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ESSENTIAL OIL OF WILD PEPPER FRUITS (*SCHINUS MOLLE*) FROM MOROCCO: CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITIES

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Abstract

Hydro distilled volatile oils extracted of wild pepper fruits (*Schinus Molle*) from Morocco were analyzed by GC/FID and GC/MS. 21 compounds standing for 95.4 % of the oil were identified of *the Schinus Molle* species. The major components of the oil were β-Myrcene (58.7 %), α-Phellandrene (11 %), Limonene (10.1 %), β-Phellandrene (8.6 %) and α-Pinene (3.1 %). The extracted essential oil was analyzed to check its radical-scavenging ability using the stable DPPH radical and the ABTS radical, for reducing power ability with a test based on the reduction of ferric cations, and for lipid peroxidation inhibitory ability using β- carotene bleaching assay. The oil showed a particularly interesting profile regarding oxidation of the fatty unsaturated substances. The antimicrobial activity of the essential oil was individually evaluated against representatives of Gram-positive and Gram-negative bacteria using the agar diffusion method. The most sensitive microorganisms are *Acinetobacter baumanii*, *Pseudomonas aeruginosa Salmonella sp*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *Citrobacter freundii*, *Enterococcus faecalis*, *Staphylococcus epidermis* and *Proteus mirabilis*.

Keywords: *Schinus Molle, antibacterial, antioxidant, essential oil.*

Introduction

The main causes of deterioration and food poisoning are oxidation processes and microbial activity [1]. Despite the use of a variety of synthetic chemical additions as well as a variety of modern preservation techniques, spoilage and food poisoning problems remain a continuing concern for consumer's health and industries [2]. In addition, the usage of a few artificial antioxidants inclusive of butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have already been documented to expose extreme facet results, which vary from intense to life threatening [3]. Therefore, it is far past query that there is an increasing need to develop a new type of nontoxic herbal antioxidant and antimicrobial compounds with significant effectiveness in preventing food-borne growth and increasing the durability of foods [4-6].

Wild pepper fruits *(Schinus Molle)* belonging to the Anacardiaceae family, is a plant family that includes numerous species of high economic importance, *e.g.* mango, cashew, pistachio, and pink pepper as well as plants that are of local importance [7-8]. Members of this family have been known as polyphenol-rich [9]. Numerous scientific studies confirm the importance of wild pepper fruits essential oil, especially as an antioxidant and antibacterial [10-12].

The main objective of this study is to evaluate the chemical composition of the essential oil of *Schinus Molle* found In Morocco. The antioxidant potential using four different techniques (DPPH, ABTS, FRAP, β-carotene), also the assessment of the antibacterial effects on nine different bacterial strains.

Material and methods

Plant material

The plant material used within this study, amounted to one kilogram of the plant gathered from the middle atlas region in Morocco.

Chemicals and reagents

Anhydrous sodium sulphate, 1,1-dipheny, l-2-picrylhydrazil (DPPH), ethanol, 6-hydroxy- 2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), potassium persulphate, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), tween 20, potassium ferricyanide, trichloroacetic acid, ferric chloride, β-carotene, linoleic acid and chloroform, were all used during this experiment.

Extraction of essential oil

Essential oil was extracted from dry wild pepper fruits by steam distillation for four hours employing a laboratory steam distillation apparatus. Once the decantation was completed, the sample obtained was dried using anhydrous sodium sulfate and kept at 4◦C until use [13].

Chemical composition of essential oils

The chemical composition of the essential oil was determined by GC / FID and GC / MS. The GC/FID analysis was established on a gas chromatography with electronic pressure control, type Hewlett Packard (HP 6890) equipped with a single injector and a flame ionization detector (FID). The FID detector set at 280°C and using a H2/Air mixture. The flow of the carrier gas Nitrogen was 1 mL/min. The initial column temperature was 60°C and programmed to increase at 4°C/min to 280°C. The injector temperature was set at 280°C. Split injection was carried out with a ratio of 1:10. Essential oil samples of 0.5 µl (5 mg/mL) injected. The apparatus was monitored and controlled by computer system. Retention indices (RI) of compounds were determined relative to the retention times of a series of n-alkanes (C5- C24) with linear interpolation using the Van den Dool and Kratz equation.

The GC/MS was done on a gas chromatograph, coupled with a mass spectrometer. The fragmentation was carried out by electron impact under a 70 eV field. The capillary column used a thickness of the film of 0.25 µm, its temperature was programmed from 60 to 280°C at a rate of 4°C/min. Helium (purity 99.99%) was the carrier gas, with a flow rate of 1 ml/ min. The injection of the sample was in split mode. The mass range was from m/z 50 to 550. The ion source and the detector temperatures were kept at 250 and 150°C respectively. The apparatus was connected to a computer system managing a NIST mass spectrum library [14].

Antioxidant activity

The free radical scavenging activity of EO was measured via 1.1-diphenyl-2-picryl-hydrazil (DPPH·).

The preparation of 0.2 mM solution of DPPH in Ethanol then the addition of 0.5 mL of this mix was introduced to 2.5 mL of the essential oil (1 mg/mL) and was left at room temperature for 30 min, after which absorbance got measured at 517 nm against blank samples. An identical procedure was executed for the Trolox of numerous concentrations (0.5-1-2.5-5-7.5 µg/ mL). The percentage inhibition (% RSA) of DPPH by the different concentrations was calculated and the antioxidant effect of the EO was represented as Trolox equivalent (mg TE/g of EO) [15].

$$\%RSA = 100 \times \frac{A_c - A_s}{A_c}$$

% RSA: Radical scavenger activity.
A_c: Absorbance of negative control.
A_s: Absorbance of the sample.

The scavenging activity of EO against ABTS radical cation was based on the use of the stock solutions of 7 mM ABTS and 2.4 mM Potassium persulphate in equal volumes then left in the dark for 12-16 hours at room temperature. Before appraise, ABTS+ solution was diluted in Ethanol to give an absorbance of 0.7± 0.02 at 734 nm. Of the obtained solutions, 2 mL was destined to react with 200 µL of EO (2 mg/ mL), then the reaction mixture was vortexed and its absorbance measured at 734 nm after 30 min. The Trolox of various concentrations (5-15-25-50-75-100 µg/mL) went through the same process. The percentage inhibition (% RSA) of ABTS+· by the different concentrations was calculated and the antioxidant power of the EO was represented as Trolox equivalent (mg TE/g of EO) [16].

$$\%RSA = 100 \times \frac{A_c - A_s}{A_c}$$

% RSA: Radical scavenger activity.
A_c: Absorbance of negative control.
A_s: Absorbance of the sample.

The capacity to diminish ferric ions was measured using the method described by Gonzalez-Rivera et al. (2018) [17]. Numerous concentrations of standard Trolox (20-60- 100-160-200-320 µg/mL) and 1 mg/mL of EO in Ethanol (1% tween 20) were mixed with 2.5 ml of Phosphate buffer (0.2M, pH 6.6) and 2.5 mL of Potassium ferricyanide (1 % w/v). The mix was then incubated at 50°C for 20 min. After that the addition 2.5 mL of Trichloroacetic acid (10 % w/v) to the reaction mixture. Afterward, it was centrifuged at 3000g for 10 min. The upper layer of the solution (2.5 mL) was mixed with deionized water (2.5 mL) and 0.5 mL of Ferric chloride (0.1 % w/v). The absorbance was measured at 700 nm after the reaction time of 30 min. The reducing power of the essential oil was represented as Trolox equivalent (mg TE/g of EO).

Antioxidant activity based on the β-carotene/linoleic acid method was evaluated by measuring the inhibition of the bleaching of β-carotene by the peroxides generated during the oxidation of linoleic acid. A stock solution of β-carotene/linoleic acid mixture was prepared adding 1ml of β-carotene, 2mg/mL of chloroform, 40µl linoleic acid and 400 µg Tween 20. Chloroform was completely evaporated using a rotary evaporator, and 100 ml of distilled water saturated with oxygen (30 min, 100 ml/min) was added and vigorously shaken to have a stable emulsion. To 2.5 mL of the emulsion, 500 µL of the EO (1 mg/mL in Methanol) was added, and 500 µl of distilled water for the test control. Absorbance of each solution was measured at 490 nm at minute zero and after 2h incubation in a water bath at 50°C. All trials were performed in triplicate. The same was done for the Trolox of various concentrations (4-10- 20-40-80-100 µg/mL). Antioxidant activity was calculated using the coefficient of antioxidant activity following the equation described by Kaplaner et al. (2017) [18]., so, the antioxidant activity of the essential oil was represented as Trolox equivalent (mg TE/g of EO).

$$\%AAC = 100 \times \frac{A_{s(120)} - A_{c(120)}}{A_{c(0)} - A_{c(120)}}$$

%AAC: Coefficient of antioxidant activity.
$A_{s(120)}$: Absorbance of the sample at t = 120 min.
$A_{c(120)}$: Absorbance of control at t = 120 min .
$A_{c(0)}$: Absorbance of the control at t = 0 min.

Antibacterial activity

Antibacterial activity was evaluated using selected pathogens and commensal strains. Following the test described by Ainane et al. (2019) [19], with some alterations. The 9 micro-organisms

tested in the realization of this experiment (*Enterococcus faecalis, Salmonella sp, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Staphylococcus epidermis, Citrobacter freundii, Proteus mirabilis, and Pseudomonas aeruginosa*) were collected clinically. These srains were maintained on slants of nutrient agar medium favorable to their development and incubated for 24 hours at 37°C. Due to the immiscibility of the essential oil and water therefore to the culture medium, an emulsification was dispensed through a 0.2% agar resolution. This makes it feasible to attain a homogeneous distribution of the EO in the medium and to maximize the contact among the germ and the compound. The Amoxicillin (25µg) and Ciprofloxacin (5 µg) had been used as positive control in the test.

To determine the minimum inhibitory concentration (MIC), dilutions were prepared 100 µL/mL, 40 µL/mL, 20 µL/mL, 10 µL/ mL, 5 µL/mL, 3.3 µL/mL, 2 µL/mL in this agar solution. To the test tubes each containing 13.5 mL of solid MH (Mueller Hinton agar) 1.5 mL of each dilution was added aseptically so as to obtain the final concentrations of 10 µL/mL, 4 µL/ mL, 2 µL/mL, 1 µL/mL, 0.5 µL/mL, 0.33 µL/mL, 0.2 µL/mL respectively. The tubes were stirred to adequately disperse the EO into the culture medium before pouring them into the Petri dishes. Controls, containing the culture medium and the 0.2 % agar solution alone, were also prepared. Seeding was done by striation using a calibrated platinum loop to collect the same volume of inoculums and to incubate at 37°C for 24 hours. Each test was repeated three times [20].

Results and discussion

Chemical composition of essential oil

Results of gas chromatographic analysis of *Schinus molle* EOs are presented in Table 1. 21 components were identified, representing 95.4% of the total composites of the wild pepper fruits. The essential oil was characterized mainly by β-Myrcene (58.7%), α-Phellandrene (11.2%), Limonene (10.1%), β-Phellandrene (8.6%) and α-Pinene (3.1%) and other 17 minor components representing a small portion of the EO.

In comparison to Do Rosário Martins *et al.* (2014) [21] results done on *Schinus molle* leafs and fruits in the Évora region (Portugal). We found a big resemblance in the chemical composition of the essential oil even though some of the technics used are different.

Antioxidant activity (DPPH, ABTS, FRAP, β-carotene bleaching)

The antioxidant potential of the *Schinus molle* EO has been tested by four testing methods, DPPH, ABTS, reducing ferric power, and β-carotene bleaching assay (Table 2). The evaluated samples were capable of reducing the stable violet DPPH radical to the yellow DPPH-H, with a value of (0.74 mg TE/g of EO. Even though DPPH and ABTS methods were based on the same principle, data gained from ABTS test are higher than those obtained from DPPH test, reaching the values (5.27 mg TE/g of EO). This difference can be traced to the steric factors that are one of the major factors for reducing of stable DPPH radicals [22]. We also evaluated the capacity of reducing the ability of the essential oil using a spectrophotometric method, to define the content of ferric ions reduced by tested oils. Tested samples were able to reduce the ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) reaching the values (7.44 mg TE/g of EO) [23]. Lipid peroxidation inhibition activity of the essential oil was evaluated using the β-carotene bleaching test. This experiment is founded on the change of the yellow color of β-carotene due to its reaction with the created radicals by the oxidation of linoleic acid in an emulsion [24]. The results obtained by this test proved the antioxidant capacity of EO (15.61 mg TE/g of EO). The antioxidant activity of EO can be explained by the presence of hydroxyl compounds. The DPPH, ABTS and FRAP tests have revealed a median antioxidant potential for the EO. However, the β-carotene bleaching test showed a high antioxidant potential of the EO. This can be explained, according to Dorman et al. (2000) [25], by the lipophilic affinity of the EO with the reaction mix.

Antibacterial activity

The evaluation of the antibacterial activity of the essential oil, was done against nine bacteria strains *Acinetobacter baumanii, Pseudomonas aeruginosa, Salmonella sp, Klebsiella pneumonia, Staphylococcus aureus, Citrobacter freundii, Enterococcus faecalis,*

Staphylococcus epidermis and Proteus mirabilis. The chosen strains are known for their ability to contaminate food, cause spoilage, and for their pathogenicity. The results of this test are presented in Table 3.

The antimicrobial activity of the essential oil against the tested microorganisms was qualitatively and quantitatively assessed by the inhibition zones and the determination of MIC. According to the results shown in Table 3, the essential oil showed a potent inhibitory effect against all bacteria strains. The diameter of inhibition zones ranging from 6.1 to 7.2 cm.

MIC values of the EO on the used strains are presented in Table 3. These results demonstrated that these oils displayed antibacterial properties. The EO showed a remarkable antibacterial potential, in particular, against the *Pseudomonas aeruginosa* and *Acinetobacter baumanii*, which could not develop even at a concentration of 0.297µL/ mL and 0.342µL/ mL respectively.

Conclusion

Based on previous observations, it can be concluded that *Schinus molle* has showed considerable antioxidant properties, especially related to the oxidation of unsaturated fatty acids, as well as the antibacterial potential shown in all tested microorganisms. These characteristics may be crucial to find organic substitutes to synthetic preservatives used by the food industry.

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Table 1. Chemical composition of *Schinus Molle* essential oil.

Compounds	RI	(%)
α-Pinene	1027	3.1
Camphene	1078	0.2
β-Pinene	1126	0.1
Sabinene	1131	0.1
β-Myrcene	1159	58.7
α-Phellandrene	1167	11.2
Isopinocamphone	1174	0.1
p-cymen-8-ol	1194	0.2
Limonene	1206	10.1
β-Phellandrene	1216	8.6
Neral	1236	0.2
p-Cymene	1268	0.9
α-Terpinolene	1281	0.4
Methyl octanoate	1390	0.1
Cubenol	1514	0.3
allo-Aromadendrene	1638	0.2
α-Terpineol	1692	0.1
Bicyclogermacrene	1721	0.5
Elemol	2074	0.1
α-Eudesmol	2205	0.1
β-Eudesmol	2214	0.1
Total identified compounds		95.4

RI : Retention Indices.

Table 2. Antioxidant activity of the essential oil of *Schinus molle fruits.*

Antioxydant activity	DPPH	ABTS	FRAP	β-carotene
***Values* (mgTE/g of EO)**	0.74	5.27	7.44	15.61

TE: Trolox equivalent.

Table 3. Antibacterial activity of the essential oil of *Schinus mole.*

Bacterial strains	DI (cm)	MIC (µl/ml)
Enterococcus faecalis	6.7	0.523
Salmonella sp.	7.1	0.425
Staphylococcus aureus	6.8	0.541
Klebsiella pneumoniae	6.9	0.498
Acinetobacter baumanii	7.2	0.342
Staphylococcus epidermis	6.4	0.511
Citrobacter freundii	6.8	0.522
Pseudomonas aeruginosa	7.2	0.297
Proteus mirabilis	6.1	0.588

DI: Diameter of inhibition.
MIC: Minimum inhibitory concentration