In Vitro Antimicrobial Activity and GC-MS Analysis of Crude Aqueous Methanolic Extract Produced from Leaves of *Eucalyptus* species

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Abstract

Objectives: To evaluate the antimicrobial activity of the aqueous methanolic extract was given by the leaves of *Eucalyptus* sp.growing in Thi-Qar Province, south of Iraq. Additionally, to detect the compounds within the extract by using the technique of gas chromatography-mass spectrometry (GC-MS).

Methodology: The healthy leaves of *Eucalyptus* sp. were collected, washed with tap water then by distilled water, dried, and pulverized through using a small mill to make the dried leaves as a powder. The leaves powder was extracted by absolute petroleum ether then aqueous methanol (80%) for obtaining a sticky crude extract of the aqueous methanol filtrate. The crude extract was tested against some microbial pathogens as well as analysis of the extract using the technique of GC-MS and phytochemical tests.

Results: The current study showed the antimicrobial activity of the crude aqueous methanolic extract obtained from Leaves of*Eucalyptus* sp. by which 40000 µg⁄ml of the extract exhibited inhibition zones around *Staphylococcus aureus*, *Streptococcus mutans*, *Escherichia coli*, and *Klebsiella* sp. besides *Candida albicans* that the inhibition zones were measured to be 30, 27, 26, 30, and 38 mm respectively. The chemical tests appeared the crude extract containsalkaloids, glycosides, flavonoids, tannins, andsaponin glycosides. GC-MS analysis detected constituents of the extract are dimethylsulfoxoniumformylmethylide, diethyl phthalate, benzene,1,1'-(1,2-cyclobutanediyl)bis-trans-,diisoctyladipate, and 2-methyl-7-phenylindole.

Conclusion: The present study concluded that the leaves of*Eucalyptus*sp. possess the bioactive products which are needed to be separatedas the pure compoundsby using techniques of chromatography in order to separately tested *in vitro* and *in vivo*, then application of spectrometry techniques for characterization of their chemical structure. Finally, these compounds can be used to treat diseasesas drugs in hospitals.

Keywords: Leaves of *Eucalyptus* sp., Antimicrobial Activity, GC-MS Analysis.

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Introduction

Medicinal plants are the producing sources of anti-infective, bioactive and anticancer compounds which developed as drugs for treating the diseases[1,2].Taxonomically, The genus *Eucalyptus* is one of family *Myrtaceae* which has the ability to produce antimicrobial, analgesic, antihypertensive agents. The genus contains different chemical compounds such as tannins, glycosides, and saponins[3-6].*Eucalyptus* is one of the Gum trees which possesses more than 500 species that represents the native plants in Australia. Also, some species of *Eucalyptus* are native in the Philippines and New Guinea [7,8].The best species of *Eucalypts* is *E.globulus* Labill that is known a Blue Gum, common name, growing in Australia, Tasmania,and Mediterranean region.The leaves of the mentioned species were used as traditional remedies for treating diseases, examples, fungal infections, pulmonary tuberculosis, influenza, and diabetes[8-13].

Previous studies showed that the leaves of *Eucalyptus* spp. have the antimicrobial activities against microbial pathogens [4,14].*Eucalyptus* spp. have the compounds used as antiseptics. The boiling leaves and roots of thesespecies are drunk used to cure for preventing colds in western Victoria. The aqueousextract producedfrom kinos of*Eucalyptus* spp. were given to treatinfections of the wounds and eyes [15-17].

Objectives

The current study was designed to achieve two aims are that thefirst aim wasto evaluate the antimicrobial activity of the aqueous methanolic extract of the leaves of *Eucalyptus* sp.growing in Thi-Qar Province, south of Iraq. The second one was an analysis of the crude extract bygas chromatography-mass spectrometry (GC-MS).

Materials and Methods

Preparation of plant sample and extraction by aqueous methanol (80%)

The healthy leaves of *Eucalyptus* sp. (Fig.1) were collected and washed with tap water then by distilled water. The leaves left at room temperature for 3 dayswith flipping until they dried. Through using a small mill, the dried leaves were pulverized to be a powder. 150 g of the plant powder placed in a clean glass beaker (1000 ml) and 700 ml of petroleum ether were added to the powder and soaked. The beaker strictly covered by two layers of parafilm, and left at room temperature for 4 days. The soaked powder was separated from filtrate through using a filter paper covered with 0.5 g of the activated charcoal as a film layer for removal of the plant chlorophyll.The soaked powder left at room temperature for 7 days until it dried and petroleum ether completely evaporated. 700 ml of aqueous methanol (20 ml of distilled water: 80 ml of absolute

methanol) poured on a clean glass beaker (1000 ml) containing the dried leaf powder (it was same powder used in step of extraction through using petroleum ether).The beaker strictly covered by two layers of parafilm, and left at room temperature for 7 days. The separation of the aqueous methanol filtrate from the soaked powder and evaporation was performed similarly to a method of the petroleum ether. Finally, a sticky crude extract of the aqueous methanol was obtained, and it was tested

Fig.1:*Eucalyptus* sp. tree

Antimicrobial screening of crude aqueous methanolic (80%) extract

Amount of aqueous methanolic extract was dissolved in dimethyl sulfoxide (DMSO) for getting 40000µg⁄ml as a stock concentration. Petri dishes of nutrient agar (NA) were prepared,and each dish inoculated by 100 µL given bymicrobial suspension (1.5 x 10^9 cell/ ml) of each tested microorganism. The inoculum was spread by asterile cotton swap.Aseptically, 100 µL of extract concentration (40000 µg⁄ml) placed in well (7 mmdiameter) which was made in a center of each dish.All dishes incubated at 37 °C for 2 daysthenthe zones of inhibition were measured around the tested microorganisms (*Staphylococcus aureus*, *Streptococcus mutans*,*Escherichia coli*,and*Klebsiella* sp.)in addition to*Candida albicans* which was tested by the same conditions except for using Petri dishes of potato dextrose agar (PDA).The test was done as triplicate against each microorganism.

Phytochemical tests of crude aqueous methanolic (80%) extract

The phytochemical chemical tests were carried out according to [18-22]. The solution of aqueous methanolic extract was performed by re-dissolving the solid extract in aqueous methanol (80%).

Detection of sterol and terpenoids

1-Salkowski's test: Treating 2- 3 ml of the plant extract solution with2 ml of chloroform and filtered.The filtrate was treated with few drops of concentrated sulphuric acid that added carefully on the side of the test tube without shacking. Formation of the reddish brown color of the interface indicates the presence of terpenoids while the appearance of golden yellow color indicates the presentingtriterpenes.

2-Libermann Burchard's test: Treating 2 ml of the plant extractsolution with chloroform and filtered. The filtrate was treated with few drops of acetic anhydride (1-2 ml) boiled and cooled. Then concentrated

sulphuric acid was added carefully on the sides of the test tube. Formation of the brown ring at the junction indicates the presence of phytosterols (however; it begins to appear red color, then blue and finally green color indicates the presence of sterols.

3- Copper acetate test: Treating 2-3 ml of plant extract solutionwith few drops of 5% copper acetate solution. Formation of emerald green color indicates the presence of diterpenes.

Detection of alkaloids

1-Wagner's test: Treating 2-3 ml of the plant extract solutionwith 2 ml of Wagner's reagent (Iodine in potassium iodide).Formation of brown/reddish brown precipitate indicates the presence of alkaloids.

2- Dragendroff's test: 2-3 ml of the plant extract solutionwere acidified with 1 drop of sulfuric acid then treated with 0.5 ml of Dragendroff's reagent (solution of potassium bismuth iodide). Formation of red precipitate indicates the presence of alkaloids.

Detection of carbohydrates

Molisch's test: Treating 2-3 ml of the plant extract solutionwith few drops of 10% alcoholic – α – naphthol solution (2-4 drops), then about 2 ml of concentrated sulphuric acid was administered carefully to the side of the test tube. If a purple color forms,it indicates to present the carbohydrates.

Detection of glycosides

1- General test: Treating 5 ml of the plant extract solutionwith few drops of 10% aqueous NaOH solution. Development of yellow color indicates the presence of glycoside.

2- Keller Kiliani test (for deoxysugar glucosides): Adding 2 ml of glacial acetic acid containing a few drops (3-4) of 5% FeCl3 solution to 2-3 ml of the plant extractsolution. Then 1 ml of concentrated H2SO4 was added to along the side of the test tube carefully. A reddish-brown ring at the interface indicates the presenting deoxysugar of cardenolides. A violet ring may appear beneath the brown ring, while in the acetic acid layer, a greenish ring may also form just gradually throughout the layer.

3- Legal test:2 ml of the plant extractsolution acidified with 1 drop of concentrated HCl, then treating with 1 ml sodium nitroprusside in 1 ml pyridine and methanolic alkali. Formation of pink to bloody color indicates the presence of cardiac glycosides.

Detection of coumaringlycosides, phenoliccompounds,flavonoids, and tannins

1- Alkaline Reagent test: Treating 2-3 ml of the plant extract solutionwith few drops of 20% sodium hydroxide solution. Formation of intense yellow color which turns to colorless by addition of few drops from dilute acetic acid indicates the presence of flavonoids.

2-Lead acetate test: Treating 2ml of the plant extract solution withfew drops of 10% lead acetate solution. Formation of white precipitate indicates the presence of phenolic compounds.

3- Ferric chloride test: about 1 ml of the plant extract solutionwas added to 2 ml of

water by using a test tube. 2-3 drops of diluted 5% ferric chloride solution were added and observed for green to blue-green (catechol tannins) or a blue-black (gallic tannins) coloration.

4- Gelatin test: 2-3 ml of the plant extract solutionwere added to a 1% gelatin solution containing sodium chloride (1%).Formation of white precipitate indicates the presence of tannins.

Detection of proteins and amino acids

1-Xanthoproteic test: Treating 3 ml of the plant extractsolution with few drops of concentrated nitric acid. Formation of yellow color indicates the presence of proteins.

2- Ninhydrin test: Treating 3ml of the plant extract solutionwith few drops of 2% (w/v) ethanolic ninhydrin reagent, and boiled for few (5-10) minutes. Formation of a blue color indicates the presence of amino acid.

3- Biuret test: Treating 3 ml of the plant extract solutionwith 1 ml of 10% sodium hydroxide solution and heated, thena drop of 0.7% copper sulfate solution was added. Formation of purplish violet color indicates the presence of proteins.

Detection of saponinglycosides

1- Foam test: Adding 1 ml of the plant extract solutionto 2-3 ml of distilled water. The mixture was shaken vigorously. Formation of foam that persists for 10 minutes indicates the presence of saponins.

2-Froth test: 1ml of the plant extractsolutionwas diluted with distilled water to be 20ml and shacked by a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam that persists for 15 minutes indicates the presence of saponins.

Gas chromatography-mass spectrometry (GC-MS) analysis of crude aqueous methanolic (80%) extract

The crude aqueous methanolic (80%) extractwas dissolved in DMSO and filtered through µM 0.45 filter syringe (Millipore) by which the filtrate subjected to the technique of GC-MSwas carried out by gas chromatography-mass spectrometry, MSDCHEM\1\METHODS\MUAFAQ.M for the determination of negative ions(m/z) through using a column characterized by HP-5MS, 5% Phenyl methyl Sillox(1629.5), 30m × 0.250 µm I.D. x 0.25 µm, SS., Inlet He, then application of the parameters in (Table 1).

Table 1. Parameters of GC-MS used to detect the compounds in aqueous methanolic (80%) extract produced from *Eucalyptus* sp.leaves.

Analysis Parameters	
EMV mode	**Gain Factor (1.00)**
Resulting EM voltage	**1306**
Power capacity	**70 EV**
Low Mass	**28.0**
High Mass	**441**
Threshold	**150**
Minimum quality for all narcotics	**97%-90**
Flow rate	**1ml/min**
Runtime	**24 min**
Hold up time	**1.5288 min**
Solvent delay	**3.00 min**
Average velocity	**36.796 cm/sec**
Temperature	**Initial 70 °C to Maximum 375 °C**
Pressure	**8.81 Psi**

Statistical analysis

The statistical analysis was carried out by using Graph Pad Prism 5.

Results

Antimicrobial screening of crude aqueous methanolic (80%) extract

The concentration of aqueous methanol (80%) extract was 40000 µg/ml exhibited the antimicrobial activity against all tested microbial pathogens. The highest zone of the inhibition was measured around *C. albicans* followed by *S. aureus* and *Klebsiella* sp. while less inhibition zone was against *E.coli* (Table 2) and (Figures:2 and 3).

Table2: Antimicrobial screening of aqueous methanolic (80%) extract produced from *Eucalyptus* sp.leaves against five isolates of the clinical microbial pathogens tested by agar well diffusion method. The used concentrations of the extract were 40000 µg/ml.

Inhibition Zones (IZ) Measured by Milliliter (mm)					
	S. aureus	*S .mutans*	*E. coli*	*Klebsiella* sp.	***C. albicans***
Ext.	30 ± 0.57	27 ± 0.57	26 ± 0.1	30 ± 0.57	**38 ± 0.57**
Tet.	50	20	0	6	**----**

Means with the same letter within the same column are significantly different at the level of $P \leq 0.05$ Inhibition Zone (Values are expressed as mean ± SD). Three independently experiments.---- : Not tested.
Note: Inhibition of *S .mutans* and *Klebsiella* sp. are not significant. Ext: IZ of plant extract. Tet.: IZ of disc contained 30 µg pure and standard tetracycline.

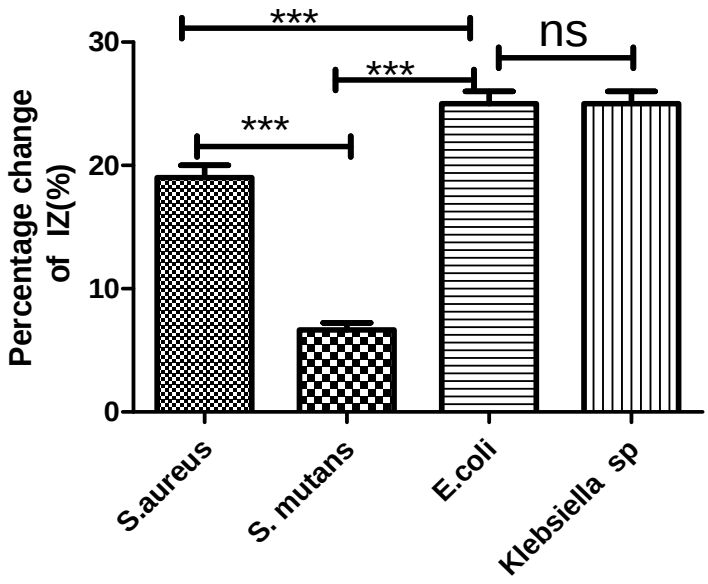
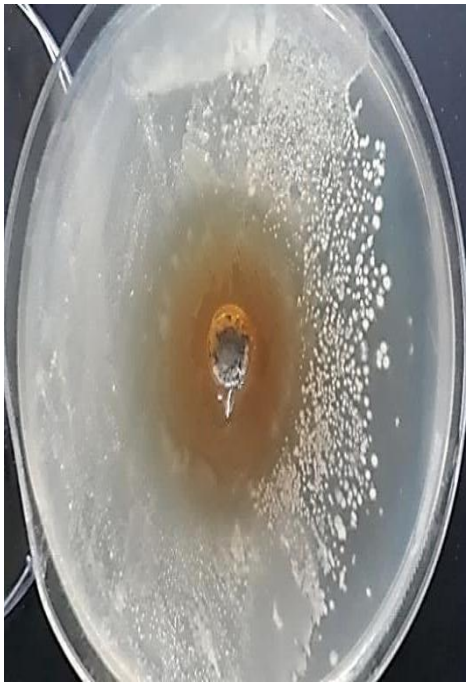
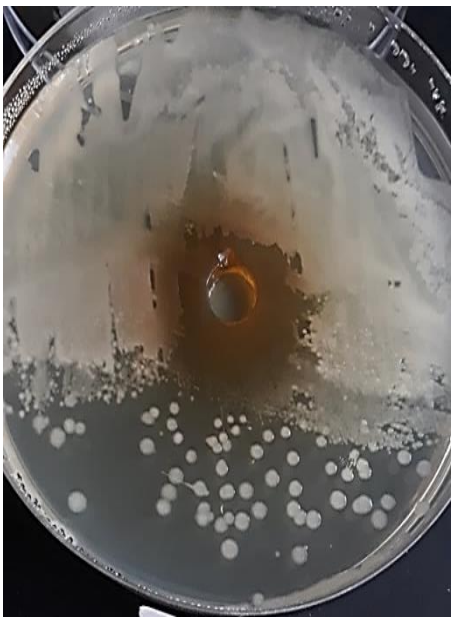


Fig.2:Therewere significant results among tested microorganisms which were inhibited by theaqueous methanolic extract from *Eucalyptus*sp. leaves. One- way ANOVA was used, and the statistical analysis was carried out by using GraphPad Prism 5.One or more stars indicates to present significant results. **ns**: The antimicrobial activity of aqueous methanolic extract against *E. coli* and *Klebsiella* sp. was not significant result.
IZ: Inhibition Zone.



A



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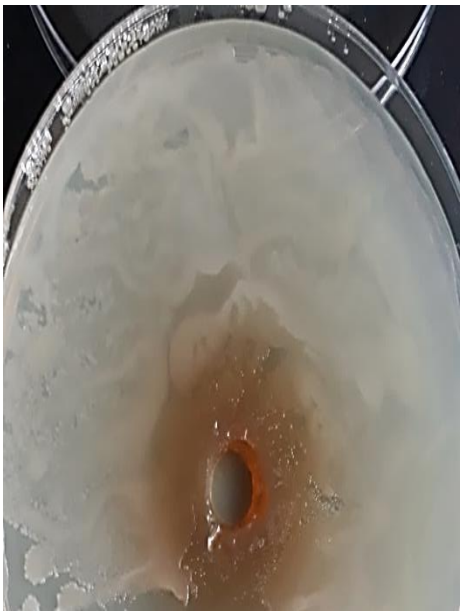

E

Fig. 3: Antimicrobial activity of aqueous methanolic (80%) extract produced from *Eucalyptus* sp. leaves against isolates of the clinical microbial pathogens tested by agar well diffusion method. The used concentration of the extract was 40000 µg/ml. **A:** Inhibition zone (IZ) against *C.albicans*. B: IZ against *S. aureus*. C: IZ against *S. mutans*. D: IZ against *E. coli*. E: IZ of disc contained pure tetracycline (30 µg) against *S. aureus*.

Phytochemical tests of crude aqueous methanolic (80%) extract

The phytochemical tests appeared that the aqueous methanol (80%) extract contains alkaloids, carbohydrates, glycosides, coumarin glycosides, flavonoids, tannins, and saponin glycosides. Table

Table 3: The compounds of crude aqueous methanolic (80%) extract produced from *Eucalyptus* sp.leaves appeared by the chemical tests.

Type of Test	Result
Tests of Sterol and Terpenoids	
Salkawski'stest	-----
Libermann Burchard's test	-----
Copper acetate test	-----
Tests of Alkaloids	
Wagner's test	+ve
Dragendroff's test	+ve
Tests of Carbohydrates	
Molisch test	+ve
Tests of Glycosides	
General test	+ve
Keller Kiliani test	+ve (cardiac glycosides)
Legal test	+ve (cardiac O-glycosides)
Tests of Coumerine Glycosides	
Alkaline reagent test	+ve
Tests of Phenolic Compounds/Flavonoids	
Alkaline test	+ve
Lead acetate test	+ve
Ferric chloride test	+ve
Tests of Tannins	
Ferric chloridetest	+ve (catechol tannins)
Lead acetate test	+ve
Gelatin test	+ve
Tests of Proteins and Amino Acids	
Xanthoproteictest	-ve
Ninhydrintest	-ve
Biuret test	-ve
Tests of Saponin Glycosides	
Foam test	+ve
Forthtest	+ve

+ve: Positive result. –ve: Negative result. -----: The solution of the test can't dissolve the extract.

Gas chromatography-mass spectrometry (GC-MS) analysis of crude aqueous methanolic (80%) extract

The GC-MS analysis detected that the crude aqueous methanolic (80%) extractcontains five compounds appeared during various time periods (minutes).The detected compounds were dimethylsulfoxoniumformylmethylide, diethylphthalate, benzene,1,1'-(1,2-cyclobutanediyl)bis-trans-, diisoctyl adipate, and 2-methyl-7-phenylindole (Table 4). Also, DMSO was detected as a solventused to dissolve the plant extract by which the sample was analyzedthroughtechnique of GC-MS (Figures: 4 a, and 4b).

Table 4: The compounds of aqueous methanolic (80%) extractdetected from*Eucalyptus*sp.leaves by GC-MS analysis besides DMSO (sample solvent).

Compounds	RT(Min.)	Abundance
Dimethyl Sulfoxide, DMSO (Sample solvent).	3.788	90%
Dimethylsulfoxonium formylmethylide	5.997	70%
Diethylphthalate	6.764	90%
Benzene,1,1'-(1,2-cyclobutanediyl)bis-trans-	10.399	90%
Diisoctyl adipate	14.801	90%
2-Methyl-7-phenylindole	17.382	90%

RT: Retention time during the minute

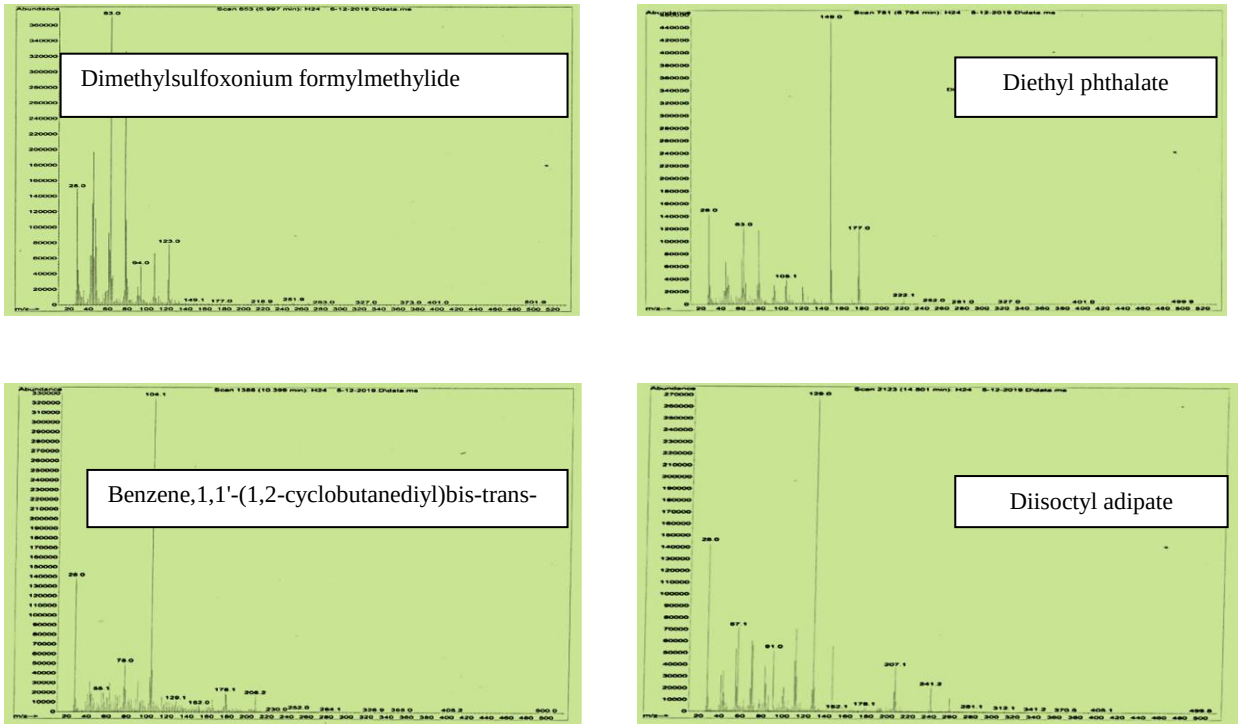

Fig.4a: The compounds of crude aqueous methanolic extractdetectedfrom leaves of*Eucalyptus* sp.by GC-MS technique.

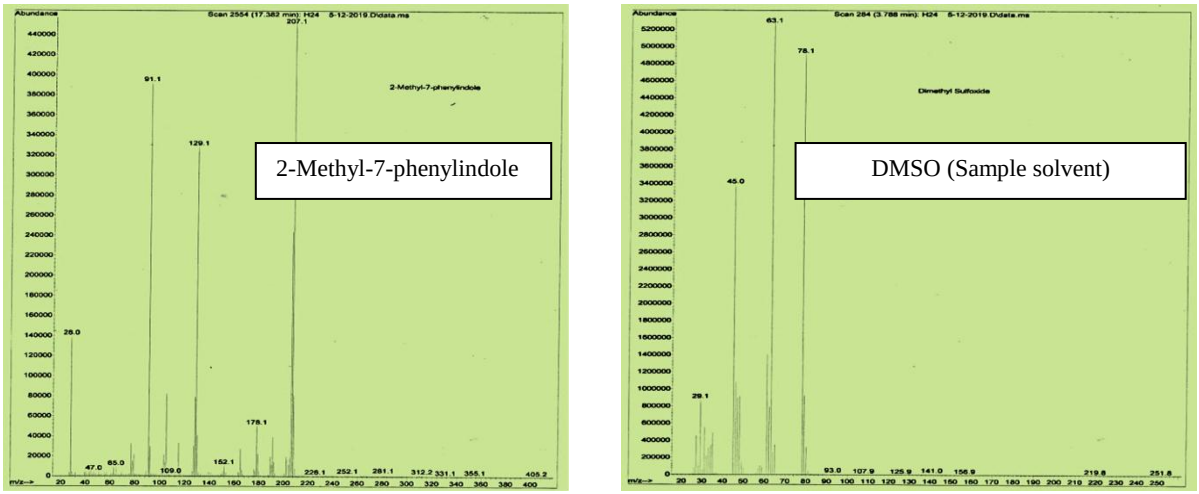

Fig.4b: The compounds of crude aqueous methanolic extractdetectedfrom leaves of*Eucalyptus* sp. by GC-MS technique.

Discussion

The selecting solventsrepresenta critical step for extraction because each plant constituentpossesses an affinity for special solvents.Of these solvents,petroleum ether is used to extract the fixed and essential oils[23].Accordingly, our study used petroleum ether as a defatting solvent by which the powder of*Eucalyptus* sp. leaves soaked in the solvent.Other investigators showed that adding water into the polar solvent will increase the polarity. Most bioactive compounds of plant matrices are medium-size molecules due to presenting aromatic delocalized π-electrons in the matrices.The compounds are highly polar ones[24, 25].Depending on the mentioned investigators, the present study manipulated powder of*Eucalyptus* sp.leaves througha mixture of distilled water and absolute methanol, (20: 80) in order to extract the polar compounds of the plant powder. The aqueous methanolic (80%) extract revealed the antimicrobial effects on the tested pathogenic microorganisms compared with control disc of tetracycline (Table 2) and (Figures: 2 and 3). Researchers noticed that the crude methanolic extract of *Eucalyptuscamaldulensis*contains the tannins and saponins whichinhibitedsome bacteria, including *S.aureus* and *E.coli*[4]. The extracts of Myrtaceae (family of*Eucalyptus* sp.) contain phytochemical compounds such asglycosides, tannins, saponins, phenols can give the antimicrobial effects against microorganisms[26-28]. The current study resulted in the crude aqueous methanolic (80%) extract containedglycosides, phenols,saponins tannins, etc. (Table 3).Aqueous methanolic extract of our studyinhibited the tested microbial pathogens. The inhibition may be attributed tothe presence of these compounds in the extract. Diethyl phthalate (DEP) is known as a colorlessliquid whichhas a slight aromatic odor. It is used in the various application such assprays of insecticides, medical treatment tubing, as an ingredient in coatings of aspirin, cosmetics, food and pharmaceutical packaging, preparation of skin careetc.[29-32].Through using GC-MS, dimethylsulfoxoniumformylmethylide was identified from leaf aqueous methanol extract of *Mundulea sericea* which exertedresults of antioxidant activity[33].

Benzene,1,1'-(1,2-cyclobutanediyl)bis-trans- was identified by GC-MS in the flavonoids from leaf methanolic extract of *Rumex vesicarius*[34].Also, 2-methyl-7-phenylindole was identified from ethanolic root extract of *Plumbago zeylanica* that the extract exhibited antimicrobial activity[35].Diisoctyladipate detected a compound in the extract of *Pleiospermium alatum*. The compound had no antimicrobial effects[36].Generally, our results of GC-MS analysis (Table 4) and (Figures: 4a and 4b) agreed with these studies which showedthat the aqueous methanol (80%) extract of *Eucalyptus* sp.leavescontained the compounds have the antimicrobial with antioxidant activity.

ConclusionThe aqueous methanol (80%) extract of*Eucalyptus* sp.leaves contains the antimicrobial compounds which may be used as medicines.

Recommendation The crude aqueous methanol (80%) extract of*Eucalyptus* sp.leavesis necessary to separate its compounds as pureones in order to test them separately *in vitro* and *in vivo* for evaluation of their antimicrobial activity and toxicity.Also, they are very needed to determine their chemical structure by using techniques of spectrometry analyses.Finally, the bioactive compounds are able to be tested via volunteers so that they are drugs as antibiotics in the hospitals. **Acknowledgment** We are grateful for our country, Iraq, and friends for supporting us to perform this paper by which we hope to be a simple step for serving life.

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الفعالية المايكروبية وتحليل كروماتوغرافيا الغاز – الطيف الكتلي، خارج جسم الكائن الحي، لمستخلص الميثانول المائي الخام الذي تم الحصول عليه من اوراق نبات الـ*Eucalyptus* species.

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المستخلص

تمثل النباتات احد المصادر المهمة للحصول على المنتجات ذات الاستخدام المفيد بما في ذلك المضادات المايكروبية. ان الدراسة الحالية اظهرت فعالية ضد مايكروبية ابداها مستخلص الميثانول المائي الخام بتركيز 40000 مايكروغرام\ مل تم الحصول عليه من اوراق نبات الـ*Eucalyptus* sp.ضد جراثيم الـ *Staphylococcus aureus*،*Streptococcusmutans*، *Escherichiacoli* و .*Klebsiella*sp فضلا عن خميرة الـ *Candida albiacns*وبأقطار مناطق تثبيط سجلت بالأرقام ٣٠، ٢٧، ٢٦، ٣٠ و ٣٨ ملم وبالترتيب.الكشوفات الكيميائية اظهرت بان مستخلص الميثانول المائي الخام يحتوي على المواد Alkaloids، Glycosides، Flavonoids، Tannins و Saponins بالإضافة الى تحليل الـ GC-MS الذي كشف عن احتواء المستخلص النباتي الخام على المركبات dimethylsulfoxonium formylmethylide, diethylphthalate, benzene,1,1'-(1,2-cyclobutanediyl)bis-trans-,diisoctyladipate, and 2-methyl-7-phenylindole.ان الاستنتاج من هذه الدراسة توضح في وجود مركبات فعالة ضد الممرضات المجهرية ما يشير الى وجوب فصلها كل على حده وبشكل نقي باستخدام تقنيات الكروماتوغرافيا لاختبار تلك المركبات النقية خارج وداخل الجسم ومن ثم تشخيص تركيبها الكيميائي كي يكون بالإمكان استخدامها كأدوية لمعالجة الامراض في المستشفيات.

الكلمات المفتاحية: اوراق نبات الـ*Eucalyptus*sp.، الفعالية المايكروبية، تحليل كروماتوغرافيا الغاز مع الطيف الكتلي.