Chemical Analysis, Antioxidant and Antibacterial Activities of Aniseeds Essential Oil

Naouel OUIS[1] (✉)
Ahmed HARIRI[2]

Summary

This study was conducted to evaluate the chemical composition, antioxidant and antibacterial activities of the essential oil of *Pimpinella anisum* L. seeds. The main constituents of the essential oil obtained by hydrodistillation using a Clevenger-type apparatus were identified by GC, GC/MS-EI, GC/MS-CI, and NMR spectroscopy. The antioxidant potential was assessed by using the DPPH method and the ferric reducing power. The antibacterial activity was determined by using the disk diffusion and the micro dilution methods against some Gram-positive and negative pathogenic bacteria. Results showed that aniseeds essential oil was characterized by a higher yield 2.6 ± 0.02% and good physic-chemical characteristics. Chemical analysis showed that the major components of the essential oil identified were anethole and estragole with percentages of 94.82 and 1.69%. Aniseeds essential oil showed a higher percentage of inhibition of DPPH 88.3 ± 0.5% and a lower value of IC_{50} 118 ± 1.5 µg mL^{-1} determined at concentration of 1000 µg mL^{-1}. This oil displayed a good ability to reduce Fe^{+3} to Fe^{+2} and provided an optical density of 1.78 ± 0.3 and IC_{50} of 60 ± 0.2 µg mL^{-1}. Tested oil showed bactericidal activity against *Pseudomonas aeruginosa* and *Escherichia coli* with report MBC/MIC of 2 and antibacterial effect against *Staphylococcus aureus* with MBC/MIC of 32. It can be concluded that aniseeds essential oil contains substances with significant biological potential such as anethole and estragole that can be exploited in different pharmaceutical and therapeutic fields.

Key words

aniseeds, antibacterial, antioxidant, essential oil, GC-MS, NMR

[1] Laboratory of Physical Chemistry of Macromolecules and Biological Interfaces, Faculty of Science the Nature and Life, University Mustapha Stambouli of Mascara (UN 2901), BP. 763, Sidi Said, Mascara, 29000, Algeria

[2] Bioconversion Laboratory, Microbiology Engineering and Health Safety, Faculty of Science the Nature and Life, University Mustapha Stambouli of Mascara (UN 2901), BP. 763, Sidi Said, Mascara, 29000, Algeria

✉ Corresponding author: naouel.ouis@univ-mascara.dz

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Introduction

Plants synthesize large quantity of bioactive substances. These compounds were used to maintain the plant's physiological properties, and to protect it against foreign agents such as bacteria, fungi, insects and animals (Schultz, 2002). Recently, the use of medicinal and aromatic plants in herbal medicine has been developed intensively by exploiting different herbs, fruits and legumes. Many studies have been focused on naturally bioactive substances that can preserve human health from oxidative stress damage caused by reactive oxygen species to replace synthetic antioxidants, which are being restricted due to their side effect (Zheng and Wang, 2001). The imbalance between reactive oxygen species and antioxidant defense system may lead to chemical modification of biologically relevant macromolecules (DNA, carbohydrates, proteins or lipids). These patho-biochemical mechanisms cause the development of different diseases (Troszynska et al., 2002). Natural antioxidants can protect the human body from free radicals and retard the progress of several chronic diseases. Hence, the studies on natural antioxidants have gained increasingly greater importance. Many antioxidant substances, naturally occurring in plant source have been identified as potential free radical or active oxygen scavengers (Duh, 1998). The frequency and spectrum of antimicrobial resistant infections have increased during the last years in hospitals due to the continued use of systematic antimicrobial agents (Rijnders et al., 2009). The side use effects of misuse and overuse of antibiotics can harm vital organs (Bocanegra-Garcia et al., 2009). The most important multidrug resistant bacteria include Gram-positive such as methicillin resistant *Staphylococcus aureus*, vancomycin resistant *Enterococci* and Gram-negative bacteria such as members of *Enterobacteriaceae* forming plasmid mediated spectrum beta lactamase. Due to their richness in various bioactive compounds, plants have been used for food preservation, pharmaceutical, alternative medicine and natural therapies (Lis-Balchin and Deans, 1997). It has long been acknowledged that some plant essential oils exhibit antimicrobial activities and it is necessary to investigate those plants scientifically (Al-Bayati, 2008). Plant substances with strong antibacterial or bactericidal capacity belong mostly to the group of phytoalexins including essential oils as the most important members (Bazargani and Rohloff, 2016). Essential oils are potential sources of novel antibacterial substances especially against pathogenic bacteria (Prabuseenivasan et al., 2006). Essential oils contain various bioactive compounds. These substances exert beneficial effects due to the synergistic action of several chemical compounds acting at single or multiple target sites and exert a good antioxidant and antibacterial activities. Derwich et al. (2011) report that the biological activities of essential oils could be associated with their chemical composition, especially of their major compounds. The knowledge of chemical composition is important for understanding the mechanism of action of essential oils. Spices considered as medicinal plants with strong flavor are used in small quantities in cooking preparation as preservative, seasoning and coloring agents. Anise (*Pimpinella anisum* L.) belonging to the Apiaceae family is an important spice and annual herb with white flowers and small green to yellow seeds, native to Mediterranean regions. This plant is used for pharmaceutics, perfumery and food industry (Ullah and Honermeier, 2013). Aniseed has been used as a condiment, mild expectorant and in treating dyspeptic complaints. Anise, which has been attributed to the rejuvenating and anti-toxic properties, is one of the oldest medicinal plants in the world. The whole plant is saturated with an aromatic essential oil. This oil is characterized by antispasmodic, antioxidant, antimicrobial, insecticidal, and antifungal properties (Tunc and Sahinkaya, 1998; Gulcin et al., 2003, Ozcan and Chalchat, 2006; Tepe et al., 2006a; Tirapelli et al., 2007). This study was conducted to determine the chemical composition by using GC, GC-MS, NMR spectrometry and to evaluate the antioxidant and antibacterial activities of the essential oil of *P. anisum* seeds.

Material and Methods

Vegetable Material

Commercially, the seeds of *Pimpinella anisum* L. were purchased from the local market (Mascara, North-West of Algeria) in 2018. The species was identified by botanist at SNV Faculty, University Mustapha STAMBOULI of Mascara. The seeds were washed with tap water to remove all impurities, then with distilled water and dried in darkness at room temperature.

Chemical Substances

All chemicals and solvents were purchased from Sigma Aldrich (Munich, Germany), unless otherwise specified.

Essential Oil Extraction

100 g of sample in 500 mL of distilled water were submitted to hydro-distillation for 3 hours, using a Clevenger-type apparatus (ST15 OSA, Staffordshire, UK) according to the method as described by the European Pharmacopoeia (2000) until total recovery of oil. The extracted oil was dried over anhydrous sodium sulfate and stored at 4 °C until tested in an opaque glass bottle sealed to protect it from air and light.

Physicochemical Analysis

The essential oil yield was determined by gravimetric method and expressed in terms of % w/w (ratio between the weight of the obtained oil and the weight of the sample to be treated). The quality of oil was verified by determination of the physic-chemical indices. The physical indices: relative density (d), refractive index (n^{20}), rotatory power [α], freezing point and miscibility with ethanol (v/v) and chemical indices: acid (Ia), ester (Ie), saponification (Is), and iodine number (Ii) were evaluated according to the European Pharmacopoeia (2000).

Identification

The chemical identification of the main compounds of essential oil of *Pimpinella anisum* seeds was carried out by GC, GC/MS-EI, GC/MS-CI and NMR spectrometry.

GC and GC-MS Analysis

GC-FID

The main constituents of the essential oil were analyzed by Typify varian 3900 Gas Chromatograph equipped with FID (Flame Ionization Detector), a capillary column [(CP-Sil5CB (30 m * 0.25 mm)), DF 0.25 µm], the flow rate of the carrier gas (N_2): 30 mL

min^{-1}. The temperature was programmed from 80 °C min^{-1} at the ramp rate of 20 °C min^{-1} to 280 °C. The temperature of injector and detector were maintained respectively at 220 °C and 300 °C. The injection volume was 1 µL. The identification of the anethole was made by its co-injection with the essential oil of *Pimpinella anisum* seeds and with the comparison of the retention indices, back with those of the various peaks revealed in the chromatogram of the tested oil.

GC-MS in Electronic Impact Mode (GC/MS-EI)

In electron impact mode, the bombardment of substances by a beam of electrons of the order of 70eV provokes ionization and fragmentation. The positive ionic fragments then form the characteristic mass spectrum of the compound. The mass spectra thus obtained are compared with those of the reference products contained in the database.

GC-MS in Chemical Ionization Mode (GC/MS-CI)

The CI method consists of ionizing the reactive gas X by electronic impact, then, the ionized reactive gas X^+ reacts with the reactive gas molecules by means of ion-molecule collisions producing the plasma reactive gas XH^+. The plasma (XH^+)-molecule (M) reaction is a proton exchange:

$$M + XH^+ \rightleftharpoons [M \ldots H \ldots X]^+ \longrightarrow [M+H]^+ + X$$

This reaction can be used to obtain information on molecular mass, ions characteristic of molecular masses are called quasi-molecular or pseudo-molecular ions regardless of their exact nature: $[M+H]^+$, $[M-H]^+$. The gas used in CI was ammonia. The GC-MS analysis was carried out on a Gas Chromatograph Varian Saturn 2100 T interfaced with a Mass Spectrophotometer with electron impact ionization (70eV). The column used was a capillary column [CB 8 (30 m * 0.25 mm), DF 0.25 µm]. The temperature of the column was programmed to rise from 80 °C min^{-1} to 280 °C min^{-1} at the rate of 20 °C min^{-1}. The carrier gas was Helium with a flow rate of 1 mL min^{-1}. The temperature of the injector and detector was respectively 280 °C and 300 °C and the injection volume was 1 µL. The constituents' identification was based on their retention indices and mass spectra were compared with the data from Baser Library.

NMR Spectrometry

With the re-crystallization phenomena, column chromatography was a method of choice for purifying and isolating the constituents of the oil, and was based on the adsorption phenomena. The stationary phase (Silica SiO_2, Alumina Al_2O_2), mixed with the eluent was poured into a cylindrical column provided with a tap at its end. The substance to be analyzed was carefully poured at the top of the column. 1 g of anise essential oil was introduced into the column filled with 30 g of silica (krongrôbe 0.063-0.200 mm) wetted with the eluent chosen by TLC. The eluent (ether/petroleum ether) was continuously added to increase the polarity. The common constituents, collected in tested tubes and detected by TLC were collected and the solvent was evaporated. Thus two fractions were obtained. The other fractions could not be recovered due to their small proportions. The identification of the compounds of the two fractions was carried out by NMR of the 1H proton and of the ^{13}C carbon. The proton and carbon 13 NMR spectra were recorded on a Bruker AC 300 apparatus in solution in deuterated chloroform. The chemical displacements were given in δ (10^{-6}) relative to $CDCl_3$ used as internal reference at 7.24 ppm in proton NMR and 77 ppm in carbon NMR. The multiplicity of signals was indicated by the following abbreviations: s, d, dd, m. meaning respectively singlet, doublet, doublet doublet and multiplet.

Evaluation of Antioxidant Activity

DPPH Free Radical-Scavenging Activity

The antioxidant activity was evaluated in terms of hydrogen donating or radical-scavenging capacity, using the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a reagent. The ability of the essential oil to scavenge DPPH was carried out by using the method described by Kirby and Schmidt (1997) with some modifications. The oil to be tested for its antiradical capacity was prepared in methanol to achieve the concentration of 1 mg mL^{-1}. Dilutions were made to obtain concentrations of 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.99, and 0.97 µg mL^{-1}. A volume of 50 µL of these solutions was added to 1950 µL of methanol solution of DPPH (6.10^{-5} M) as free radical source. The mixtures were stirred for 30 seconds and then incubated in the dark for 30 min at room temperature. The absorbance was measured using UV/Vis spectrophotometer model Hitachi 4-2000 at 517 nm against pure methanol (Shimada et al., 1992). Ascorbic acid and catechin were used for comparison as positive control. A lower absorbance of the reaction mixture indicated a higher free radical-scavenging activity. The inhibition percentage of DPPH was calculated using the equation:

% of inhibition = 100 * (Absorbance of control – Absorbance of sample)/Absorbance of control

where absorbance of the control containing all reagents except the oil and absorbance of the sample (presence of the essential oil). IC_{50} (concentration of substrate that inhibits 50% of the DPPH radicals present in the reaction medium) values were estimated from the % inhibition versus concentration plot, using a non-linear regression algorithm.

Ferric-Reducing Power

The ferric reducing power was determined by using the potassium ferricyanide-ferric chloride method described by Yildirim et al. (2001). 1 mL of oil of different concentrations was mixed with 2.5 mL of 0.2 M phosphate buffer pH 6.6 and 2.5 mL of potassium ferricyanide solution K3Fe(CN)6, 1%. After incubation for 20 min at 50 °C, 2.5 mL of trichloroacetic acid 10% was added and the reaction mixture was centrifuged for 10 min at 3000 rpm (Sigma labrzentrifugen D-37620 Osterode am Harz, Germany). An aliquot of 2.5 mL of the supernatant from each sample mixture was mixed in a test tube with 2.5 mL of distilled water and 0.5 mL of ferric chloride solution (0.1%) prepared freshly in distilled water. After 20 min of reaction time at 35 °C, the absorbance was recorded at 700 nm against a blank that contains all reagents except the essential oil solutions and ferric chloride. The control was achieved by different concentrations of ascorbic acid and catechin. Higher absorbance of the reaction mixture indicated higher reducing power. Tests were carried out in triplicate. The concentration providing 0.5 of absorbance (IC_{50}) was calculated by plotting absorbance at 700 nm against the corresponding sample concentration.

Evaluation of Antibacterial Activity

Tested Bacteria and Inocula

The essential oil of *P. anisum* seeds was tested against referenced pathogenic bacteria now involved in nosocomial infectious and acquired resistance to the antibiotics. Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923) and Gram-negative bacteria *Pseudomonas aeruginosa* (ATCC 25853) and *Escherichia coli* (ATCC 25922) were kindly provided by the Laboratory of Medical Analysis located in the hospital of Oran (west of Algeria). All microbial strains were stored on nutrient agar slope at 4 °C and sub cultured for 24 hours at 37 ± 1 °C before use. The oil was dissolved in dimethyl sulfoxide (DMSO) and sterilized by filtration through a filter paper 0.45 mm. The inocula were prepared according to the method as described by Atwal (2003) by culture of 18-24h of the tested bacteria on agar medium equivalent to 0.5 McFarland (10^8 CFU mL^{-1}). The absorbance was measured using UV/Vis spectrophotometer at 625 ± 0.1 nm. The inocula were diluted to 1/100 in sterile saline to a final concentration of 10^6 CFU mL^{-1}. The tests were performed on agar and liquid medium Muller Hinton (Okigbo et al., 2009). The antibiotics selected were: Amoxicillin (AMX 25 µg), Ampicillin (AM 10 µg), Erythromycin (E 15 IU), Gentamicin (G 10 IU), Spiromycine (SP 100 µg), and Tetracycline (TE 30 µg). Different disks of selected antibiotics were placed on the Mueller-Hinton agar, and then the boxes were dried for 30 minutes at room temperature to get a good spread of antibiotics. The plates were incubated at 37 ± 2 °C for 18 to 24 hours. The measure of the diameter of zones of inhibition allows classifying bacteria in three categories: sensitive, intermediate or resistant.

Agar Diffusion Method

The antibacterial activity was determined by the disk diffusion method using Mueller-Hinton agar (Okigbo et al., 2009). The sterile disks were separately impregnated by the essential oil for a few hours. In sterile *Petri* dishes, culture media in Muller-Hinton were poured, left for 15 min to solidify, then 1 mL of inocula was deposited beforehand prepared and inoculated with a rake. The disks containing the test products were transferred to the inoculated box. After incubation at 37 ± 1 °C for 24h, the results were determined by measuring the diameter of the zone of inhibition in mm including disk diameter of 6 mm (Lino and Deogracious, 2006). The antibacterial activity was considered positive from a diameter greater than 6 mm according to the antibiogram committee of the French Society of Microbiology (Rios et al., 1988).

Determination of MIC and MBC

The MIC and MBC of the tested oil was evaluated using agar dilution method (Akomo et al., 2009). 100 µL of oil was added to the first well, and then a serial dilution was carried out (1/2) to achieve concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 and 0.39 mg mL^{-1}. 100 µL of the bacterial suspension of 18 hours in the broth Muller-Hinton (10^6 CFU mL^{-1}) were added to each well. The plates were shaken and incubated at 37 °C for 24 hours. The lowest concentration required to completely inhibit the growth of the tested microorganism was designed as MIC and was expressed in µg mL^{-1}. The MBC was defined as the lowest concentration that kills 99.99% of the burden of the initial inocula. For the determination of the MBC, 100 µL of each well that showed no change in culture were inoculated on nutrient agar and incubated at 37 °C for 48 hours and the lowest concentration showing no growth after incubation was considered as MBC.

Data Analysis

All determinations were conducted in triplicates and results for each measured parameter were expressed as mean ± SD. Data were statistically determined by analysis of variance ANOVA using the confidence level ($P \leq 0.05$) using Microsoft Excel and SPSS statistics software 8.1.

Results and Discussion

Results of Physicohemical Characterization

The essential oil of aniseeds presents a limpid liquid aspect, volatile, mobile, with yellow color, strong and fresh smell. The studied seeds were characterized by a higher yield 2.6 ± 0.02%. According to Tabanca et al. (2005) anise fruit contains around 1.5-5.0% of essential oil mainly composed of volatile phenylpropanoids like trans-anethol with 90%. According to the literature, the essential oil yields of aniseeds range from 2.69 to 3.30% (Ullah and Honermeier, 2013; European Pharmacopeia, 2000). The physicochemical analysis (relative density 0.961 ± 0.002 g g^{-1}, refractive index at 20 °C 1.554 ± 0.002, rotatory power +2.0 ± 0.1, miscibility with ethanol at 96% 1/23 (v/v), freezing point 15 to 19 ± 0.5 °C, acid index 1.68 ± 0.03 mg KOH gEO-1, ester index 8.70 ± 0.04 mg KOH gEO^{-1} at 20 °C, saponification index 7.01 ± 0.03, and iodine number 1.27 ± 0.05) showed the good quality of the studied oil.

Results of Chemical Identification

GC and GC-MS Analysis

The major constituents of aniseeds essential oil analyzed by GC method (Fig. 1, Table 1), GC/MS-EI method (Fig. 2) and by GC/MS-CI method (Fig. 3) are estragole and anethole, whereas other peak analyzed by GC method at percentage of 2.05% was not identified.

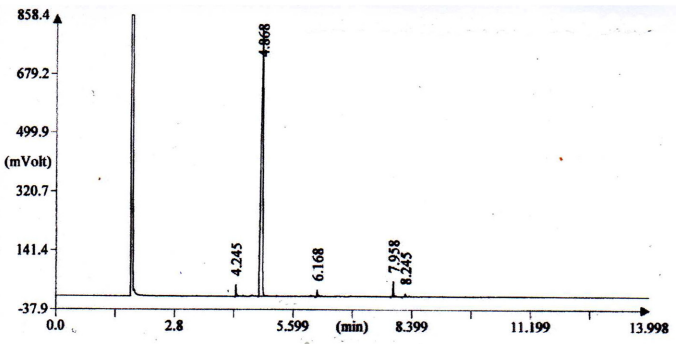


Figure 1. The GC spectrum of aniseed essential oil

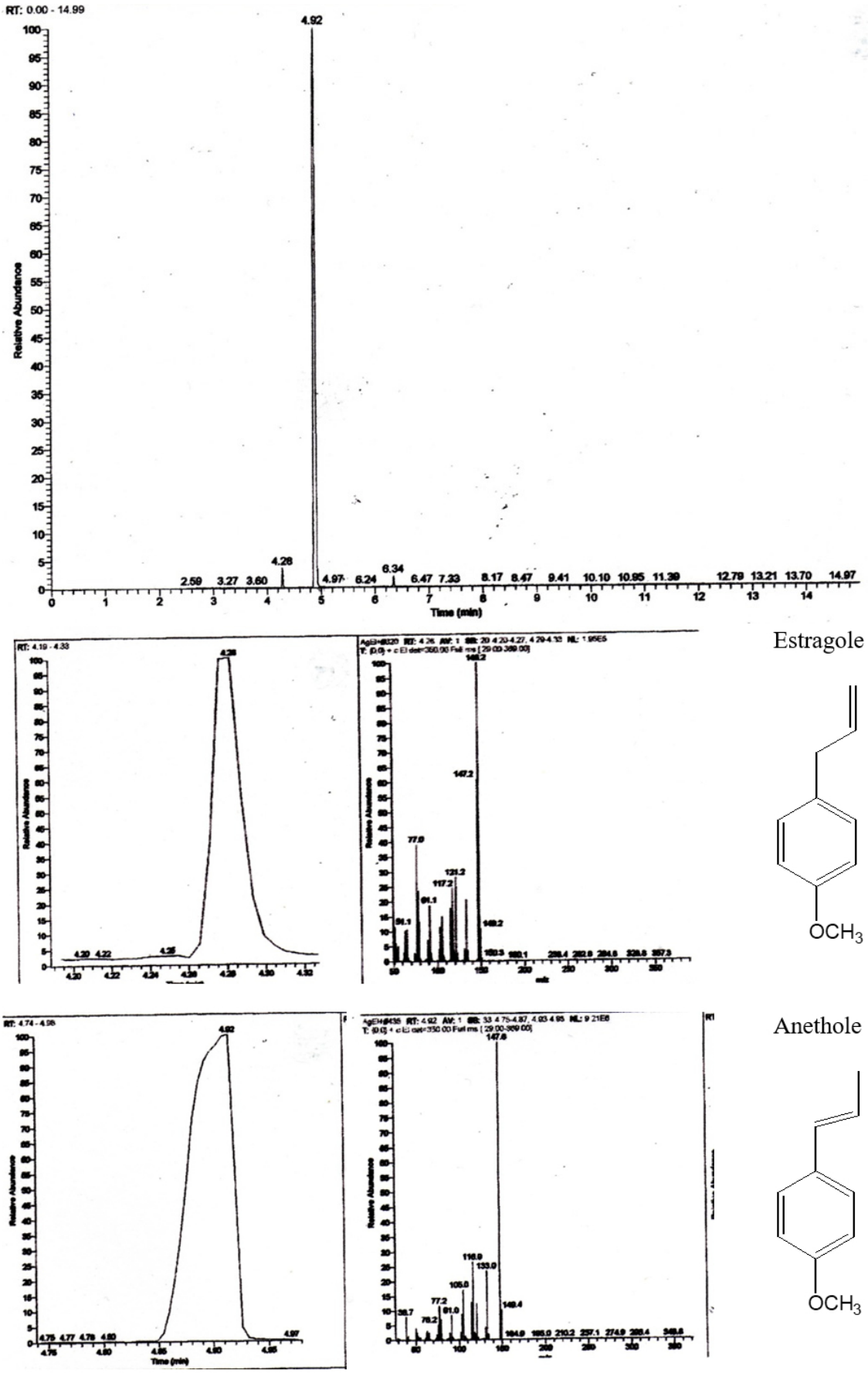


Figure 2. Composition of essential oil analyzed by GC/MS-EI method

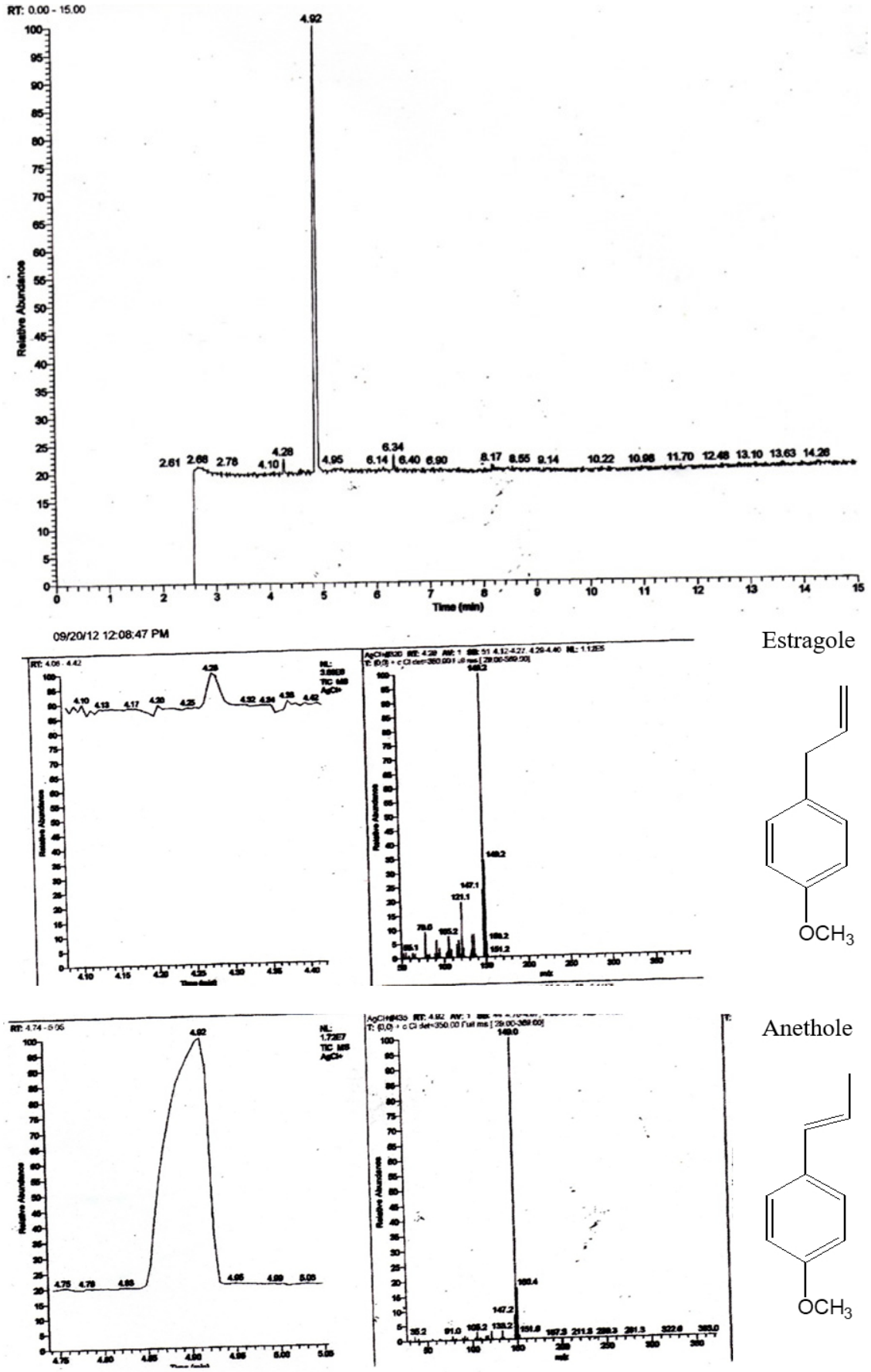


Figure 3. Composition of essential oil analyzed by GC/MS-CI method

Table 1. Composition of essential oil analyzed by GC method

Peak number	Area %	Retention time	Identified compound
1	1.6974	4.25	Estragole
2	94.8259	4.87	Anethole
3	0.9255	6.17	/
4	2.0576	7.96	/
5	0.4936	8.25	/
Total	100.0000		

Our results of chemical analysis are in line with several other studies demonstrating that *Pimpinella anisum* seeds oil contains trans-anethole 75-90% responsible for the characteristic taste and smell (Ullah and Honermeier, 2013; Ponte et al., 2012; Samojlik et al., 2012; Chandler and Hawkes, 1984), estragole 1-2% (Ullah and Honermeier, 2013; Zargari, 1989), eugenol 1.99% (Samojlik et al., 2012; Monod and Dortan, 1950), β-himachalene 12.3% (Pavela, 2014) coumarins and terpene hydrocarbons (Burkhardt et al., 1986; Kartnig et al., 1975) as the major compounds and which are good antibacterial agents (Cowan, 1999).

NMR Spectrometry

As shown in Fig. 4, the NMR^{1}H spectrum of aniseed essential oil reveals the presence of several peaks:

A multiplet peak of 6.82 to 6.86 ppm attributable to the proton C<u>H</u>=(CHCH$_3$).

Two doublets peak at 6.33 and 6.38 ppm corresponding to the protons of benzene.

A multiplet peak between 6.06 and 6.13 ppm attributable to the methylene proton (CH$_3$)<u>CH</u>=C.

A singlet peak at 3.76 ppm relating to the methoxy protons (OC<u>H</u>$_3$).

A doublet peak appears at 1.87 ppm attributable to the chemical displacement of the methyl protons (C<u>H</u>$_3$) linked to the group C=C.

As shown in Fig. 4, the NMR-13C spectrum of aniseed essential oil shows chemical displacements values of the carbons corresponding to anethole.

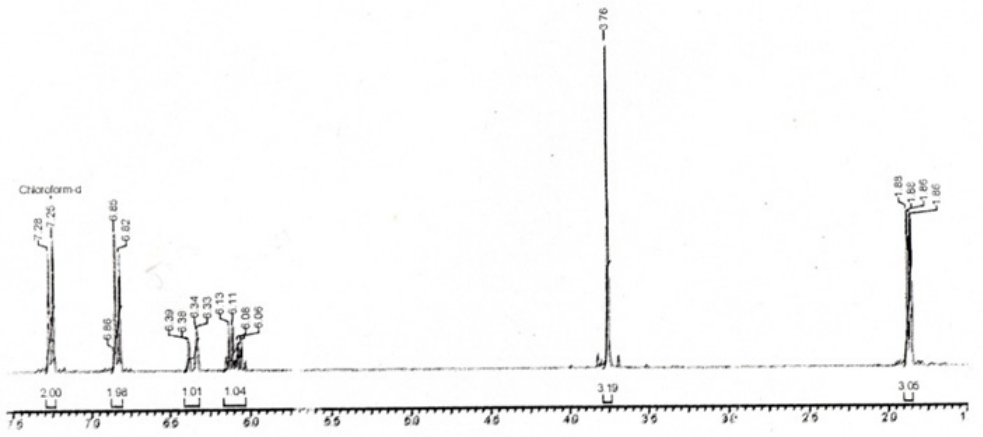
Results of Antioxidant Activity

As shown in Fig. 5-A, essential oil of the seeds of *Pimpinella anisum* showed a higher scavenging activity of free DPPH 88.3 ± 0.5% at concentration 1000 µg mL^{-1}. This result was close to those found with ascorbic acid 97.40 ± 3.65% and catechin 93.96 ± 2.63% at the same concentration. The antioxidant capacity of this oil directly depends on the concentrations used. The scavenger effect of active compounds on free radicals depends on the presence of free OH groups, in particular 3-OH, with a configuration 3',4'-rthodihydroxy (Heim et al., 2002). Anethole as major constituent of the studied oil can play a key role for antiradical activity of aniseeds oil. The obtained results are in agreement with the findings of several authors who reported that the efficiency of an antioxidant to reduce DPPH essentially depends on its hydrogen donating ability, which is directly related to the presence of phenolic compounds (Hazzit et al., 2009) and the abundance of monoterpenes hydrocarbons (Ruberto and Baratta, 2000) and oxygenated monoterpenes (Tepe et al., 2004). The IC$_{50}$ parameter commonly used to evaluate the antioxidant capacity is necessary for each essential oil to reduce 50% of free DPPH concentration in a defined time. A low IC$_{50}$ value corresponds to a higher antioxidant activity. The studied oil presents IC$_{50}$ of 118 ± 1.5 µg mL^{-1}, which value is higher than the values obtained by positives control (catechin 14.26 ± 0.2 µg mL^{-1} and ascorbic acid 17.21 ± 0.3 µg mL^{-1}). According to the IC$_{50}$ values, the antioxidant activity of aniseeds essential oil was lower compared to the antioxidant effect of catechin and ascorbic acid.

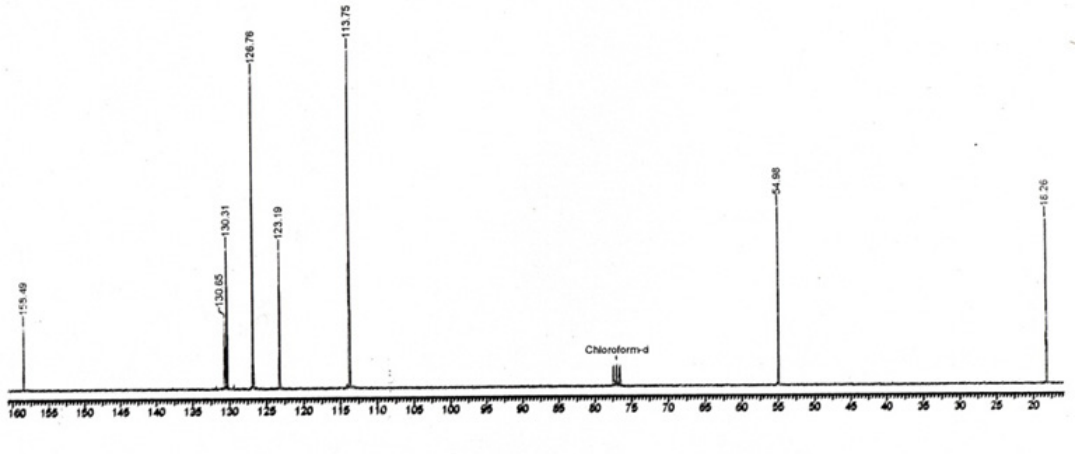
The ferric reducing power is often used to measure the ability of natural antioxidant to donate an electron or hydrogen to form a more stable product. The reducing power of the *Pimpinella essential* oil increased in a concentration-dependant manner (Fig. 5-B). The capacity of the studied essential oil to reduce Fe^{+3} to Fe^{+2} provides an optical density of 1.78 ± 0.3. This value was much lower tthan the optical densities obtained by ascorbic acid 2.07 ± 0.2 and catechin 2.67 ± 0.1 at the same concentration 1000 µg mL^{-1}. We can classify the power reduction of iron as follows: catechin, ascorbic acid, and *Pimpinella essential* oil. Similar values of IC$_{50}$ were obtained by catechin 53 ± 0.2, ascorbic acid 58.3 ± 0.3 and essential oil 60 ± 0.2 µg mL^{-1}. Several studies reported the positive correlations between antioxidant activity and reducing power of certain bioactive compounds. Shimada et al. (1992) reported that the reductive potential might be related to the presence of phenolic compounds, such as isothymol and carvacrol, due to the hydroxyl substitutions in the aromatic ring which possess potent hydrogen-bonding abilities. The antioxidant capacity has been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Yildirim et al., 2001). Phenolic compounds act as chelators of metal ions that induce oxidation (Han and Baik, 2008). The antioxidant capacity of essential oil may be associated with multiple systems, as they possess chemical mixture with diverse functional groups, polarity and chemical behavior (Tepe et al., 2006b).

Results of Antibacterial Activity

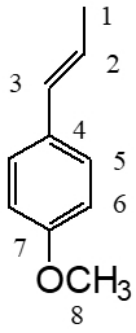
As shown in Table 2, among the most active antibiotics on Gram-negative bacteria, first came Spiramycin which acts against *Pseudomonas aeruginosa* and *Escherichia coli*, followed by Gentamycin, Tetracycline and then Erytromycine. Amoxicillin and Ampicillin were inactive against all two strains. For Gram-positive bacteria, *Staphylococcus aureus* was sensitive to most antibiotics except Ampicillin. All bacterial strains were resistant to the antibiotics. Our results are in line with the work of Alsalim et al. (2017). Essential oil of *Pimpinella* seeds showed significant activity against all tested bacteria, the diameter of the zones of inhibition exceeding 11 mm for all tests. The highest activity was noted against *Pseudomonas aeruginosa* with an average value of 13.2 ± 0.5 mm. According to Kalemba and Kunicka (2003), the sensitivity of a microorganism to essential oils depends on the properties of this oil and the resistance of the microorganism.



A. NMR[1]H spectrum of aniseed essential oil



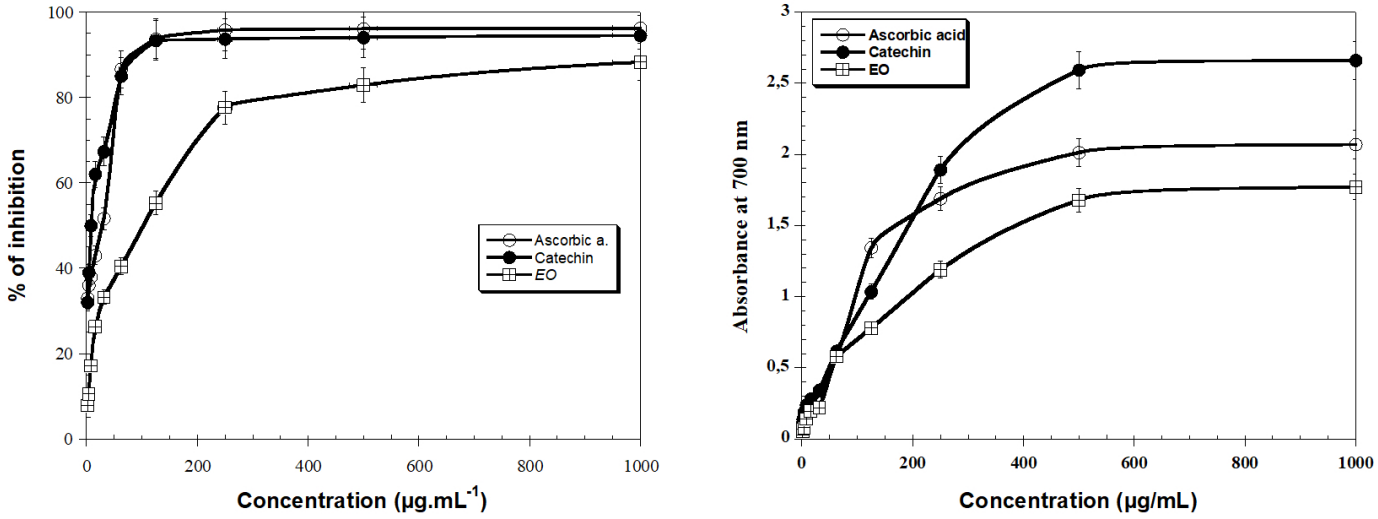
B. NMR spectrum of carbon 13 of aniseed essential oil



Anethole

NMR-[1]H (δ ppm, 300MHz)	NMR-[13]C (δ ppm,300MHz)
H_6 = 7,3 (d, 1H)	C_7 = 158,56
H_5 = 6,86 (d, 1H)	C_4 = 130,78
H_3 = 6,34 à 6,40 (m, 1H)	C_6 = 130,33
H_2 = 6,07 à 6,15 (m, 1H)	C_5 = 126,83
H_8 = 3,81 (s, 3H)	C_3 = 123,37
H_1 = 1,89 (d, 3H)	C_2 =113,86
	C_8 = 55,17
	C_1 = 18,33

Figure 4. Results of NMR analysis of aniseed essential oil



A: DPPH method

B: Ferric reducing power method

Figure 5. Antioxidant capacities of positive control (ascorbic acid, catechin) and essential oil of *Pimpinella anisum* seeds. Values represent Mean±SD; n=3; Confidence level P≤0.05

Table 2. Zone of inhibition in mm for antibiotics and essential oil of *Pimpinella anisum* seeds against tested bacteria. Values represent Mean±SD; n=3; Confidence level P≤0.05, *ZI: zone of inhibition*

	Tested bacteria		
	Escherichia coli	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Antibiotics			
Amoxicillin	1.0 ± 0.1	0	15.0 ± 0.3
Ampicillin	0	0	0
Erythromycin	4.0 ± 0.1	5 .0± 0.2	6.0 ± 0.1
Gentamicin	9.0 ± 0.1	8.0 ± 0.3	16.0 ± 0.3
Spiromycine	15.0 ± 0.3	18.0 ± 0.5	7.0 ± 0.1
Tetracycline	4.0 ± 0.2	3.0 ± 0.2	5.0 ± 0.3
Aniseed essential oil			
ZI	11.4 ± 0.5	13.2 ± 0.5	12.6 ± 0.4
MIC (mg.mL^{-1})	50 ± 0.1	25 ± 0.4	6.25 ± 0.2
MBC (mg.mL^{-1})	100 ± 1.2	50 ± 1.5	200 ± 2.5
MBC/MIC	2	2	32

Zaika (1988) and Hussein (1990) showed that Gram-positive bacteria were more resistant to essential oils compared to the Gram-negative bacteria, whis is contrary to the results found by Smith-Palmer et al. (1998). Inouye et al. (2001) revealed that Gram-positive bacteria were generally more sensitive to essential oils than Gram-negative bacteria. In addition, among Gram-positive bacteria greater resistance has been detected for those which produce lactic acid. Chao et al. (2000) explained that Gram-negative bacteria had a layer of peptidoglycan wedged between the plasma membrane and an outer layer made up of lipopolysaccharides and proteins. This structure can prevent the uptake of oils or protect the peptidoglycan layer from oils. The outer lipopolysaccharide membrane of Gram-negative bacteria constitutes a barrier to impermeability to hydrophobic substances, which can enter and prevent the growth of Gram-positive bacteria. In the latter, the peptidoglycan layer is located outside, thus allowing these bacteria to be more available to come into contact with the oils. Elgayyar et al. (2001) and Delaquis et al. (2002) disagree with this report, because for these authors it is very difficult to make such generalizations as each essential oil is unique in its composition and each Gram-positive bacterium differs considerably from one another in structure and functionality. Deans et al. (1995) and Dorman and Deans (2000) propose that the susceptibility of bacteria to essential oils appears to have only a small influence on the inhibition of microbial growth. In this work, the essential oil tested on the different Gram-positive and negative bacteria showed a similar action. The obtained results are generally in agreement with the studies previously reported in the literature. In contact with Gram-negative and Gram-positive, the essential oil extracted from *Pimpinella* seeds showed a slight inhibitory action on the growth of *Pseudomonas aeruginosa*, *S. aureus* and *E. coli* with a fairly high inhibitory effect on *Pseudomonas aeruginosa*. The antimicrobial activity of essential oils is linked to the nature of the majority of constituents. In fact, oils with high contents of monoterpene hydrocarbons are reported to be very active toward microorganisms. Hypotheses favoring harmful effects on the integrity of the bacterial membrane were reported by Lorber and Muller (1976). However, the rupture of the membrane by terpenes has been shown on Gram-positive and negative bacteria (Helander et al., 1998; Lis-Balchin et al., 1998; Griffin et al., 1999). Furthermore, the mode of action of the minor components of essential oils is unknown. Delaquis et al. (2002) suggest that these compounds induce differences in the cell envelope of Gram-positive bacteria. Their action is strongly limited in Gram-negative bacteria.

In the second step, the antibacterial property was determined by micro dilution method. This antibacterial activity was not due to the presence of a particular substance but only to the synergistic or antagonistic effect of each component of the natural product (Cheng et al., 2001). Aligiannis et al. (2001) proposed a classification of plant material on the basis of MIC results as follows: *Strong inhibition*: MIC less than 500 µg mL^{-1} and *Moderate inhibition*: MIC ranges from 600 to 1500 µg mL^{-1}. The MIC was determined on a sterile micro plate and was accompanied by transplanting of the cells showing no growth in order to determine the MBC and to demonstrate an antibacterial or bactericidal effect of the tested oil. The essential oil of *Pimpinella* seeds displayed a good *in vitro* antibacterial activity with MIC of 50 ± 0.1 mg mL^{-1} against *E. coli*, 6.25 ± 0.2 mg mL^{-1} against *S. aureus* and 25 ± 0.4 mg mL^{-1} against *P. aeruginosa*. Our results are in line with the work of Bazargani and Rohloff (2016) and the findings of Al-Bayati (2008). The author explains that the Gram-positive bacterium *S. aureus* was more sensitive to the essential oils compared to the Gram-negative bacterium. The tolerance of Gram-negative bacteria to essential oils was due to the presence of hydrophilic outer membrane that blocs the penetration of hydrophobic essential oils into target cell membrane. The report MBC/MIC was 2 (less than 4), which indicates, according to Spencer and Spencer (2004) the bactericidal effect of this oil against *E. coli* and *Pseudomonas* and the value of 32 indicating the antibacterial effect against *S. aureus*. Some significant infections should be treated with single antibiotics, due to the rapid resistance developed by bacteria. Essential oils contain various bioactive compounds; these substances exert beneficial effects due to the synergistic action of several chemical compounds acting at single or multiple target sites.

Conclusion

In the present study, essential oil of *Pimpinella anisum* seed was chemically analyzed. Results have shown that anethole and estragole are the major components with percentage of 94.82 and 1.69%. Essential oil of aniseed has shown *in vitro* antioxidant and antibacterial activities through inhibition of free radical DPPH, higher reducing power and inhibition of Gram-negative and positive bacteria. This oil has displayed a good antibacterial activity against pathogenic bacteria such as *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. This study has revealed the potential role of aniseed oil as antioxidant and antibacterial agent.

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