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Antimicrobial properties of *Hyssopus officinalis* extract against antibiotic-resistant bacteria in planktonic and biofilm form

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Abstract

Introduction: Extensive use of antibiotics led to the development of bacterial resistant to antibiotics. Medicinal plants can be alternative choice for antibiotics. The plant (*Hyssopus officinalis*) belongs to *Lamiaceae* family recently were attracted as a source for antimicrobial agents. The aim of this study was to evaluate the antibacterial and inhibitory activity of *H. officinalis* extract on the growth of six antibiotic-resistant bacteria.

Materials and methods**:** In this study, ethanolic and methanolic extracts of *H. officinalis* were prepared. Antibacterial activity of the ethanolic and methanolic extracts was evaluated by paper disc diffusion method. Also, MIC and MBC of these extracts were determined for six pathogenic bacteria. The effect of these extracts on biofilm of bacteria (biofilm formation and destruction) was evaluated by microtiter plate method. The chemical composition of the extract was identified by GC-MS.

Results: The results of study showed the maximum inhibitory effect of these extracts against planktonic forms belong to *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Between all studied bacteria, *Acinetobacter baumannii* showed the greatest sensitivity to *H. officinalis* extracts in Muller Hinton broth (MIC= 3.125 mg ml^{-1}). The highest inhibitory effects of *H. officinalis* ethanolic extract on biofilm formation were observed against *Escherichia coli* (95 %). The results of biofilm destruction showed that *Klebsiella pneumoniae* biofilm had a resistant biofilm structure between all tested bacteria (16.41 %). The GC-MS analysis revealed that five active compounds were present in the extract of this plant.

Discussion and conclusion: The data obtained in this study confirmed that *H.officinalis* extract inhibit growth and biofilm formation of some pathogenic bacteria. It can be proposed for future studies that the compounds of this plant used for design a antimicrobial agent.

Key words: Antimicrobial, Biofilm, Extract, *Hyssopus officinalis*, Pathogenic bacteria

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Introduction

Despite extensive progress in scientific knowledge and medicine, infectious diseases remain a leading cause of worldwide morbidity and mortality. The main chemical agents for eradication of pathogenic fungal and bacteria are antibiotics. Antibiotics were gold key for the treatment of pathogens for long times. However, in recent years, antibiotic resistant bacteria were increased and the use of antibiotics for treatment of pathogenic microbes had less efficiency (1). Bacterial biofilms are complex and vast-structured communities of adhesive microorganisms enclosed in a self-produced extracellular matrix of exopolysaccharide and irreversibly connecting to various surfaces (2, 3). The capability of microorganisms to attach to both biotic and abiotic surfaces and to form biofilm are accountable for a number of diseases of chronic nature, indicate high resistance to antibiotics (4, 5). Accordingly, biofilms can cause important problems in very areas, both in medical settings and in non-medical settings (6, 7).

In recent years, attentions drawn on herbs, because they increase in antibiotic-resistant bacteria and side effect of synthetic antibiotics. Medicinal plants are known for having synthetica diversity of compounds to protect themselves against many of their own pathogens and therefore can serve as antimicrobial materials (8).

Hyssopus officinalis is a flowering plant, which belongs to *Lamiaceae* family (9) and a herb widely growing in Iran (10). This plant has been used in folk medicine for various aims such as anti-inflammatory, anti-bacterial, anti-pyretic, anti-spasmodic, antihypertensive and anti-hyperlipidemic purposes (11). The aim of this research was to evaluate the antibacterial and antibiofilm activity of *H. officinalis*. Also the effective compounds of this plants were determined.

Materials and Methods

Plant samples collection and extraction: The fresh plant of *H. officinalis* was prepared in May, 2015, from Kerman, Iran. The taxonomical identification of the plant was confirmed by a plant taxonomist and the DH9541 code were registered as herbarium code for this plant. The plants dried in air and powdered. Fifty grams of *H. officinalis* powder were soaked in 500 ml of ethanol and methanol (96 %) solvents. Different extracts were prepared using the cold maceration process for 72h at room temperature under constant shaking and filtered with Whatman No.1 filter paper. The residue was further macerated and filtered twice with the same solvent overnight. The filtrates obtained from each extraction were mixed and concentrated under vacuum. The extracts obtained were kept at 4°C for further use (12, 13)

Bacterial strains: Six pathogenic bacteria were used in this study. The Gram-positive bacteria were *Bacillus cereus* (ATCC1298) and *Staphylococcus aureus* (ATCC1189), and Gram-negative bacteria were *Pseudomonas aeruginosa* (ATCC27853), *Escherichia coli* (ATCC35218), *Acinetobacter baumannii* (ATCC1611) and *Klebsiella pneumonia* (ATCC700603).

Disk Diffusion Method: Antimicrobial activity of the ethanolic and methanolic extracts was evaluated by paper disc diffusion method. Saved culture of test bacteria was grown in Notrient Broth (NB) medium at 37 °C for 24 h. Final cell concentrations were adjusted to 10^8 cfu ml^{-1} with reference to the McFarland standard (OD= 0.5). This inoculum 1ml was added on the surface of Mueller-Hinton Agar (MHA, Oxoid) plates by sterile cotton swab. Then, 6 mm paper discs (0.15 mg ml^{-1} of each extract) were placed into each of these concentrations for 1 h. The disc was put for 30 minutes at room temperature and transferred to the medium. Disc solvent-free extract used as negative control. The

inhibition zone around each disc was measured in millimeter and the assay was carried out three times for each extract (14, 15).

MIC and MBC determination: Determination of the Minimal Inhibitory Concentration (MIC) was carried out using the macrobroth dilution method as recommended by the Clinical and Laboratory Standards institution (CLSI). The concentration of bacterial culture was serialy diluted to reach 5×10^5 cfu ml^{-1}. Different concentrations of extract were prepared by dissolving extract stock concentration (100 mg ml^{-1}) in sterile culture medium (NB). Then, 1 ml of standardized inoculum (5×10^5 cfu ml^{-1}) was added to 1 ml of each extract concentration. Then, all tubes were incubated at 37°C for 18 h. MIC was calculated as the lowest concentration able to inhibit bacterial growth. Two controls were used for this experiment: 1) tube with extract and growth medium, and 2) the tube with growth medium and inoculum. Minimal Bactericidal Concentration (MBC) was determined by inoculate negative tubes (tubes that had not any visible growth) into MHA plates and subsequent incubation at 37°C for 24 h. The minimum concentration inhibited colony formation on agar was considered as MBC for the extract (16).

Inhibition of biofilm formation: Biofilm formation in polystyrene microtiter plates was assayed according to O'Toole and Kolter (1998) method with some modifications. Three concentrations of each extract (12.5, 25 and 50 mg ml^{-1}) were pipetted (100 µl) into the wells of the microtiter plates. For biofilm assessment, bacteria were cultured at 37°C overnight and re-suspended at an optical density at 600 nm (OD_{600}) of 0.2 in 1 ml of TSB. Afterwards microtiter plates were incubated for 24 h at 37°C. In this experiment, three controls were used in microplate including: 1) Extract control: this well inoculated with NB medium and extract 2) Negative control: this well inoculated with bacteria without any extract 3) Media control: only NB pour into this well (17).

The formation of biofilm was quantitated by measuring the absorbance of crystal violet stained biofilm at 630 nm and by total viable counts (18). After incubation, the media were aspirated and non-adherent cells were removed by washing the wells three times with sterile phosphate buffer saline (PBS). Adherent microorganisms were fixed by adding 150 µl of methanol 96% for 15 min. The microtiter plates were stained with 200 µl of crystal violet (1%) (Merck, Germany) for 20 min, excess stain rinsed off with running tap water. To measure the absorbance of adherent cells, the crystal violet was re-solubilized with 160 µl of acetic acid glacial 33% (Merck, Germany) and the absorbance was measured with a microtiter plate reader (ELX-800, Biotek, USA) at 630 nm. Percent prevention of biofilm formation was calculated using the ratio between the values of $OD630_{nm}$ wells with and without the extracts.

% inhibition = [(OD negative control-OD media control)-(OD test-OD extract control)] /(OD negative control-OD media control) ×100

Disruption of established biofilm: Disruption of established biofilm structures was measured as described by Sandasi (2008) with some modifications. 100 µl of the standard bacterial culture (OD_{600}=0.2) was transferred into sterile 96-well polystyrene microtitre plates and incubated at 37°C for 24 h. After incubation, the medium was aspirated and the planktonic cells were removed by washing the biofilms three times with sterile PBS. Thereafter, three different concentrations (12.5-50 mg ml^{-1}) of *H. officinalis* extracts were added to each well and plates were then placed back into the 37°C for 24 h. The control wells were the same as described in section 2.5. The percentages of reduction biofilm structures in the presence of different concentrations of

extracts were calculated employing the formula as described in section 2.5.

Assessment of biofilm metabolic activity: The effect of the *H. officinalis* upon the metabolic activity of pre-formed biofilm was measured according to Ramage and Lopez-Ribot (2005). Briefly, pre-formed biofilms were washed twice with PBS, extracts (12.5-50 mg ml^{-1}) were added to each well and plates were incubated for 24 h at 37◦C. Afterwards, 50 µl of a Triphenyl Tetrazolium Chloride (TTC, Merck, Germany) solution was added to each well and allowed the reaction to occur in the dark at 37°C for 3 h. Final absorbance was read at 490 nm using microtiter plate reader. The percentages of reduction of biofilm metabolic activity in the presence of different concentrations of extracts were calculated by comparing the absorbance of control bacteria and treated inoculation with each extract (19).

Gas Chromatography–Mass Spectrometry (GC/MS): The chemical compound of *H. officinalis* were determined by GC-MS. The chromatograph (Agilent 6890 UK) was equipped with an HP-5MS capillary column (30 × 0.25 mm ID × 0.25 mm film thickness) and the data were taken under the following conditions: initial temperature 50°C, temperature ramp 5°C/min, 240°C/min to 300°C (holding for 3 min), and injector temperature at 290°C. The carrier gas was helium and the split ratio was 0.8 ml/min. For confirmation of analysis results, essential oil was also analyzed by GC/MS (Agilent 6890 gas chromatograph equipped with an Agilent 5973 mass-selective detector; Agilent UK) and the same capillary column and analytical conditions as above. The MS was run in electron ionization mode with ionization energy of 70 eV (20).

Statistical analysis: Differences for individual parameters between control and treated groups were tested by Duncan analysis of variance (ANOVA) using SPSS version 18.0. Differences were considered significant if the *P* value was less than 0.05, 0.01 and 0.001. All experiments were performed in triplicate and repeated three times.

Results

The inhibitory effects of *H. officinalis* xtracts against planktonic forms of bacteria: The zone of inhibition (ZOI) for methanolic and ethanolic extracts of *H. officinalis* were shown in Table (1), also the MIC and MBC of these extracts were illustrated in this table. The big and small ZOI for methanolic and ethanolic extracts related to *S. aureus* and *P. aeruginosa*, respectively.

The inhibitory effects of *H. officinalis* extracts in broth medium (MHB) were more than solid medium (MHA). This result can be expected because the concentration of extract used in solid medium (paper disks) were lower than broth medium (3.125-50 mg ml-1). Between all pathogenic bacteria, *A. baumannii* show the greatest sensitivity to *H. officinalis* extracts in broth medium (MIC=3.125 mg ml^{-1}). Among six pathogenic bacteria studied in this study the maximum MIC (25 mg ml^{-1}) belonged to *K.pneumonia*.

Table 1- The antimicrobial effect of *H. officinalis* against six planktonic bacteria and MIC/MBC values

Bacteria	Disk Diffusion		MIC		MBC	
	Methanolic extract (mm)	Ethanolic extract (mm)	Methanolic extract (mg/ml)	Ethanolic extract (mg/ml)	Methanolic extract (mg/ml)	Ethanolic extract (mg/ml)
S. aureus	20±0.8	18±0.3	0	6.25	50	25
B. cereus	12±0.4	10±0.6	0	6.25	12.5	25
p. aeruginosa	8±0.7	7±0.2	12.5	12.5	25	50
A. baumannii	13±0.5	8±0.1	3.125	12.5	25	25
E. coli	12±0.2	15±0.3	0	25	6.25	50
K pneumoniae	12±0.9	13±0.2	25	25	0	50

The inhibitory effects of *H. officinalis* extracts against biofilm structures: The efficacy of each concentrations of H. officinalis extract to inhibit biofilm formation, demolished of biofilm, and metabolic activity were shown in Figures (1) and (2). The maximum inhibitory effects of *H. officinalis* ethanolic extract on biofilm formation were observed on *E. coli* (95 %), although methanolic extract had low efficiency to inhibit biofilm formation of *B. cereus* (28.03%) (Fig1-a).

The eradication of bacterial biofilm treated with different concentrations of *H. officinalis* extracts were varied. The *P. aeruginosa* biofilm was susceptible (95.4 %), Between six pathogenic bacteria that studied in this research *K. pneumoniae* had the minimum sensitivity to inhibitory effect of *H. officinalis* (Fig.1-b).

The effect of *H. officinalis* extracts on inhibition of dehydrogenase enzyme of pathogenic bacteria were illustrated in Figure (2). As shown in this figure metabolic activity of bacteria in biofilm dramatically decreased, the maximum reduction observed in *P. aeruginosa* (OD=0.7) and minimum reduction were recorded for *K. pneumoniae* (OD=2.1).

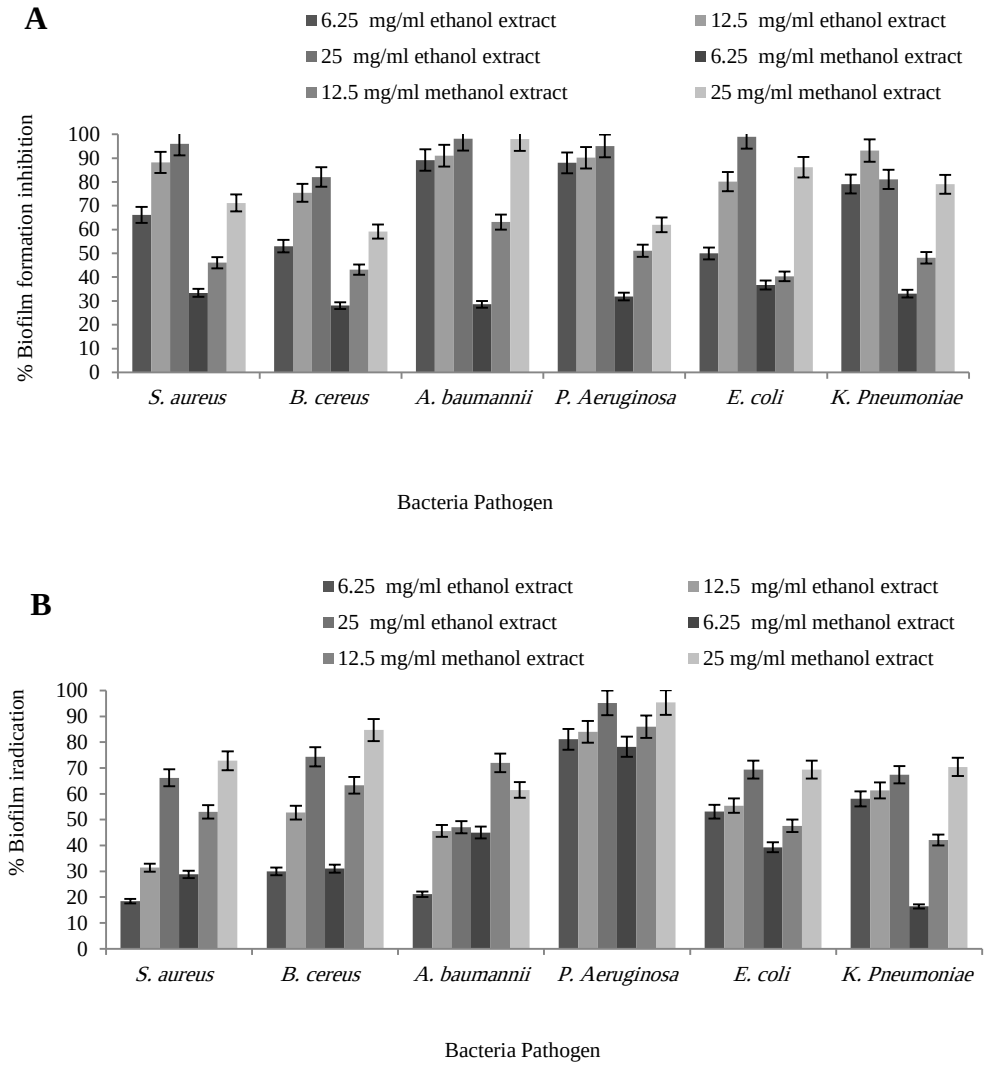

Fig. 1- The effect of *H. officinalis* extract against biofilm of pathogenic bacteria (a) inhibition of biofilm formation (b) disruption of biofilm structures with different concentrations of *H. officinalis* extracts.

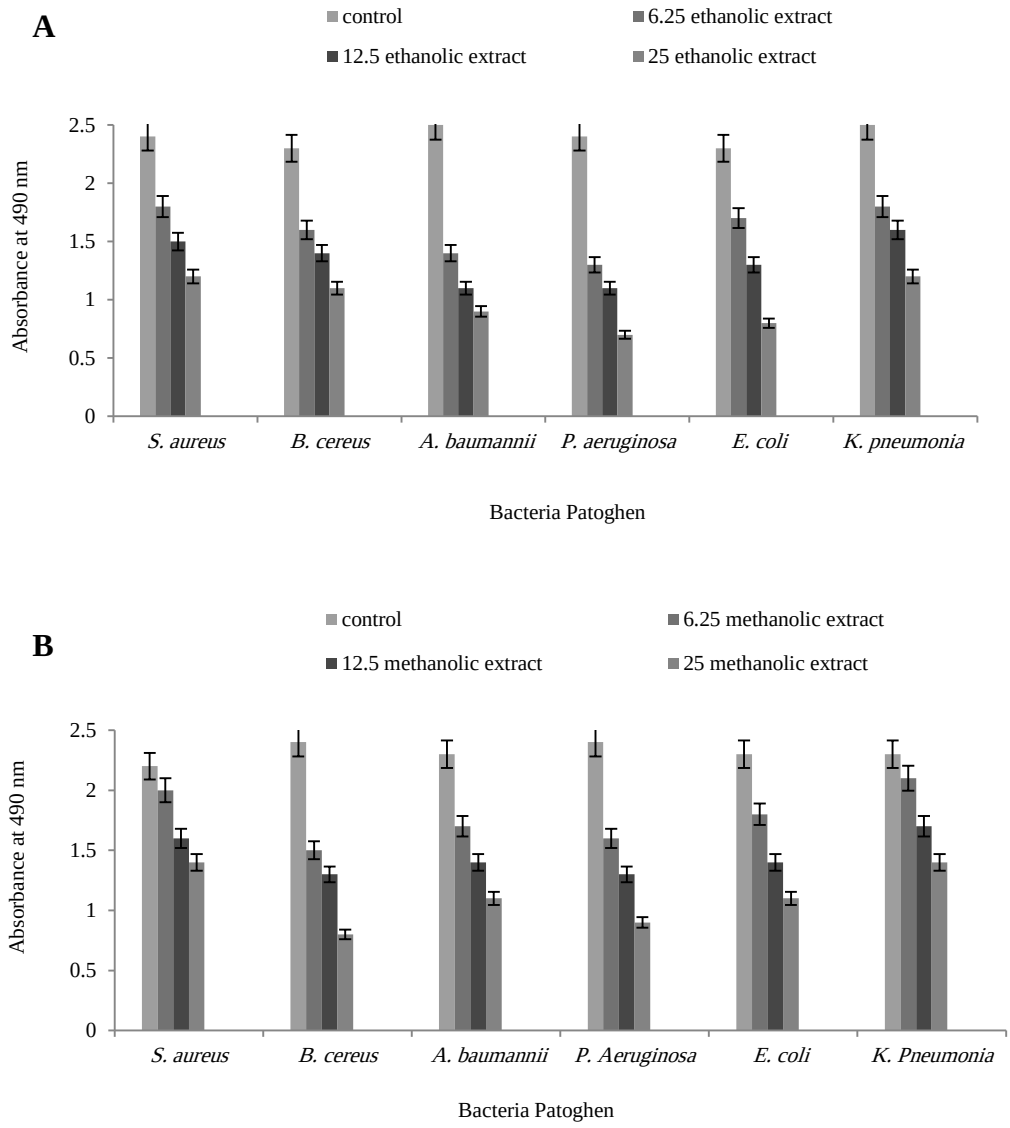

Fig. 2- The effect of *H. officinalis* extract on dehydrogenase enzyme activity of six pathogenic bacteria (a) ethanolic extract (b) methanolic extract

According to ANOVA analysis, it was confirmed that inhibitory efficiency of *H. officinalis* extracts were significant at 0.05, 0.01 and 0.001 level (Table 2). As shown in Table 2, the inhibitory effect of extracts on formation, destruction and metabolic activity of bacterial biofilm was significant at 0.05, 0.01, and 0.01 percent levels, respectively. The inhibition and destruction of bacteria biofilm in treatment with extract of *H. officinalis* was independent from solvent used for extraction.

Chemical composition of *H. officinalis* extract: The chemical mixture of *H. officinalis* extract was determined by GC-MS. The results were shown in Table (3) and also the chromatographs were presented in Figure (3). As shown in Table (3), the main active compounds illustrated in the extract of this plant was: Verbenone, Pyrane, Phenol, Furan, and Cyclopropan. All detected compounds in the extract of this plant have aromatic ring. In other hands, the phenolic compounds were predominat in this medicinal plant.

Table 2- The results of biofilm inhibition and destruction by extract of *H. officinalis* were statistically analyzed by Duncan's test

Sources Change	DF			MS			Sig		
	Biofilm formation	Destruction biofilm	Inhibition enzyme	Biofilm formation	Destruction biofilm	Inhibition enzyme	Biofilm formation	Destruction biofilm	Inhibition enzyme
Bacteria	5	5	5	0.028	0.135	0.150	*	***	**
Extract	1	1	1	0.862	0.006	0.083	*	-	-
Concentration	2	2	3	0.322	0.288	3.671	-	**	***
Bacteria*Extract	5	5	5	0.008	0.032	0.018	-	**	-
Extract* Concentration	2	2	3	0.050	0.009	0.040	*	-	*
Bacteria* Concentration	10	10	15	0.007	0.013	0.025	-	-	-
Total	25	25	32						

DF: Degree of Freedom
MS: Min Square
Sig: Signification

Table 3- Chemical composition of *H. officinalis* extracts analysis by GC-MS

No.	Compounds	Formula	RT	Area	%Total
1	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	C6H8O4	9.443	5.858e+4	0.266
2	D-Verbenone	C10H14O	10.495	2.001e+4	0.091
3	2-Furancarboxaldehyde, 5-(hydroxylmethyl)	C6H6O3	10.657	2.181e+5	0.990
4	Phenol, 2,6-dimethoxy	C8H10O3	12.413	6.317e+4	0.287
5	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene	$C_{15}H24O$	15.503	8.681e+4	0.103

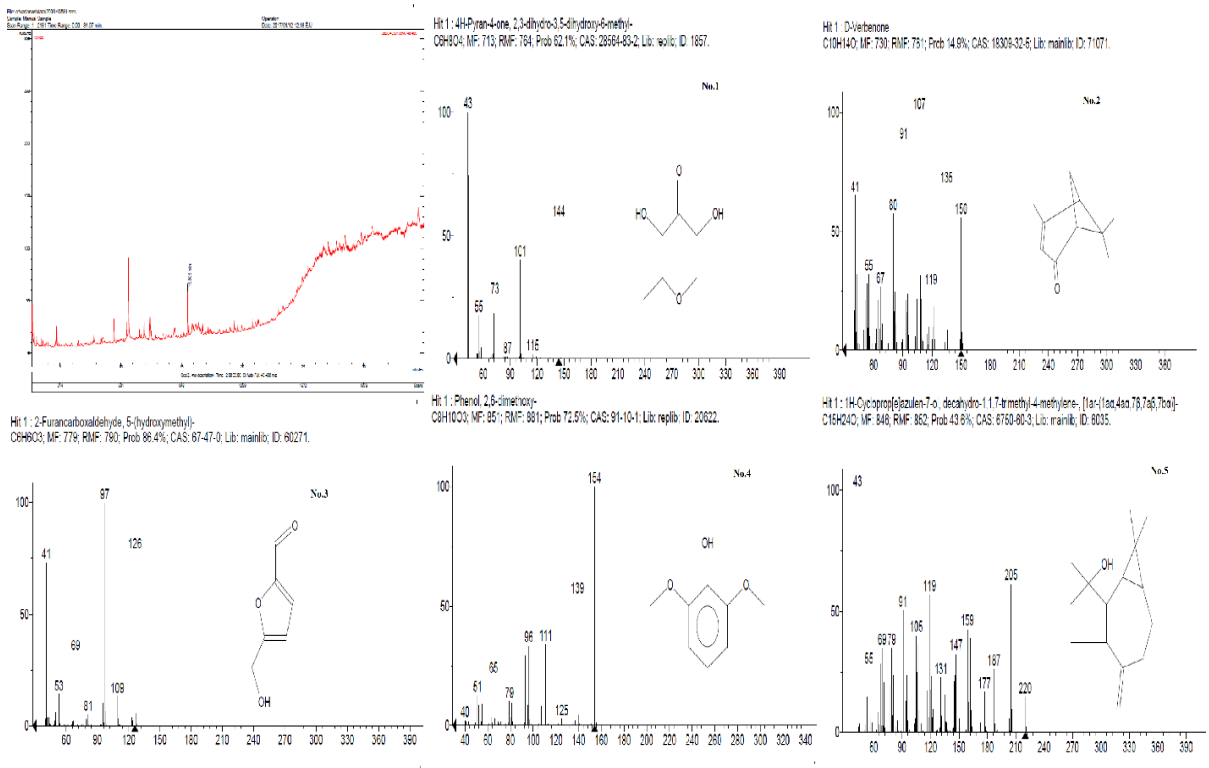

Fig. 3- The GC-MS Chromatogram of *H. officinalis* extract. Five main compounds were detected in the GC-MS analysis include (from left to right): Verbenone, Pyrane, Phenol, Furan and Cyclopropan

Discussion and conclusion

Widespread use of antibiotics in the recent years causes the emergence and spread of drug-resistant bacteria resistant to treatment of infections. One of the most important reasons for antibiotic resistance is the remaining of microbes in biofilm structures. This structure prevents the penetration of antimicrobial agents and also inhibits the effective function of these agents. So, acquiring new pathway to deal with bacteria, especially in the biofilm forms is necessary (21, 22). Research in this field especially focused on biological compounds, because the biological nature of these materials reduced side effects in comparison to conventional chemical agents. Between biological derivatives, the medicinal plant as options draw more attention as a good way for dealing with pathogenic bacteria (23, 24).

In this study, antimicrobial properties of *H. officinalis* were investigated against six pathogenic bacteria. Disk diffusion test confirmed that *H. officinalis* extracts had high ability to prevent the growth of selected bacteria. As shown in Tables (1) and (2) the methanolic extract better inhibited planktonic forms of bacteria than ethanolic extract, except that the effects of ethanolic extract against *E. coli* and *K. pneumonia* were better than methanolic extract. In disk diffusion test, *S. aureus* was the most sensitive bacterium, while the inhibitory effect of this plant extracton *P.aeruginosa* was weak.

Since the inhibitory effects of *H. officinalis* on all tested bacteria in broth medium (MHB) are higher than solid medium (MHA), it can be concluded that the active compounds of the extracts, like many other herbal extracts, has lower diffusion in Muller Hinton agar medium and for display optimum inhibitory effects in solid medium, it is necessary to use higher concentration of extract than broth medium.

Khaled et al (2014) studied antioxidant and antibacterial activities of Lamiaceae family. Their results confirmed that these extracts have antibacterial effect against *E. coli* and *S. aureus.* However, *E. coli* was more sensitive to the extracts than *S. aureus* and the most effective extract was *H. officinalis* (25).

Our results are in accordance with those results reported by Khaled et al (2014). This contradiction between these two studies can be related to geographic areas where these plants have been collected (26).

The ability of *H. officinalis* ethanolic extract in the inhibition of biofilm formation was more than the demolish of biofilm. These results revealed that the components of H. officinalis extract had better efficacy to inhibit biofilm formation compared to biofilm destruction.

There was very low publication that studied the antibiofilm activity of *H. officinalis*. Elmasri et al (2014) assessed the chemical composition and antibacterial activity of *H. officinalis*. They isolated sixteen compounds from methanolic extract of the aerial parts of the plant. Antibacterial activity of the crude extract as well as four of the isolated metabolites were measured. Also, they studied the antibiofilm activity of these metabolit against *S. aureus*. Their results confirmed that these metabolits from *H. officinalis* dramatically inhibited *S. aureus* biofilm formation. Our results in the current research also confirmed methanolic extract of *H. officinalis* had sufficient inhibitory effect against biofilm formation of *S. aureus* (%70). Thus, our results are in accordance with the results reported by Elmasri et al (2014) (27).

The chemical composition of *H. officinalis* extract were determined in this study. Similar to other medicinal plants, the chemical constitute of this plant revealed phenolic and aromatic metabolites. The antibacterial activity of these metabolites

were described in some publication in other herbs. For example, Lin-fang et al (2013) by GC-MS analysis of *Picrasma vassioides* identified 46 compounds. The major compounds in volatile oil are terpenoids, hydroxyl compounds and other acyclic alkane compounds (28).

Kamel and Sanidra (1994) analyzed the volatile oil of two *H. officinalis* varieties. They detected 93 compounds in the two oils. Their results confirmed that the major part of the extract was sesqui terpenoids. In our chemical analysis, we reach these metabolits and our results are in accordance with the mentioned study (29-31).

In this current research, the antimicrobial and antibiofilm activity of *H. officinalis* extracts were confirmed. The extracts of this plant can be used for treatment of some bacterial infections especially against bacteria that acquired resitance to conventional antibiotics.

The results of current study confirmed antibacterial and anti-biofilm activity of H. officinalis. It can be concluded that the extract of this plant has potential to design new antimicrobial agent against pathogenic bacteria that are resistance to conventional antibiotics.

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