative in this field. However, some studies have evaluated the potential of this plant to improve wound healing and to protect the skin against induced damage. There is a close link between an agent's ability to promote wound healing and its dermatoprotective effect. The skin constitutes the first protective barrier against external aggressions, and healthy skin is important for the rapid healing of wounds. It has been demonstrated that certain molecules, in addition to their healing potential acting through several mechanisms (reducing inflammation, promoting collagen synthesis, stimulating cell regeneration, etc.), exert dermatoprotective effects by solidifying the skin barrier and protecting the skin against damage caused by pollutants, UV rays, and other environmental aggressions.

Several experiments performed on rabbits have suggested that *P. lentiscus* oil may have beneficial effects on skin healing through various mechanisms, including promoting wound contraction, improving wound overall appearance, reducing the epithelization period, accelerating wound healing, and promoting collagen deposition [78–80].

Regarding the protective effects of α-pinene, Fraternale *et al*. [81] showed that this molecule, alone and combined with limonene, exerts an inhibitory effect on elastase activity (IC_{50}: 161.61 ± 5.23 and 153.91 ± 4.81 µg/mL, respectively, compared to the standard, 52.62 ± 3.47 µg/mL). Moreover, Karthikeyan and colleagues performed two successive studies to assess the role of this monoterpene in preventing DNA damage, inflammation, and UVA radiation in human [82] and mouse skin [83]. In the first study, α-pinene protected epidermal keratinocytes from cytotoxicity induced by apoptotic cell death, single- and double-stranded DNA breaks, as well as UV radiation. In addition, it modulated nucleotide excision repair (NER) proteins and inhibited inflammatory mediators. The second study used UVA-irradiated mice, , α-pinene inhibited lipid peroxidation, activated NF-κB p65, and was pro-angiogenic. Additionally, it inhibited matrix metalloproteinase (MMP) mRNA expression and apoptotic mediators, thereby preventing dermal tissue damage in mice. The findings of both studies suggest that α-pinene has potential as a protective agent for skin cells and tissues.

For the dermatoprotective activity of limonene, Kulig *et al*. [84] recorded an important anti-tyrosinase inhibitory activity. Additionally, several studies have evaluated other mechanisms that may contribute to skin protection. Uddin *et al*. [85] investigated the ability of $_{D}$-limonene to prevent sunburn in mouse skin exposed to UV rays. After four days of oral treatment of this molecule at different doses, reductions in cell proliferation and sunburn were reported.

4. Conclusion

In conclusion, our study highlights the potential health benefits of PLEO, particularly its antioxidant, antidiabetic, and dermatoprotective properties. Moreover, our findings suggest that the main compounds of PLEO, namely limonene, and α-pinene, exhibit similar beneficial effects. The *in vitro* assays revealed that PLEO, α-pinene, and limonene possess potent antioxidant activity, as evidenced by their ability to scavenge free radicals and inhibit lipid peroxidation. In addition, PLEO, α-pinene, and limonene showed significant α-amylase and α-glucosidase inhibitory activity, suggesting their potential as antidiabetic agents. Furthermore, our results indicate that PLEO and its main compounds have dermatoprotective effects, reflected in their ability to inhibit tyrosinase and elastase activity. These findings suggest that PLEO, α-pinene, and limonene could be interesting options for the development of natural and safe cosmetic products. In this regard, it is important to continue investigations in order to better understand the mechanisms underlying the observed beneficial effects of PLEO, α-pinene, and limonene. Additionally, *in vivo*, studies are needed to confirm the potential health benefits of these compounds and assess their safety profiles. Overall, our study provides promising evidence for the use of PLEO, α-pinene, and limonene in the prevention and treatment of

various diseases, as well as for the development of natural and safe cosmetic products. Further research in this area opens exciting new perspectives.

Availability of Data and Materials

All data were cited in this manuscript.

Author Contributions

Conceptualization, KWG and AB; Data curation, NEO and AA; Formal analysis, NEO; Funding acquisition, ADIA; Investigation, RU, ADIA and AB; Methodology, NEO, AA and AB; Project administration, AB; Resources, KWG and HNM; Software, KWG; Supervision, ADIA and AB; Validation, TB and RU; Visualization, TB; Writing—original draft, NEO, AA, ADIA and AB; Writing—review & editing, TB and KWG. 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 wish to thank Princess Nourah bint Abdulrahman University Researchers Supporting Project number (PNURSP2023R33), Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia, for the financial support.

Funding

This research work is supported by Researchers Supporting Project number (PNURSP2023R33), at Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia.

Conflict of Interest

The authors declare no conflict of interest.

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