



***In Vitro* Antimicrobial Activity of Aqueous-Methanolic Extract of *Populus* sp. Leaves**

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Abstract: Plants are a rich source of giving benefit natural products, including antimicrobial agents. The current study was designed to evaluate the antimicrobial activity of *Populus* sp. leaves that the aqueous methanolic extract (200 mg.ml⁻¹) of the leaves revealed antimicrobial effects against some microbial pathogens in which the highest inhibition zone was recorded against *Candida albicans* followed by *Staphylococcus aureus* but no effects on the growth of both *Streptococcus mutans* and *Klebsiella* sp. The chemical tests appeared that the extract contains sterols, terpenoids, carbohydrates, glycosides, flavonoids, tannins, proteins, amino acids, and saponins glycosides while alkaloids were not detected. GC-MS analysis detected the aqueous methanolic extract has four compounds are {2-Pyridineacetaldehyde,[2-(2-pyridinyl) ethylidene]hydrazone}, {n-Propylamine, N-acetyl-3-[2-acetyl-3,4,5-trimethoxyphenyl]-}, {1-(Methyl propyl)-4-(1',1',2'-trichloro-3'-ethyl allyl)benzene} and {1H-Indole, 5-methyl-2-phenyl-}.

Keywords: *Populus* sp. leaves, Methanolic Extract, Antimicrobial Activity, GC-MS Analysis.

Introduction

Medicinal plants are one of the living sources that possess the ability to produce the agents which inhibit the microbial pathogens and cancer cells. (Wolfender *et al.*, 2011; Newman & Cragg, 2012). The genus of *Populus* represents the deciduous trees of the angiosperms, Salicaceae family, (Ferreira *et al.*, 2009). *P. euphratica* is commonly known as Euphrates poplar, Salt poplar, and Firat poplar. Ecologically, the species grows along

riversides during summertime especially in the environments that are characterized by salinity (Ferreira *et al.*, 2006; Han *et al.*, 2013). Another species of the genus is *P. nigra* L. has ecological and economic importance (Ballian *et al.*, 2006).

Species of the genus *Populus* were studied to extract the natural products which have antimicrobial and antioxidant activities. The

extracts of *P. euphratica* leaves gave antimicrobial effects on the growth of some bacteria (Badi *et al.*, 2016). In Iraq, researchers extracted polyphenolic compounds from the leaves of that species (Kredy *et al.*, 2015). Native peoples in North America and Eurasia used *P. tremuloides* to be as traditional medicines for treating their diseases such as venereal diseases and arthritis (Royer *et al.*, 2012; St-Pierre *et al.*, 2018). Other investigators noted that the extracts of *P. alba* had antibacterial and antioxidant effects (Al-Hussaini & Mahasneh, 2011; Boudkhili *et al.*, 2012; Haouat *et al.*, 2013).

The present study was conducted to get two aims. The first aim was to evaluate the antimicrobial activity of the crude aqueous methanolic extract from *Populus* sp. leaves while the second was to detect the constituents of the extract by using chemical tests and GC-MS analysis.

Material and Methods

Preparation of Sample and Its Extraction

Healthy leaves of *Populus* sp. (Fig. 1), growing in Thi-Qar Province, Iraq, were collected on August 2018. The leaves were



Fig. (1): Healthy leaves of *Populus* sp. from Thi-Qar Province-Iraq.

Antimicrobial Activity of Aqueous Methanolic Extract

Amount of the aqueous methanolic extract was dissolved in the dimethyl sulfoxide (DMSO) for getting a concentration was 200 mg.ml⁻¹. Petri dishes of both Muller Hinton

washed with tap water, then by distilled water, and left at room temperature for 3 days until they dried. By using a small mill, the dried leaves were pulverized and grinded to be a powder. 200 g of the powder were placed in a clean glass beaker (1000 ml-size), and 700 ml of petroleum ether were poured on the powder to be soaked. Through using two layers of parafilm, the beaker was strictly covered and left at the room temperature for 4 days. The soaked powder was separated from the filtrate by a filter paper containing 0.5 g of activated charcoal as a film to remove the chlorophyll pigment. At the room temperature, the filtrate and powder were separately left until petroleum ether evaporated completely for obtaining the powder had no petroleum ether. At the same conditions of petroleum ether method except for time period, the same powder (powder had no petroleum ether) was soaked with 700 ml of aqueous methanol (20 ml of distilled water: 80 ml of absolute methanol) for 7 days. The filtrate was separated from the soaked powder and evaporated like a filtrate of petroleum ether for giving sticky extract that was tested against some microbial pathogens.

agar (MHA) and potato dextrose agar (PDA) were prepared. The MHA dishes were used to test the bacteria while the dishes of PDA were prepared for testing *Candida albicans*. Each dish of both MHA and PDA was inoculated with 100μL of 1.5 x 10⁹ cell.ml⁻¹ from the microbial suspension. The inoculum was

spread on the dish by using a sterile cotton swab. After drying the dishes, well (7 mm in size) was done in each dish. 100 µL of the concentration was placed in the well and incubated at 37 °C for 2 days until the zones of inhibition were given around the tested microorganisms. *C. albicans* incubated at both 25 and 37 °C. Also, a disc contained 30 µg/ml of the pure tetracycline was used a control agent against bacteria.

Chemical Tests of Aqueous Methanolic Extract

The chemical tests were performed according to (Chang *et al.*, 2002; Shah and Seth, 2010; Sabri *et al.*, 2012; Gupta *et al.*, 2013; Rahman *et al.*, 2013) and as follow:

Detection of Sterol and Terpenoids

1-Salkowski's Test: 2-3 drops of the plant extract solution were treated with 2 ml of chloroform, then filtered to obtain a filtrate. Without shaking, few drops of concentrated sulphuric acid were carefully added on the side of a test tube containing the filtrate. If the reddish brown color of the interface appears, it indicates the presenting terpenoids while the appearing golden yellow color indicates the presenting triterpenes.

2-Libermann Burchard's Test: 2 ml of the plant extract solution were treated with chloroform, then filtered to obtain a filtrate. Few drops of acetic anhydride were added into the filtrate which boiled and cooled. Then few drops of concentrated sulphuric acid were carefully added on the sides of the test tube. If brown ring at the junction forms, it indicates the presenting phytosterols (however; the color begins to appear red, then blue and finally green color indicates the presence of sterols).

3- Copper Acetate Test: 2-3 ml of plant extract solution were treated with few drops of 5% copper acetate solution. If emerald green color appears, it indicates the presenting diterpenes.

Detection of Alkaloids

1-Wagner's Test: 2-3 ml of the plant extract solution were treated with 2 ml of Wagner's reagent (Iodine in potassium iodide). If

brown/reddish brown precipitate forms, it indicates the presenting alkaloids.

2- Dragendroff's Test: Acidifying 2-3 ml of plant extract solution with 1 drop of sulfuric acid. The acidified extract was treated with 0.5 ml of Dragendroff's reagent (solution of potassium bismuth iodide). If red precipitate forms, it indicates the presenting alkaloids.

Detection of Carbohydrates

1-Molisch's Test: 2-3 ml of the plant extract solution were treated with few drops of 10% alcoholic – α – naphthol solution (2-4 drops), then about 2 ml of concentrated sulphuric acid were carefully added to the side of the test tube. If purple color appears, it indicates presenting carbohydrates.

2- Benedict's Test: 2-3 ml of the plant extract solution were treated with 0.5-1 ml of commercial Benedict's reagent. The mixture was boiled by using a water bath for 4-10 minutes, then cooled. If a precipitate of reddish brown color forms, it indicates the presenting carbohydrates while orange-red precipitate indicates the presenting reducing sugars.

Detection of Glycosides

1- General Test: 5 ml of the plant extract solution were treated with few drops of 10% aqueous NaOH solution. If yellow color develops, it indicates the presenting glycoside.

2-Raymond's test: 3 ml of the plant extract solution were treated with hot methanolic alkali (sodium hydroxide). If a violet color appears, it indicates the presenting glycoside.

3- 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 extract solution. Then, adding 1 ml of concentrated H₂SO₄ along the side of the test tube carefully. If a reddish-brown ring at the interface forms, it indicates the presenting deoxysugar of cardenolides. Appearing violet ring may form beneath the brown ring, while in the acetic acid layer, a greenish ring may also form just gradually throughout the layer.

4- Legal Test: Acidifying 2 ml of the plant extract solution with 1 drop of concentrated HCl, then treatment with 1 ml sodium nitroprusside in 1 ml pyridine and methanolic alkali. If pink to bloody color appears, it indicates the presenting cardiac glycosides.

Detection of Coumarin Glycosides, Phenolic Compounds, Flavonoids, and Tannins

1- Alkaline Reagent Test: 2-3 ml of the plant extract solution were treated with few drops of 20% sodium hydroxide solution. If intense yellow color appears (that turns to colorless by adding few drops of dilute acetic acid), it indicates the presenting flavonoids.

2-Lead Acetate Test: 2 ml of the plant extract solution were treated with few drops of 10% lead acetate solution. If white precipitate forms, it indicates the presenting phenolic compounds.

3-Ferric Chloride Test: about 1 ml of the plant extract solution was added to 2 ml of water by using a test tube. Adding 2-3 drops of diluted 5% ferric chloride solution. If green – bluish green appears, it indicates Catechic tannins while blue-black indicates Gallic tannins.

4-Gelatin Test: 2-3 ml of the plant extract solution were added to a 1% gelatin solution containing sodium chloride (1%). If white precipitate forms, it indicates the presenting tannins.

Detection of Proteins and Amino Acids

1-Xanthoproteic Test: 3 ml of the plant extract solution were treated with few drops of concentrated nitric acid. If yellow color appears, it indicates the presenting proteins.

2-Ninhydrin Test: 3 ml of the plant extract solution were treated with few drops of 2% (w/v) ethanolic ninhydrin reagent, and boiled for 5-10 minutes. If a blue color appears, it indicates the presenting amino acid.

3-Biuret Test: 3 ml of the plant extract solution were treated with 1 ml of 10% sodium hydroxide solution and heated, then a drop of 0.7% copper sulfate solution was

added. If purplish violet color appears, it indicates the presenting proteins.

Detection of Saponine Glycosides

1- Foam Test: 1 ml of the plant extract solution was added to 2-3 ml of distilled water. The mixture was shaken vigorously. If foam forms (persists for 10 minutes), it indicates the presenting saponins.

2-Froth Test: Diluting 1 ml of the plant extract solution with distilled water to get 20 ml. The diluted solution of the extract was **shook** by a graduated cylinder for 15 minutes. If 1 cm layer of foam forms (persists for 15 minutes), it indicates the presenting saponins.

Gas Chromatography-Mass Spectrometry Analysis (GC-MS) of Aqueous Methanolic Extract

Dissolving an amount of the crude aqueous methanolic (80%) extract in DMSO, and filtered by using μ M 0.45 filter syringe (Millipore). The filtrate was subjected to be analyzed by gas chromatography-mass spectrometry, MSDCHEM\1\METHODS\MUAFQA.M to determine the negative ions(m/z) by which the used column was characterized by HP-5MS, 5% Phenyl methyl Sillox(1629.5), 30m \times 0.250 μ m I.D. \times 0.25 μ m, SS., Inlet He, then applying the parameters showing in (Table 1).

Preliminary Assay of Cytotoxicity Against Red Blood Cells (RBCs)

The assay of the extract against human red blood cell (RBCs) was performed according to Nair *et al.*, (1989). 1 ml of human blood (O+) was mixed with 20 ml of normal saline for preparing a blood solution. Three plastic tubes were used that each tube contained 2 ml of the solution. Two of them were used as control tubes where one tube was negative control contained 2 ml of the blood solution only while the second one was positive control had 100 μ L of DMSO mixed with the 2 ml of the solution. The third tube contained blood solution and 100 μ L of 200 mg.ml⁻¹ obtained from an aqueous methanolic extract.

Table (1): Used parameters of GC-MS to detect the compounds within aqueous methanolic (80%) extract from leaves of *Populus* sp.

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	90-97%
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

All tubes were incubated at room temperature for 1 hr. If the color of blood solution changed to be pale color compared with control tubes, it indicates the hemolysis due to a concentration of the extract.

Statistical analysis

The statistical analysis was performed by using program of Graph Pad Prism 5 and One- way ANOVA.

Results

Antimicrobial Activity of Aqueous Methanolic Extract

The aqueous methanolic extract (200 mg.ml⁻¹) had antimicrobial activity against a growth of *C. albicans* followed by *S. aureus*, then *E. coli*, while the extract could not inhibit the growth of both *S. mutans* and *Klebsiella* sp. The antibacterial activity of the extract was compared with a disc contained 30 µg/ml of the pure tetracycline. Statistical analysis showed significant differences among tested microorganisms (Table 2, Figs. 2 & 3).

Table (2): Antimicrobial activity of aqueous methanolic extract obtained from *Populus* sp. leaves.

Inhibition Zones (IZ) Measured by Milliliter (mm)					
	<i>S. aureus</i>	<i>S. mutans</i>	<i>E. coli</i>	<i>Klebsiella</i> sp.	<i>C. albicans</i>
Ext.	10	0	7	0	20
Tet.	36	36	0	5	----

Ext.: 200 mg.ml⁻¹ aqueous methanolic extract. Tet.: disc contained 30 µg/ml of the pure tetracycline.

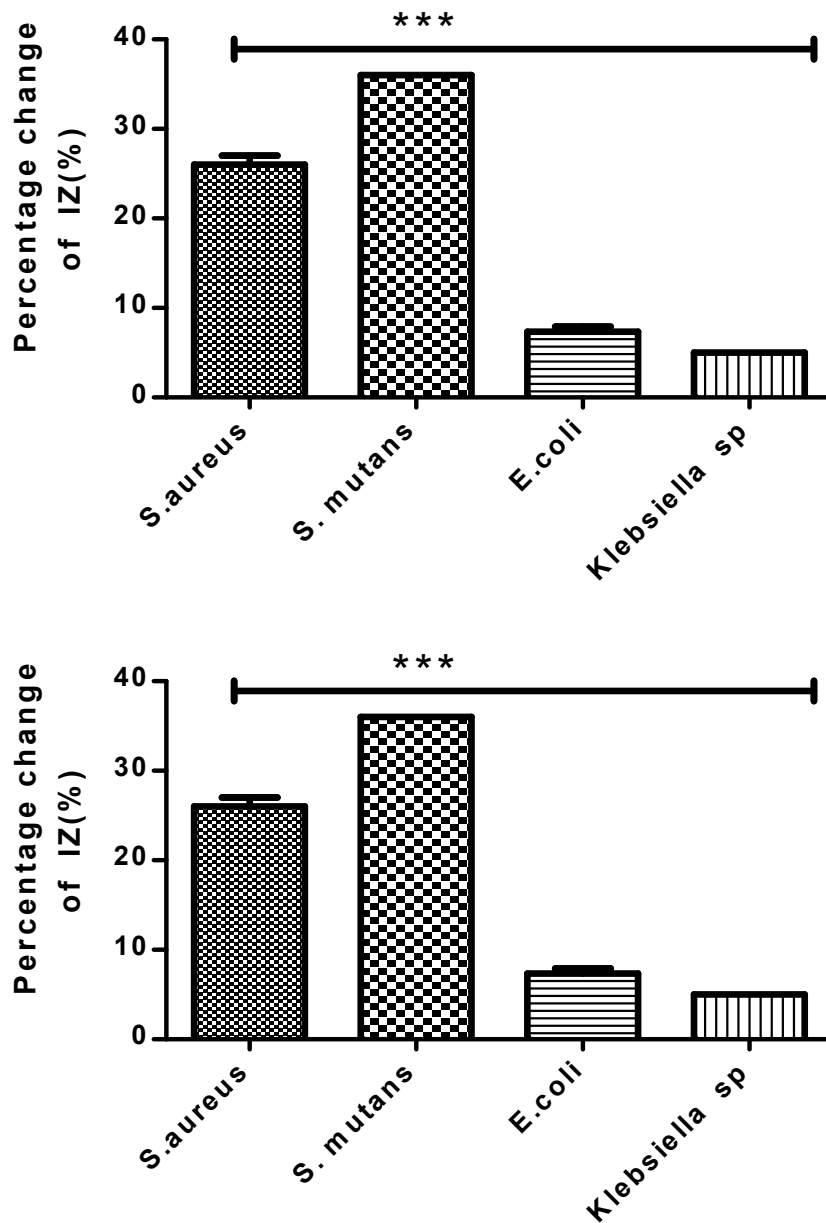


Fig. (2): There were significant differences among tested microorganisms. One-way ANOVA was used, and application of Graph Pad Prism 5 program. One or more stars indicate to present the significant results. IZ: Inhibition zone.



Fig. (3): Antimicrobial activity of aqueous methanolic extract from *Populus* sp. leaves. The used concentration was 200 mg.ml⁻¹. (A): Against *C. albicans*. (B): Against *S. aureus* compared with a disc of tetracycline (30 µg/ml).

Chemical Tests of Aqueous Methanolic Extract

The chemical tests resulted in the various compounds within the aqueous methanolic extract of *Populus* sp. leaves. These compounds are sterols, terpenoids, carbohydrates, glycosides, flavonoids,

tannins, proteins, amino acids, and saponins glycosides but alkaloids were not detected in the extract (Table 3). Relating to the chemical tests, the solid extract of petroleum ether filtrate obtained from the leaves of *Populus* sp. contains sterols and terpenoids besides alkaloids.

Table (3): Compounds of aqueous methanolic and petroleum ether filtrate extracts of *Populus* sp. leaves were detected by chemical tests.

Type of Test	Methanolic Extract	Petroleum Ether Extract
Sterol /Terpenoids		
Salkawski's test	-ve	+ve
Liebermann's test	+ve	+ve
Copper acetate test	+ve (diterpene)	
Alkaloids		
Wagner's test	-ve	+ve
Dragendroff's test	-ve	-ve
Carbohydrates		
Molisch test	+ve	-----
Benedict test	+ve	-----
Glycosides		
General alkaline test	+ve	-----
Raymond's test	+ve	-----
Keller Kiliani test	+ve (cardiac glycosides)	-----
Legal test	+ve	-----
Coumarin Glycosides		
Alkaline test	+ve	-----
Phenolic Compounds		
Flavonoids		
Alkaline test	+ve	-----
Lead acetate test	+ve	-----
Ferric chloride test	+ve	-----
Tannins		
Ferric chloride test	+ve (catechol tannins)	-----
Lead acetate test	+ve	-----
Gelatin test	-ve	-----
Proteins and Amino Acids		
Xanthoproteic test	+ve	-----
Ninhydrin test	-ve	-----
Biuret test	+ve	-----
Saponine Glycosides		
Foam test	+ve	-----
Forth test	+ve	-----

+ve: Positive result. -ve: Negative result. -----: The extract could not dissolve in the solution of the test.

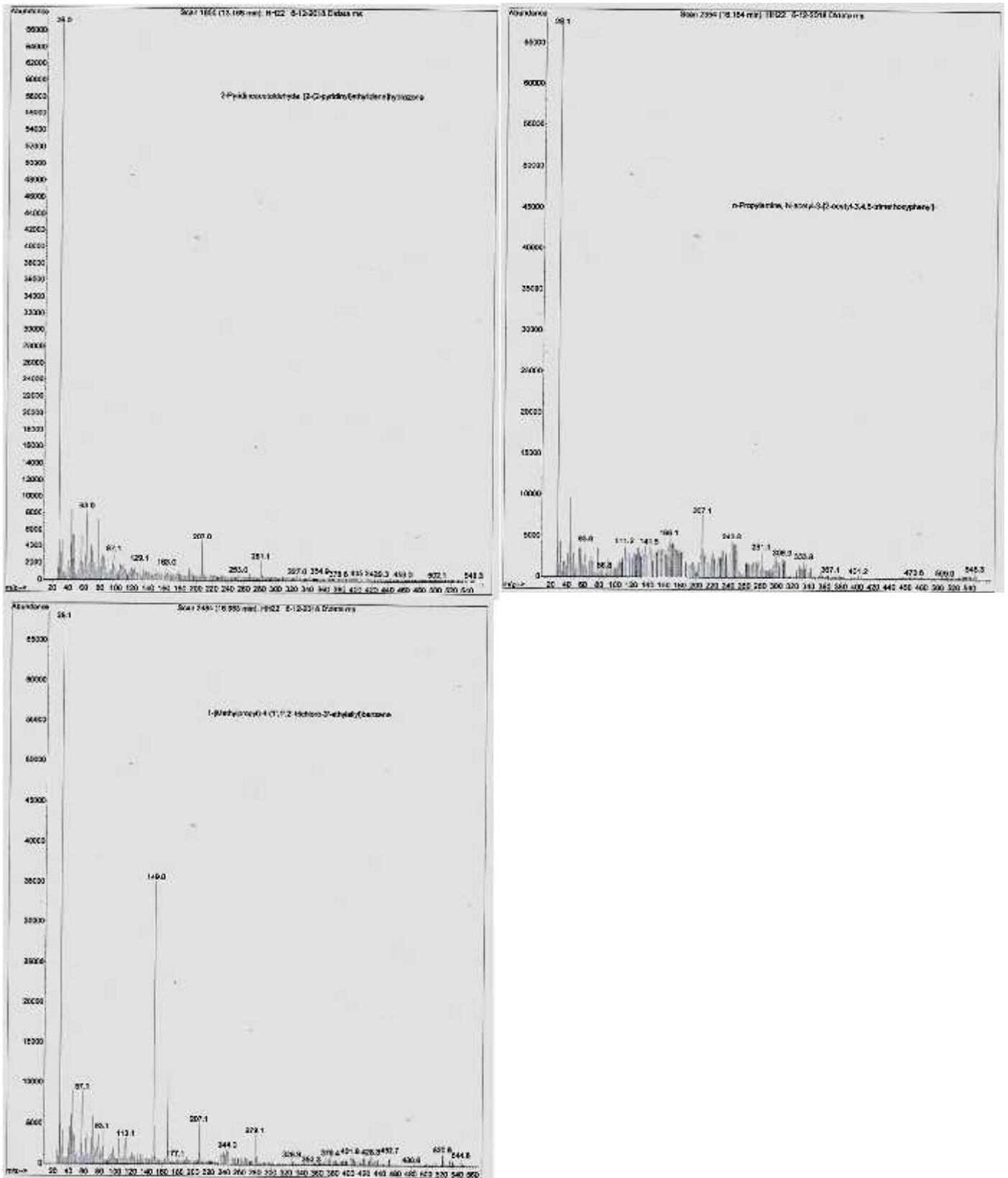


Fig. (4a): The compounds of aqueous methanolic extract produced from leaves of *Populus* sp. detected by GC-MS analysis.

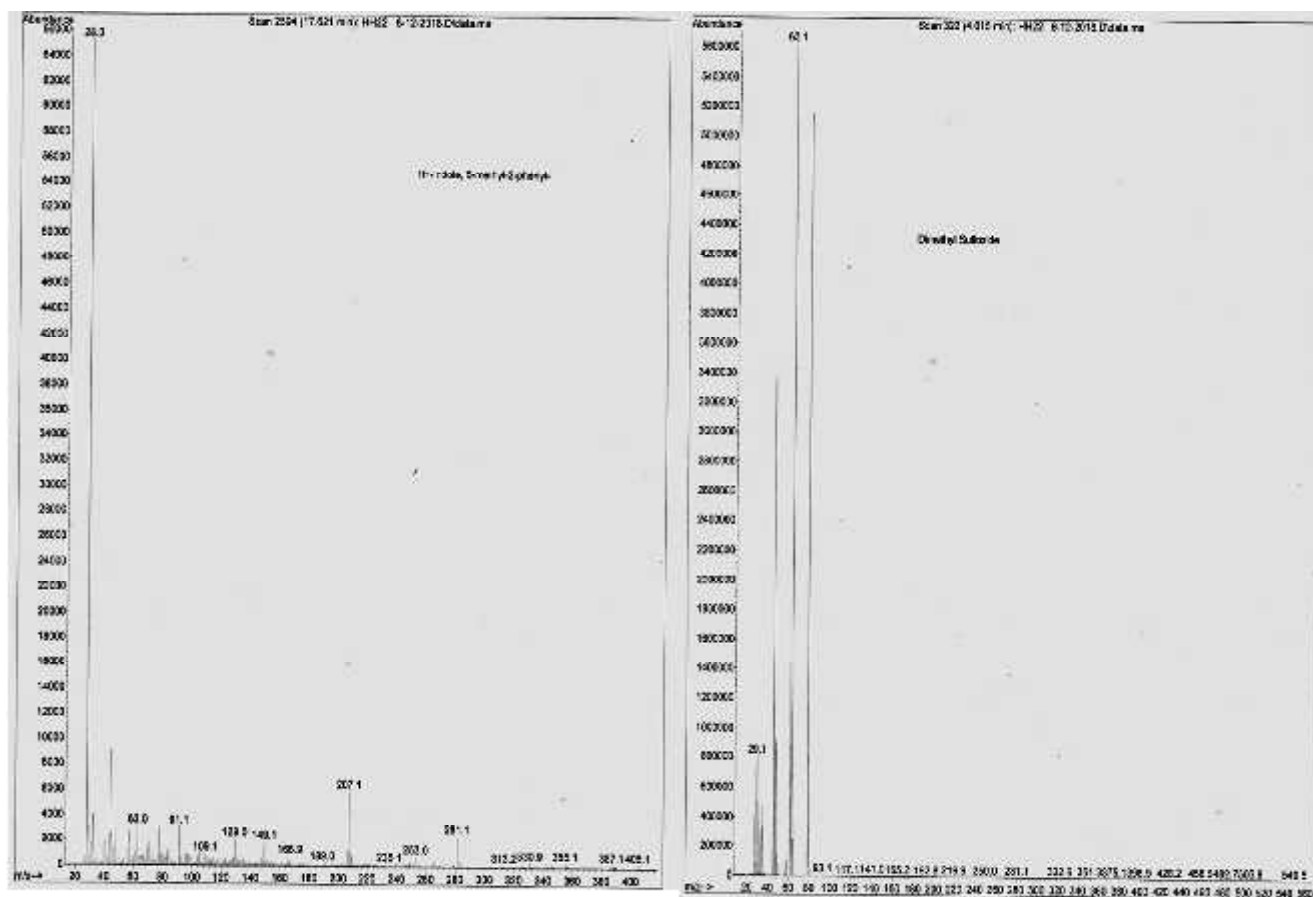


Fig. (4b): The compounds of aqueous methanolic extract produced from leaves of *Populus* sp. detected by GC-MS analysis. DMSO was detected as a sample solvent.

Extract

The GC-MS analysis detected four compounds in the aqueous methanolic extract obtained from *Populus* sp. leaves. Also, the analysis of GC-MS detected DMSO as the

sample solvent by which the extract dissolved. The retention times of the compounds were recorded 4.015- 17.621 minutes (Table 4, Figs. 4a & 4b).

Table (4): The compounds of the aqueous methanolic extract obtained from leaves of *Populus* sp. detected by GC-MS analysis. (RT: Retention time by the minute).

	Compounds	RT (min.)
1	2-Pyridineacetaldehyde,[2-(2-pyridinyl) ethylidene] hydrazone	13.166
2	n-Propylamine, N-acetyl-3-[2-acetyl-3,4,5-trimethoxyphenyl]-propyl)-4-(1',1',2'-trichloro-3'-ethylallyl)benzene 1-(Methyl	16.184
3		16.963
4	1H-Indole, 5-methyl-2-phenyl-	17.621
5	Dimethyl Sulfoxide, DMSO, (Sample solvent)	4.015

Preliminary Assay of Cytotoxicity Against Red Blood Cells

The used concentration of the aqueous methanolic extract did not give any change in the color of the human blood solution in Comparison with the control tubes during 1h

of the testing time period but the same concentration changed the color of blood solution into slightly blackish red after 24 hours of the testing time (Fig. 5).

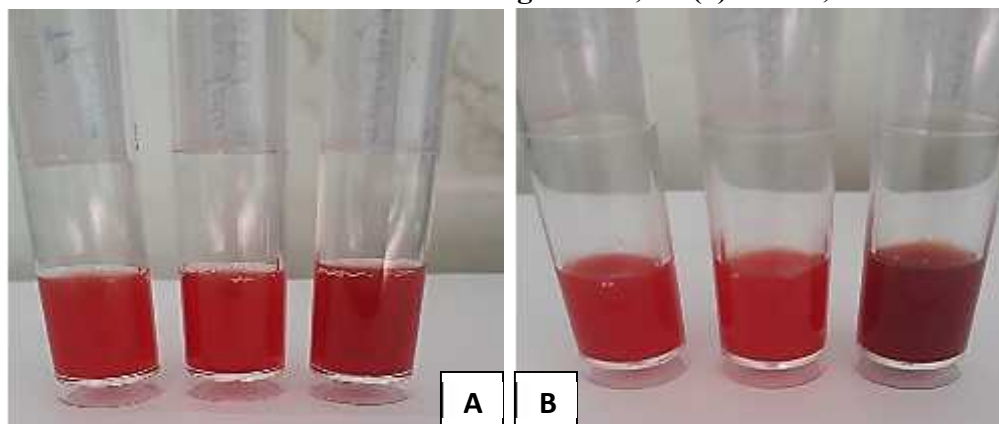


Fig. (5): Preliminary assay of the aqueous methanolic extract against RBCs cytotoxicity. From left to right: The first tube represented negative control tube containing blood solution only (blood mixed with normal saline). The second tube was positive control tube containing 100 μL of DMSO mixed with blood solution. The third tube contained 100 μL of 200 $\text{mg}\cdot\text{ml}^{-1}$ from the extract. A: After 1 hr. of the testing time period. B: After 24 hrs. of the testing time period.

Discussion

Selection of the solvents represents an important and critical step for the extraction because the compounds within the extracts of plants differ in their affinity for the solvents. Petroleum ether is used to extract the fixed and essential oils, as well as sterones (Saroya, 2011). The addition of a polar solvent to the polar one will increase the polarity of the solvents. The plant matrices contain the bioactive compounds which are medium-size molecules due to their aromatic delocalized π -electrons. This phenomenon indicates that the matrices are highly polar which need to be extracted by polar solvents (Andri *et al.*, 2009; Kumoroa *et al.*, 2009; Altemimi *et al.*, 2017; St-Pierre *et al.*, 2018). For these reasons, our study used two polar solvents were a mixture of absolute methanol and distilled water (80:20) for extraction the bioactive compounds from leaves of *Populus* sp. while the petroleum ether was used before aqueous methanol (80%) in order to remove the fixed and essential oils besides sterones from the plant pulverized leaves (Table 3).

The plant extracts contain the bioactive compounds such as glycosides, tannins, saponins, phenols which inhibit the growth of microorganisms (Ahmad *et al.*, 1998; Pamplona-Roger, 1999; Shariff, 2001). The effects of these compounds varied according to susceptibility and strains of bacteria (Karou *et al.*, 2006). Based on the mentioned

researchers, our results may attribute why the crude extract of aqueous methanol exhibited the antimicrobial effects on some of the tested microorganisms (Tables 2 & 3, Figs 2 & 3).

Hydrazone-derivatives are able to be the antimicrobial, antitumor, anti-inflammatory, antiplatelet agents. These derivatives resulted in the antimicrobial activity against *S.aureus*, and *Mycobacterium tuberculosis* (Küçükgülzel *et al.*, 2002; Rollas *et al.*, 2002; Kaymakçioğlu *et al.*, 2006). Other scientists showed that indole derivatives such as 4-(5-methyl-3-phenyl 1H-indole) inhibited the microbial pathogens such as *S. aureus* and *C. albicans* (Yukseket *et al.*, 2013). Our study showed that GC-MS detected derivatives of both hydrazine indole in the aqueous methanolic extract (Table 4, Figs 4a & 4b). The GC-MS analysis may explain why the aqueous methanolic extract inhibited some tested microorganisms. It may be thought that the microbial inhibition was due to derivatives of both hydrazine indole within the extract.

Conclusions

The aqueous methanolic extract of *Populus* sp. leaves has antimicrobial compounds which very needs to be separated by using techniques of chromatography to extract them as the pure compounds. *In vitro* and *in vivo*, the purified compounds can be tested for evaluation of their half lethal concentrations

(LC₅₀) and half lethal dose (LD₅₀). Finally, the elucidating chemical structure of these compounds can be given by using techniques of spectrometry such as NMR, GC-MS etc. After applying these biological and chemical analyses, the identified pure compounds are tested on volunteers in order to apply them as drugs for treating microbial diseases in hospitals.

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