FIRST REPORT ON ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF ESSENTIAL OIL OF *Persea americana* FROM ALGERIA

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ABSTRACT

The present investigation describes the chemical profile and biological activities of *Persea americana* essential oil (EO) isolated by hydrodistillation. The chemical composition was determined using Gas Chromatography-Mass Spectrometry (GC/MS). The main compounds found were represented by estragole (65.5%) and methyleugenol (10.94%). The antibacterial activity was evaluated against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. Results revealed the effectiveness of the essential oil on both Gram-positive and Gram-negative bacteria. The screening of antifungal activity was performed using poisoned food and broth-microdilution assays. *P. americana* EO was capable of inhibiting the mycelial growth of all tested fungi, with *Cladosporium* sp. and *Alternaria* sp. being the most sensitive species. The DPPH test performed to determine the antioxidant activity showed a low IC_{50} value of 0.22 mg/mL. Overall, *P. americana* EO proved to be an effective antimicrobial and antioxidant agent, that could be used as a natural perspective to control foodborne pathogens and lipid peroxydation.

Keywords: Antibacterial, antioxidant, antifungal, essential oil, *Persea americana*.

1. INTRODUCTION

Fungal spoilage of food crops represents a relevant issue to food security worldwide. This contamination is the main cause of post-harvest losses in the vegetable industry, as it affects the quality and reduces the shelf life of the products [1-2]. Nowadays, synthetic fungicides are the main treatments used in the management of phytopathogens contamination. However, because of the excessive use of fungicides, many species are becoming resistant, resulting in the increase of the incidence and severity of plant diseases [3].

Bacterial infection and lipid oxidation are also considered as important mechanisms resulting in food deterioration. Therefore, the control of the lipid oxidation process and the microbial proliferation is of great interest to the food industry. Lipid oxidation affects the sensory properties, color attributes and the nutritional values [4]. Moreover, the development of pathogenic bacteria responsible for food deterioration are responsible of negative impacts on human health and economy [5]. Since many microbial species are becoming resistant to antimicrobial agents, the development of alternative methods of preventing food deterioration is needed. In recent years, there has been a growing interest in the development of biocides and food preservative agents using natural products as raw materials. The antimicrobial and antioxidant properties of plant extracts have been widely reported in the literature. Essential oils have been described as a promising solution in the control of microbial pathogens. In addition to their biological properties, Eos have been proven to have a low toxicity and less environmental impacts than chemical substances.

Persea americana Mill. (Lauraceae) known as "avocado" is an evergreen tree native from Mexico and Central America. It is botanically classified into three races, West Indian, *Persea americana* Mill. var. *americana* (*P. gratissima* Gaertn.); Mexican , *P. americana* Mill. var. *drymifolia* Blake (P. *drymifolia* Schlecht. & Cham.), and Guatemalan, *P. nubigena* var. *guatemalensis* L. [6]. Avocado is widely cultivated for its fruits, known for their health-promoting properties and nutritional potential. These health benefits are due to the presence of high levels of nutrients including vitamins, minerals, proteins and monounsaturated fatty acids [7]. In traditional medicine, this plant is reported for the management of skin problems, hypertension, diabetes, bronchitis and diarrhea [8-9]. Furthermore, it has been shown to exhibit several pharmacological properties, including antibacterial, antifungal, antiviral, antitubercular, antiinflammatory, antihypertensive, hypocholesterolemic, vasorelaxant, analgesic, anticonvulsant, wound healing, antiulcer, antihepatotoxic, antioxidant, and insecticidal activities [10].

Avocado is cultivated in countries with subtropical and tropical climates all over the world, and is grown commercially in more than 60 countries worldwide [11]. Recently, this plant is being cultivated in the Mediterranean region, mainly in Spain, Morocco, Algeria and Tunisia. In Algeria, the culture of exotic fruits like avocado and mango has been introduced few years ago. However, this production still very low when compared with the producer countries such Peru or Mexico. To our knowledge, no study has reported the biological activities of *P.americana* essential oil growing in Algeria. Thus, this study was conducted in order to evaluate the antioxidant, antibacterial and antifungal activities of the essential oil of avocado leaves and to determine its chemical composition.

2. EXPERIMENTAL

2.1. Plant material and extraction procedure

The leaves of *Persea americana* were collected from Algiers (Algeria) between March and April 2023. The fresh leaves were washed with tap water and air-dried at room temperature. The dried plant was ground in order to obtain a fine powder with a uniform particle size. Essential oils were extracted by hydrodistillation in a Clevenger-type apparatus for 2 hours. The obtained oil was dried under anhydrous sodium sulphate, and stored in amber flasks at 4 °C until use. The extraction yield was calculated by dividing the weight of the essential oil by the weight of the dried plant material.

2.2. Antioxidant activity

The DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical method was used to determine the scavenging activity of *P.americana* EO, following the method previously described by Zielińska et al. [12]. Volumes of 50 µL of the EO at various concentrations prepared in ethanol were added to 1.95 mL of DPPH solution (0.1 mM). The test tubes were vortexed and kept for 30 min in the dark. The absorbance values were taken at a wavelength of 517 nm. A positive control was prepared under the same conditions by replacing the EO with ascorbic acid. The antioxidant activity was estimated based on the following formula**:**

DPPH Scavenged (%) = (A control - A sample /A control) x 100

Where A sample is the absorbance of the DPPH˙ solution with the EO, and A control is the absorbance of the DPPH˙ solution without the EO.

The scavenging activity was expressed as IC_{50} values (the half-maximal inhibitory concentration). The concentration of EO needed to scavenge 50% of the free radicals (IC_{50}) was determined based on a linear equation obtained from the graph.

2.3. Antibacterial activity

The antibacterial activity was tested against five pathogenic strains (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 43300, *Escherichia coli* ATCC 87393, *Pseudomonas aeruginosa* ATCC 9027, *Klebsiella pneumoniae*) on Mueller-Hinton Agar (MHA) using the disc diffusion assay [13]. All microorganisms were sub-cultured on Nutrient agar at 37°C for 24h. After incubation, bacterial suspensions were prepared in sterile physiological saline

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and the opacity was adjusted to 0.5 McFarland with a spectrophotometer. A volume of 20 µL of a diluted EO (10 and 40 mg/mL) in DMSO (dimethylsulfoxide) was spotted into sterile paper discs. The discs containing the EO were allowed to dry than placed on the surface of Mueller-Hinton Agar plates inoculated with the bacterial suspensions. Inhibition zones (mm) were determined after 48 hours of incubation at 37°C. Chloramphenicol (concentration of 30 µg/mL) was used as positive growth control.

2.4. Antifungal activity

2.4.1. Radial growth inhibition

In this study, the poisoned food technique was performed for the analysis of the antifungal activity [14]. *P.americana* EO was tested against eight fungal isolates involved in food spoilage (*Aspergillus carbonarius, Aspergillus flavus, Aspergillus ochraceus, Aspergillus terreus, Penicillium chrysogenum, Acremonium* sp., *Cladosporium* sp., *Alternaria* sp.). Isolates were procured from the microbial culture collection of the faculty of natural sciences, Bouira (Algeria). The essential oil was prepared by dissolving the requisite amounts in DMSO and added into CYA medium to give a final concentration of 0.5 mg/mL. All strains were first cultred in Czapek yeast extract agar (CYA) for 7 days at 25°C. Subsequently, mycelial agar plugs (6 mm diameter) were cut from the outer edge of each colony and inoculated into CYA medium supplemented with the EO. Fluconazole (0.25mg/mL) used as a conventional antifungal treatment served as a positive control. The negative control was prepared in the same conditions using DMSO. After 7 days of incubation at 25°C, colony diameters were measured and the radial growth inhibition expressed as a percentage was calculated with the following formula.

$$\text{Inhibition (\%)} = [1 - (A/B)] \times 100$$

A: radial growth diameter of the colony in treatment.

B: radial growth diameter of the colony in control.

2.4.2. Micro-well dilution assay

The minimum inhibitory concentrations (MIC) of *P.americana* essential oil were determined by the broth microdilution method [15]. Serial two-fold dilutions of the EO were prepared in DMSO (10 to 0.019 mg/mL). Aliquots of 100 µL of each dilution were introduced to wells of a 96-well microtiter plate containing 100 µL of PDB (Potato Dextrose Broth) medium inoculated with different fungal suspensions (in sterile 0.1% Tween-80) at a final concentrations of 10^4 spores/mL. Wells containing PDB and inoculated PDB were prepared as negative and positive controls, respectively. After an incubation of 72h at 30°C, the MIC defined as the lowest concentration of the extract that inhibited microorganism growth was determined visually. To evaluate the minimum fungicidal concentration (MFC), 50 µL from wells showing negative growth were subcultured on PDA (potato dextrose agar) medium and incubated for 72h at 30°C. The lowest concentration necessary to totally prevent a visible growth was defined as the MFC.

2.5. GC/MS analysis

The characterization of the EO composition was carried out by gas chromatography system (Agilent, American) coupled with a mass spectrometer. The HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 µm) was used for the compounds separation. Helium (He) served as a gas vector, at a flow velocity of 1.0 mL/min. The oven temperature was maintained at 40 °C for 3 min then increased to 250°C at 10°C/min, and held for 5 min. A volume of 1 µL was injected, with a split ratio of 1/50. The temperature of injector was 270°C. The mass spectrometer operated in positive electron-impact ionization (EI) mode at 70 eV. The identification of EO components was achieved by comparing the spectral mass of each compound with those stored in databases of the National Institute of Standards and Technology (NIST). Results were expressed as relative percentages derived from the pics area.

2.6. Statistical analysis

All experimental studies were carried out in triplicates and the results were expressed as mean ± standard deviation (SD).

3. RESULTS AND DISCUSSION

3.1. Extraction yield

In the present work, the essential oil of *P. americana* was extracted from the dried leaves by hydrodistillation. Based on the obtained data, the yield of extraction showed a value of 0.8%. The EO was characterized by a pale yellow color with a spicy and a sweet aniseed aroma.

The yield obtained in our investigation was higher than that obtained by other authors. In fact, Nasri et al. [16] reported a yield varying between 0.014% and 0.5% in different varieties of avocado. In another study, Granados-Echegoyen et al. [17], observed an extraction yield of 0.24% using the same procedure of extraction. These differences can be influenced by numerous factors, including cultivation location, stage of maturity, drying time and extraction temperatures [18- 19].

3.2. Chemical composition

The analysis of the essential oil by GC/MS showed the presence of compounds belonging to different groups. The most frequent chemical group was represented by phenylpropanoids. Twenty components accounting for 98.72% of the oil composition were identified. Estragole (65.5%) and methyleugenol (10.94%) were the most abundant compounds (Table 1).

Table 1. Relative percentages of the components found in *Persea americana* essential oil from Algeria.

	Retention time (min)	Compounds	Relative abundance (%)
1	8.21	α-Pinene	1.77
2	9.05	β-Phellandrene	0.36
3	9.11	β-Pinene	3.14
4	9.38	β-Myrcene	0.19
5	10.12	Limonene	0.15
6	10.17	Eucalyptol	0.23
7	10.46	β-Ocimene	0.16
8	12.27	Anisole	0.15
9	**13.03**	**Estragole**	**65.5**
10	14.31	Anethole	4.27
11	15.62	α-Copaene	0.45
12	**15.88**	**Methyleugenol**	**10.94**
13	16.24	β-Caryophyllene	5.79
14	16.57	Isoeugenol methyl ether (Z)	0.11
15	17.03	Germacrene D	0.37
16	17.09	Isoeugenol methyl ether (E)	0.48
17	17.44	γ-Muurolene	0.1
18	17.5	Trans-Cadina-1(6),4-diene	0.14
19	17.89	Nerolidol	2.42
20	18.31	Caryophyllene oxide	2
Monoterpene hydrocarbons			5.77
Oxygenated Monoterpenes			0.23
Phenylpropanoids			**81.45**
Sesquiterpene hydrocarbons			6.85
Oxygenated sesquiterpenes			4.42
Total identified			98.72

Literature surveys have revealed the diversity of avocado essential oil composition. The predominance of estragole in the Mexican race of avocado has been previously established by many researchers. In fact, Sagrero Nieves & Bartley, [20] identified 30 components from which estragole was the major compound. Also, Granados-Echegoyen et al. [17] reported the predominance of estragole (61.86%), sabinene (15.16%) and α-pinene (14.25%) in *P.americana* EO from Mexico. Estragole is a phenylpropanoid compound found in a large number of plants (like basil, star anise, anise, and fennel) commonly employed in herbal medicinal products, and flavourings [21]. This molecule is also considered as a useful taxonomic marker that can be used to distinguish the Mexican avocado cultivar from others [22]. In other surveys, different observations in the chemical composition have been reported. Larijani et al. [23]

observed a predominance of methyl eugenol (31.2%), caryophyllene (16.9%) and estragole (9.0%) in avocado Eos from Iran. In Nigeria, the chemical composition of the avocado essential oil obtained from the leaves showed the predominance of Caryophyllene (43.9%) and valencene (16.0%) [24]. These differences in the type and percentage of components are usually related to several factors such as the variety, environmental factors and ecological conditions [25].

3.3. Antioxidant activity

The antioxidant potential of *P. americana* essential oil has been evaluated using the DPPH method. This assay is frequently used to determine the capacity of antioxidants to scavenge free radicals. In this work, the tested EO exhibited high scavenging activity levels with an IC_{50} of 0.22 mg/ml. The IC_{50} of ascorbic acid used as a positive control was slightly lower with a value of 0.18 mg/ml, indicating a better antioxidant activity (Table 2).

Aqueous and alcoholic extracts of *P.americana* leaves have been described by many researchers as antioxidant agents capable of decreasing oxidative stress [26-27-28]. However, little information is available on essential oils from avocado in the literature. The data reported by several authors showed a positive correlation between the antioxidant potential and the chemical composition of Eos. The predominance of estragole and methyleugenol in the studied essential oil could explain the scavenging activity observed in this work. In fact, phenylpropanoids are a class of secondary metabolites known for their biological activities involved in plant responses to biotic or abiotic stresses. The presence of such components confers an inhibitory effect for oxidative stress and reactive oxygen species [29]. Moreover, some factors, as drying temperature is also an important parameter for maintaining compounds integrity and their biological effect [30].

Table 2. Yield of extraction and antioxidant activity of the essential oil of *P. americana* leaves using DPPH assay.

	Yield (%)	DPPH test	
		% DPPH Inhibition	IC_{50} (mg/mL)
P. americana	0.8 ± 0.06	89.8±0.13	0.22±0.07
Ascorbic acid	-	93.6±1.05	0.18±0.09

3.4. Antibacterial activity

The antibacterial activity was evaluated against two Gram positive (*B. cereus, S. aureus*) and three Gram negative bacteria (*E. coli, P. aeruginosa, K. pneumoniae*). The obtained data expressed as diameters of inhibition are summarized in table 3. Our results showed that the tested strains were all susceptible to *P. americana* EO. *B. subtilis* was the most sensitive with an inhibition diameter reaching 18mm at a concentration of 40 mg/mL. *K. pneumoniae* and *S. aureus* were the most resistant isolates with respective inhibition diameters of 12 mm and 13mm.

Table 3. Inhibition zones (mm) against the studied bacterial strains.

	***P. americana* EO**		**Chloramphenicol**
	10 mg/mL	**40 mg/mL**	**30 µg**
Bacillus subtilis	13.3±1.15	20.3±1.52	12±0
Staphylococcus aureus	8.3±0.57	12.5±0.5	11±0
Escherichia coli	11.6±0.57	12.3±1.15	10±0
Pseudomonas aeruginosa	0±0	9.6±0.57	14±0
Klebsiella pneumoniae	11.25±0.35	13.5±0.7	13±0

Previous investigations reported the Eos' ability to control the growth and development of microorganisms including bacterial species. Our observations are in agreement with those reported by other researchers showing the antibacterial activity of *P. americana* EO against Gram-negative and Gram-positive bacteria [16- 31]. The biological activities of EO are related to their chemical composition. It has been reported that their effects depends on their major compounds but also to the synergistic effects between major and minor compounds [32-33]. The main mechanism of action of Eos involves the increase of bacterial cell permeability. Because of their hydrophobic property, Eos can easily pass through the phospholipid bilayer of the cell membrane and of the mitochondria, and cause a rupture of their structure resulting in a leakage of cell contents and a release of vital intracellular components [34].

3.5. Antifungal activity

The antifungal activity was evaluated against eight fungal strains by measuring the mycelial growth inhibition, MIC and MFC. This investigation showed that *P. americana* EO affected the radial growth of all fungi at a concentration of 0.5 mg/mL. Nevertheless, fluconazole was found to be more active than the EO in inhibiting the selected species (Table 4).

Table 4. Effect of *P.americana* essential oil on mycelial growth inhibition (%) of the fungal species cultivated on CYA medium.

	Essential oil (0.5 mg/mL)	**Fluconazole (0.25mg/mL)**
A.carbonarius	46.51±0.31	36.08±0.63
A.flavus	51.6±1.36	45.45±1.5
A.ochraceus	53.12±1.04	46.8±1.04
A.terreus	43.29±0.6	24.53±0.46
P. chrysogenum	42.34±1.4	48.17±0.6
Acremonium sp.	63.8±0.2	54±2
Cladosporium sp.	100±0	100±0
Alternaria sp.	100±0	100±0

The EO had not a similar effect on all fungi. Variations in the reduction of the growth was observed. In fact, *Cladosporium* sp. and *Alternaria* sp. were completely inhibited while *Acremonium* sp., *A.flavus* and *A.ochraceus* showed a high susceptibility to the EO, with a mycelial growth reduction levels exceeding 50%. *A.carbonarius, A.terreus* and *P. chrysogenum* were the most resistant isolates and showed inhibition levels ranging from 41.8% to 44.2%. In addition, an inhibitory effect on sclerotia production of *A. flavus* and *A.ochraceus* and their pigmentation has been noticed. After incubation, the colonies were characterized by a production of white sclerotia on CYA medium containing Eos. This absence of pigmentation is probably due to the inhibition of melanin synthesis. Melanin plays an important role in protecting mycelia and sclerotia from ultraviolet radiations and other adverse environmental conditions [35]. Melanin synthesis inhibition causes the sclerotia, mycelia, and spores to be less environmentally persistent [36]. Moreover, in some species like *Aspergillus,* the presence of this pigment can also provide a resistance to the degrading enzymes [37].

The microdilution method was used to determine the MIC and MFC values. As shown in table 5, *A.terreus* and *P. chrysogenum* showed MIC values of 0.62 µL/mL in contrast to the rest of the tested species showing an MIC of 0.31 µL/mL. The EO was more active against *A.ochraceus, Acremonium* sp., *Cladosporium* sp. and *Alternaria* sp. with MFC values of 0.62 µL/mL.

Table 5. Minimum inhibitory concentrations and fungicidal concentrations of *P.americana* essential oil against the tested filamentous fungi.

	MIC	**MFC**
A.carbonarius	0.31	5
A.flavus	0.31	5
A.ochraceus	0.31	0.62
A.terreus	0.62	5
P. chrysogenum	0.62	2.5
Acremonium sp.	0.31	0.62
Cladosporium sp.	0.31	0.62
Alternaria sp.	0.31	0.62

Previous studies have shown that the fungitoxic properties of Eos are closely related to their ability to inhibit the chitin synthesis, known as is an essential component of the cell walls and septa of all pathogenic fungi. It has been suggested that the EO components can interfere with cell wall structures, affecting morphogenesis and hyphal growth. In addition, the hydrophobic compounds of Eos may interact with ergosterol, an essential molecule that maintains the cellular integrity, viability, function, and normal growth of the fungus [38-39].

CONCLUSIONS

The antimicrobial and antioxidant activities of *P.americana* EO were investigated trough this research. Results revealed a promising effect of the

studied EO against numerous pathogenic fungal species and showed its ability to reduce the growth of Gram-positive and -negative bacteria. The EO exhibited a remarkable antioxidant activity with an IC_{50} value of 0.22 mg/ml. The GC/MS analysis indicated the presence of high amounts of estragole (65.5%) and methyleugenol (10.94%) that can be responsible of these biological activities. According to our results, *P.americana* EO can be considered as a valuable plant extract with potent compounds that can be employed as a natural agent in controlling food deterioration. However, further research is required to explore the toxicity of this EO before its application.

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