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Biological Activity of Eugenol Acetate as Antibacterial and Antioxidant Agent, Isolation from *Myrtus communis* L. Essential Oil

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Abstract

The fundamental goals of this study is to determine the chemical composition of *Myrtus communis* (*M. communis*) essential oil the local and to assess its antimicrobial activity against *Esherichia coli*, *Proteus mirabilis* isolated from urine, *Staphylococcus aureus* from Ear, *Pseudomonas aeruginosa* and *Acinetobacter sp.* from burns and *Salmonella typhi* from blood. Column Chromatography, Thin Layer Chromatography (TLC), Infra (IR) Red, Nuclear Magnetic Resonance Spectroscopy (H^1NMR) and Gas chromatography-mass spectrometry (GC-MSS) were used to determine the chemical composition of essential oil from *M. communis* leaves. A disk diffusion method was applied to evaluate the antibacterial activity and microdilution susceptibility assay method was utilized to evaluate the (minimum inhibitory concentration) (MIC) and antioxidant activity was analyzed using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assays. Column Chromatography, (TLC), (IR), (H^1NMR) and (GC-MS) revealed that *M. communis* contained eugenol acetate compound. The results showed broad antibacterial activity against pathogenic bacteria isolates tested with ranged between (13.5-36.5) mm except *P. aeruginosa* and less minimum inhibitory concentration ranged between 25-100 µg\ml. The antioxidant activities have shown high rates of inhibition. A verification of non-toxicity of eugenol acetate compound against human blood revealed a negative test.

Keywords

Myrtus communis L, Essential Oil, Antibacterial, Antioxidant, Pathogenic Bacteria

1. Introduction

Until now, essential oils are increasing interest for their potential multipurpose use as antioxidant, antibacterial, and cancer prevention agent [1, 2, 3]. Essential oils are volatile organic compounds found in various plant tissues. The quality of essential oils depends on the several factors including the part of the plant used, the plant variety and its country of origin, the method of extraction and the refining process [4]. The volatile oil from aromatic plants isolated Provides a number of ecological benefits to the plant. Until recently, essential oils have been studied mostly from their flavor and fragrance viewpoints only for flavoring foods, drinks and other goods [5]. In reality, however, essential oils and their components are gaining interest due to their relatively safe status، their wide acceptance by consumers and their exploitation for potential multi-purpose functional use [6] Particularly، essential oils isolated of Myrtle (*Myrtus communis* L.).

Myrtus communis L. (Myrtle) (Myrtaceae) is an evergreen shrub which grows mainly in Mediterranean climates and has long been used by locals for its culinary and medicinal properties [7, 8]. *M. communis* is an important medicinal and aromatic plant,because of the high essential oil content in its leaf, flower and fruit glands. Many authors have described the

chemical composition of the myrtle essential oil [9, 10, 11].

Recently, more researches about the pharmacological activities of *M. communis* oil including its anti-inflammatory, antimicrobial [12, 13, 14, 15, 16]. Antioxidant, hypoglycemic and antimutagenic properties have been widely investigated [9,17,18]. Even today, more than 50 active ingredients of *M. communis* oil have been identified and the major components as determined by gas chromatography-mass spectrometry are α-pinene, limonene, 1, 8-cineole, 4- terpineol, α-terpineol, linalool, geranyl acetate, methyl eugenol, phenolic and acetate compounds [19].

Pharmacological properties of different parts of *Myrtus communis* have been greatly reported, but there has been very little information on antioxidant and medicinal properties in study zone (Iraq). Hence, the present study attempts to investigate the chemical composition of *Myrtus communis* (*M. communis*) essential oil and to assess its antimicrobial activity against clinical pathogenic bacteria and antioxidant activity, Column Chromatography, Thin Layer Chromatography (TLC), Infra (IR) Red, Nuclear Magnetic Resonance Spectroscopy (H1NMR) and Gas chromatography-mass spectrometry (GC-MS) were used to determine the chemical composition of essential oil from *M. communis* leaves.

2. Materials and Methods

2.1. Plant Material

Leaves of *Myrtus communis* L. were obtained from Agri cultural and Natural Resources Center Misan, Iraq. They were dried in shade, powdered in miller, and stored in an airtight bottle for future work.

2.2. Essential Oil Extraction

The leaves of M. communis L. were dried at room temperature (20–25°C). The essential oils were extracted by hydro distillation using a Clevenger-type apparatus. The essential oil from myrtle leaves used for the antibacterial and antioxidant tests were extracted as follows: 70 gm of grinded leaves + 700 mL of distilled water for 3 hours 30 minutes. The extracted oils were dried and stored at 4°C until use [15]. Essential oils were collected in vials. These fractions were tested for purity by using thin layer chromatography (TLC) [20].

2.3. Separation and Isolation by Column Chromatography

This technique was used a glass column with 1.5 x 35 cm then filled with 15 gm of silica gel(c-60) as a stationary phase, and after a solvent of 0.1 gm of essential oil was used and eluted by petroleum: chlorophorm ration (7:3) v\v as a mobile phase with flow rate of 0.5 ml\minutes, until the separation was finished. The fractions of essential oil were collected in vials. These fractions were tested for purity by using thin layer chromatography (TLC) [20].

2.4. Thin Layer Chromatography (TLC) Test

According to [21] thin layer chromatography was carried out for fractions of myrtle essential oil, 10µl of myrtle essential oil was used on a thin layer chromatography as a stationary phase and Petroleum: chlorophorm ration was (7:3) V/V, as a mobile phase. This layer was left in eluted by the previous ration for few minutes and then was dried with hot air drier. Then spots were detected y iodine steam. Finally retardation factor (R_F) a spots were calculated by using the following formula:

$$\text{RF value} = \frac{\text{Distance from the base line to the centre of the sparated zone}}{\text{Distance from the base line to the solvent front}}$$

2.5. Infrared Spectroscopy (IR)

The IR spectrum of the essential oil was obtained using prinks correlation charts. The IR spectra were reported in percentage transmittance. The wave number region for the analysis was 4000-400 cm-1 (in the mid-infrared range).

2.6. Nuclear Magnetic Resonance Spectroscopy (H^1NMR)

Essential oil sample under study was dissolved in dimethyl sulphoxide (DMSO) solvent for H^1NMR Determination. H^1NMR spectra was recorded on a Varian Gemini 300 BB NMR Spectrometer (USA) with 300 MHz NMR Magnet, Gemini 2000 Console and 5 mm probe, Observing 1 H at 300 MHz at temperature of 25°C, H^1NMR spectra were acquired using spectral width of 8000 Hz, relaxation delay of 1 s, 64 repetitions, acquisition time of 1.998 s and with a total time of 3 min, 37 s. Chemical shifts are expressed in d units (ppm).

2.7. Gas Chromatography Mass Spectroscopy (GC-MSS)

The GC-MSS analysis of the essential oil was carried out using Varian 4000 Ion trap GC/MS/MS with Fused silica 15m x 0.2 mm ID x 1µm of capillary column. The instrument was set to an initial temperature of 110°C, and maintained at this temperature for 2 min. At the end of this period the oven temperature was rose up to 280°C, at the rate of an increase of 5 ºC/min, and maintained for 9 min. Injection port temperature was ensured as 250°C and Helium flow rate as 1 ml/min. The ionization voltage was 70eV. The samples were injected in split mode as 10:1. Mass spectral scan range was set at 45-450 (m/z). Using computer searches on a NIST Ver.2.1 MS data library and comparing the spectrum obtained through GC-MS-MS compounds present in the essential oil were identified.

2.8. The Test Microorganism

All clinical bacterial isolates were obtained from AL-Sadder hospital which include *Esherichia coli* and *proteus mirabilis* from urine, *Staphylococcus aureus* from Ear, *Pseudomonas aeruginosa* and *Acinetobacter sp.* from burns and *Salmonella typhi* from blood and has been collected all

bacterial isolates were maintenance by culturing on specific media until use.

2.9. The Antimicrobial Activity Test

The antimicrobial activity was performed with the disk diffusion method [22]. A test culture of each bacterial strain was prepared in a concentration of (1×10^6) cell per ml depending on the McFarland opacity standard. Mueller Hinton agar plates were inoculated with 0.1 ml of each bacterial isolates (1×10^6) cell per ml by spreading method and left inoculated plates for 10 min then one well of 6 mm diameter was punched into agar, of each Petri dish. Volumes of 10 microliter of essential oil was inoculated wells. They kept at 30 minute before they were transferred to incubator. The plates were incubated at 37°C for 24 h. the diameter of inhibition zones was measured. Each experiment was done in three replicate.

2.10. Micro Dilution Method of Determination of the Minimal Inhibition Concentration (MIC)

The MIC values were determined for the inoculums of the bacteria strains were prepared from 12 Mueller- Hinton broth cultures and suspensions were adjusted to The McFarland opacity standard, the 96-well plates were prepared by dispensing into each well 100 µl of nutrient broth and 5µl of the inoculums. A 100 µl aliquot from the stock solutions of each plants extract was added into the first wells. Then, 100 µl from the serial dilutions were transferred into eleven consecutive wells. The last well containing 195µl of nutrient broth without essential oil and 5 µl of the inoculums on each strip were used as the negative control. The final volume in each well was 200 µl. The plates were incubated at 37°C for 18 to 24 h. The essential oil tested in this study was screened two times against each strain. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of the microorganisms.

2.11. Antioxidant Activity

The DPPH radical scavenging capacity was measured according to [23]. 1 ml of essential oil was mixed with 0.5 ml of 0.2 mM methanolic DPPH solution. The reaction was allowed to stand at room temperature in the dark for 30 min and the absorbance was recorded at 517 nm against a blank (methanol solution). Dilutions were made to obtain concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90 and 1.95 µg/ml. Tests were carried out in triplicate. The ability to scavenge the DPPH radical was calculated using the following equation:

Scavenging effect (%) = [(A0-A1)/A0] × 100. Where A0 and A1 are the absorbance of the control and the sample, respectively.

2.12. Cytotoxicity Studies by Hemolytic Activity

The cytotoxicity was studied by examining hemolytic activity against human red blood cells (RBCs) using Running tap water as positive control. The positive control showed about 100% lysis, whereas the phosphate buffer saline (PBS) showed no lysis of RBCs as Negative control. When the effect of extract plant was compared with the controls [24].

2.13. Statistical Analysis

Statistical analysis of data. Analysis of variance (ANOVA) was used to determine the significance ($p\leq0.05$) of the data obtained in all experiments.

3. Results and Discussion

The results of chemical composition of the myrtle leaf essential oil by column chromatography showed that contain four distinct components (A, B, C and D), these component were purity according to TLC plates test. The antibacterial activity of components (A, B, C and D) showed that component (B) a higher antibacterial activity against bacterial isolate (Tab. 1). On the other hand, many studies have confirmed that the methods used for testing the antimicrobial activities of plants species and their components affect the levels of inhibition [25]. So it was conducted chemical analysis for the purpose of identification the chemical composition by IR (Fig. 1, Tab. 2), H^1NMR (Fig. 2) and GC-Mass spectroscopy (Fig. 3). Which indicated that the compound was Eugenol acetate owned the molecular 206.26 weights and formula $C_{12}H_{14}O_3$ (Fig. 4).

Myrtle leaves contain tannins,cineol, linalool, terpineole, linalyl acetate, terpineole and flavonoids compounds [16].

Table. 1. *The antibacterial activity of components (A, B, C and C) isolated from essential oil of M. communis L..*

Bacterial strains	A	B	C	D
E. coli	10.0	25.5	12.5	20.0
S. aureus	12.0	36.5	19.5	21.0
Ps. a	0.0	0.0	0.0	0.0
S. typhi	8.0	25.0	28.0	23.5
Acinetobacter sp.	8.0	13.5	12.5	10.0
*P. mirabilis*10.0		23.0	20.0	18.0

Numbers represent average of three replicates P ≤ 0.05

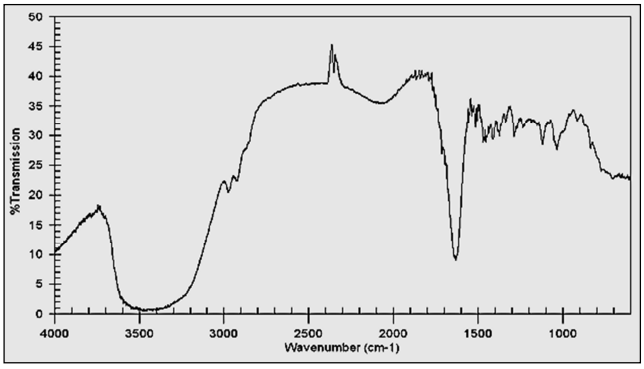

Fig. 1. *Infrared spectroscopy (IR) of component (B).*

Table. 2. *Absorption bundles and effective synthetic groups belonging to her in the spectrum of infrared (IR) of component (B).*

Functional groups	Frequency cm^{-1}
CH st	2990
C=C st	1650
CH_2 b	1400
C-O st	1280

St= stretching, b= bending

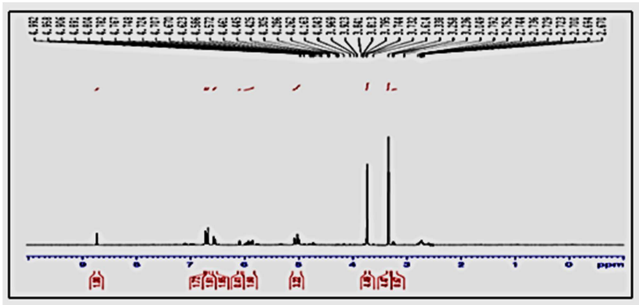

Fig. 2. *NMR spectrum (H^1NMR) of Eugenol acetate.*

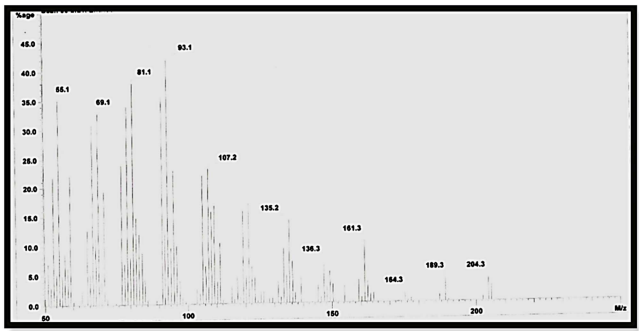

Fig. 3. *Mass spectrometer (GC / Mass) of Eugenol acetate.*

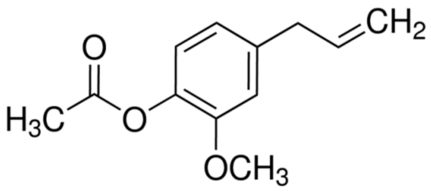

Fig. 4. *Chemical structure of the compound 4-allyl-2-methoxyphenyl.*

The antibacterial activity of Eugenol acetate against selected bacterial strains was assessed (Tab. 1). The results from the disc diffusion method revealed that the Eugenol acetate showed higher antibacterial activity. The maximum inhibition zone was 36.5 mm against *S. aureus*. followed by *E. coli* 25.5 mm while the minimum zone of inhibition was found to be 13.5 mm in diameter against *Acinetobacter sp*. While *Ps. aeruginosa* was resistant (Fig. 5), the antibacterial activity of Eugenol acetate perhaps because contain on the active phenol (OH) group [26]. In addition, may be due to the chemical composition containing a group Esters, and which has a deadly effect of microorganisms [27]. The Eugenol acetate showed antimicrobial activity with a MIC ranged between 25.0-100 ug/ml (Tab. 3). These results are similar to another study reporting that essential oil exhibited antibacterial activity against a large number of bacteria reported the MIC [28].

Table. 3. *Inhibition zone diameter and (MIC) by Eugenol acetate.*

Bacterial isolates	MIC(ug/ml)
E. coli	50.0
S. aureus	25.0
Psa	-
S. typhi	50
Acinetobacter sp.	100
P. mirabilis	100

The current results were also confirmed by previous studies. Oyedemi et al. [29]. showed that essential oils components(eugenol, -terpineol and -terpinene) have a bactericidal effect against the both gram positive and gram negative bacteria by disrupting their membrane systems. Its important characteristic their hydrophobicity, enables them partition the lipids of bacterial cell membrane, disturbing the cell structure and rendering them more permeable. Amensour et al. [12] confirmed that The mode of myrtle extract and essential oil activity affect mainly cell wall and membrane structures. It was reported that the permeability of bacterial cell wall and cell membrane are affected by these extracts, leading to the release of intracellular contents outside of cell. This can be accompanied with the disruption in the membrane function such as electron transfer, enzyme activity or nutrient absorption.

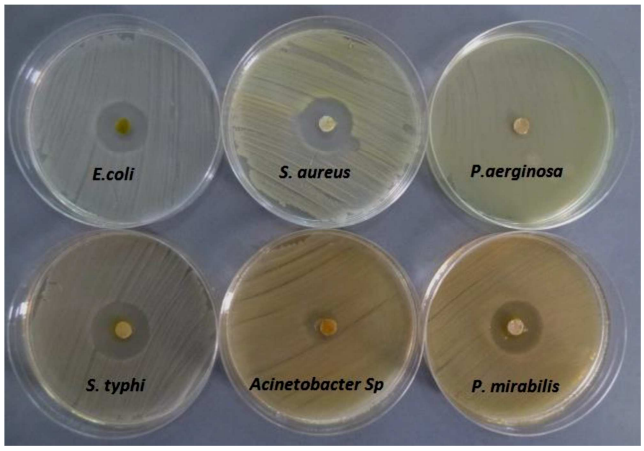

Fig. 5. *Antibacterial activity component (B) Eugenol acetate.*

The free radical scavenging activity of the Eugenol acetate tested was determined through the DPPH method and results are showed at all concentrations from1.95 to 1000µg/ml is rather strong (10-80%). The radical scavenging capacity of essential oil of M. communis were significantly lower compared to commercial antioxidants (BHT) examined in this study. (Fig. 6). The current results were also confirmed with the previously published data which describe the antioxidant activities of different extracts and compounds obtained from myrtle leaves [30, 19, 18, 31]. As demonstrated in several studies the antioxidant capacity of plant extracts is strongly related to phenolic content [32, 33].

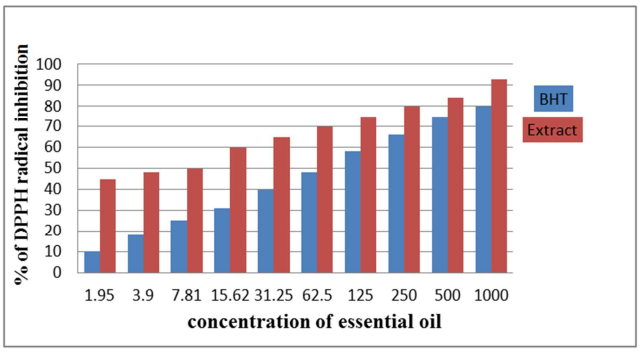

Fig. 6. *Antioxidant (DPPH scavenging) activity of investigated component (B) Eugenol acetate presented as percentage of DPPH radicals inhibition.*

4. Discussion

Data presented in this study showed strong in vitro inhibitory activity of *M. communis* on all the clinical bacterial isolates tests. We were determined phenolic compound (4-allyl-2-methoxyphenyl, eugenol acetate) was abundant in this extract in results obtained we can conclude that myrtle essential oil showed considerable and antibacterial potential (in a non-toxic concentration range), DPPH-tests show that eugenol acetate is the compound responsible for the scavenging activity of the entire oil. To conclude, *M. communis* essential oil could be a promising source of natural antioxidants and antibacterial. Nevertheless, additional in vitro and in vivo studies are needed to unequivocally demonstrate this.

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