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Original Article

Evaluating Antibacterial Effects of Alcoholic Extracts and Essential Oil of *Althaea officinalis* Against Two Types of Gram-positive and Gram-negative Bacteria (*Bacillus cereus* and *Klebsiella pneumonia*)

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ABSTRACT

Regarding the emergence of microbial resistant strains to chemical drugs, it was important to make efforts for finding new antimicrobial factors with fewer side effects for substituting chemical drugs. This study investigated the antibacterial effects of alcoholic extracts (ethanol and ethyl acetate). *Althaea officinalis* L. parts (flower, leaf, stem and root) were considered against two positive and negative bacteria types of pneumonia (*Bacillus cereus* and *Klebsiella pneumonia*) under laboratory conditions. In order to investigate anti-microbial activities of alcoholic extracts and *A. officinalis* essence in different concentrations, they were affected over mentioned bacteria by using the Diffusion Disk method. Penicillin, Ampicillin, Gentamicin, and Vancomycin were used as a positive control and Ethanol, Ethyl acetate and DMSO solvents as negative control and Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) have been determined. Taking essence has been conducted by the Clevenger system. Its chemical combinations were identified by GC-MS. Results showed that stem (total) ethanol extracts and *A. officinalis* essences (in the concentration 100 mg/ml) have the highest microbial properties on *K. pneumonia*. The ethanol extract was affected on all components of *A. officinalis* and Ethyl acetate extracts of leaf and stem over *K. pneumonia* and *A. officinalis* essence (in the concentration 12.5 µl/ml) over both bacteria. Gentamicin as an anti-biotic had good inhibitory power against bacteria as a positive control in comparing other antibiotics. 56 combinations of *Althaea officinalis* essence were extracted by more than 93% of main combinations consisted of Thymol, p-Cymene, γ-Terpinene, β-pinene, Terpineol, Carvacrol. The more extract and essence concentration increased antibacterial properties and inhibitory halo diameter. *A. officinalis* extracted combinations with anti-bacterial properties were considered as the main factor for consuming *A. officinalis* in different industries as an herbal drug by natural origin and anti-bacterial effects.

INTRODUCTION

The emergence of pathogenic microorganisms by multiple antibiotic resistance treated the clinical efficiency of many common antibiotics, globally. In addition, the side effects of consuming chemical drugs made researchers pay more attention to obtain plants with anti-microbial properties more than ever [1,2].

Infection with a bacterial source is the most identified part of diseases. So, many efforts have been conducted to recognize, control and treat the pathogenic factors. Herbal medicines have long been regarded as a treatment for different diseases. Although treatment by chemical and synthetic drugs could be effective, they have some side effects and might create medicine resistance. Therefore, using herbal medicines could be a good solution for this

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problem. Lack of medicine resistance, health and environmental hygiene are such drug's benefits [3-5]. Different parts of *Althaea officinalis* such as leaf, flower and stem are used as drugs [6,7]. *A. officinalis* has some properties such as anti-cough, anti-heartburn, anti-gastritis, anti-tumor, anti-virus, and anti-biotic effects, anti-bacterial, and anti-inflammatory [6, 8]. Flower and root are applied as skin wound disinfectants [6]. Flower and leaf relieve constipation and respiratory diseases [7]. The alcoholic extract of the root has the property for relaxing smooth muscle [9]. *A. officinalis* including root and flower have a different amount of flavonoids of polyphenols, polysaccharides, mucins, fibers, unsaturated fatty acids, minerals and albumin [10-12]. *A. officinalis* has starch, fat, essence, anthocyanin, althea, de-oxy-benzoic acid, and cyaniding. The most important combination of *A. officinalis* root is mucilage (the plant viscous material) with 10% sugar which created rhamnose, galactose and galacturonic acid due to hydrolysis. Flower and leaf have 6 to 9% mucilage. Its leaves consisted of coumarin and scapolite. Flavonoids combination and a little amount of essence are the other flower and leaf material of *A. officinalis* [6,7].

This study has been conducted by the target of evaluating anti-bacterial effects of alcoholic extracts in *A. officinalis* (flower, stem, leaf and root) and *A. officinalis* essence over *Klebsiella pneumoniae* as negative gram bacteria and *Bacillus cereus* as positive gram bacteria.

MATERIAL AND METHODS

Applied plant preparation: *A. officinalis* was collected in spring from the northern part of Iran in Akand village in Sari city, Mazandaran Province, Iran. It was identified by Razi University Herbarium with voucher number 693. It was dried in moderate weather, free air and shadow without direct light of sun and heating after identification and confirmation. After cleaning and drying, it was powdered and kept in covered dishes for keeping away from the light and heating until testing time. The basic special parts of this plant are its anti-bacterial properties based on existing reports.

Standard microbial strains preparation: In this study, *K. pneumoniae*, negative gram bacteria strain (PTCC 1053) and *B. cereus*, positive gram (PTCC 1154) were prepared from pastor Institute research Centre (Iran, Tehran); with a complex of global standard bacteria and fungus in a lyophilized form.

Preparation of microbial solution: A suspension by MC far land 5% dilution prepared from cultured strains for 24 hours in Moller Hinton Agar culture medium over Moller Hinton Broth culture medium. Then standard strains were investigated relating to ampicillin, Gentamicin, vancomycin and penicillin anti-biotic.

Extraction of *A. officinalis* alcoholic extracts: In order to find suitable solvent to extract herbal effective combinations, ethanol and ethyl acetate solvents were used by applying the soaking method for extraction. The purpose is that the plant phase enters an alcoholic phase in a desirable way 1000^{cc} of ethanol 96% solvents and ethyl acetate 99% added to 50g of dried powder in any studied components of (flower, leaf, stem and root). The resulted combination has put in a shaker after 48 hours by the speed of 100 cycles per minute after filtrating paper in 3 stages, the pure extract obtained in free weather far away from direct light - by solvent – scattering rotary system and concentrated. (Total) pure extract, ethanol and ethyl acetate obtained with different, special colors, smell and flavors.

Extract dilution and preparation of Discs with extracts: At first, extract dilution was prepared by ethanol and ethyl acetate solvents in 50, 100, 200, 400, and 800 mg/ml. Blank discs were put on pipes with mentioned extracts dilutions to prepare discs with extracts and were prepared after 3 to 4 minutes and complete absorption of discs were dried at 37 °C degrees and were prepared for putting disk.

A. officinalis essential oil preparation: Extraction of *A. officinalis* essential oil was done by distillation method with water and Clevenger system for 5 to 6 hours. Dilution and also identification of essence formulating combination were considered until determination of its anti-microbial properties for decreasing the rate of volatility rate, and then they were put in the freezer.

Dilution and preparation of disks with essence: Regarding lack of essence solubility in the culture medium, Dimethyl Sulfoxide (DMSO) was used as solvent [13]. For dilution, 50µl of essence was diluted by using 50µl of DMSO and 12.5, 25, 50 and100 µl/ml concentrations were prepared. To prepare the discs containing the extract, the blank discs were placed in tubes containing the mentioned

dilutions of the extract and after 3 to 4 minutes and complete absorption of the discs, they were dried at 37 °C and directed for disking.

A. officinalis essence and alcoholic extracts diffusion disk method: In order to measure ethanol and ethyl acetate extracts (flower, stem, leaf and root) and *A. officinalis* essence of 24 hours culture of any bacteria, microbial suspension equals McFarland standard no. 0.5 were prepared over monotonous culture agar Moller Hinton culture level. Discs with different dilutions were placed over culture medium level in suitable seasons and the plates were incubated for 24 hours at 37 °C. The bacteria resistance with extract and essence were determined by measuring lack of growth halo diameter around discs by millimeter special and standard ruler in 3-times sensitivity repetitions. Antibiotic disc used as positive control and blank discs smeared with ethanol and ethyl acetate used as a negative control.

Determination of MIC and MBC in alcoholic and essence extracts: Determination of MIC and MBC values have been done by pipe dilution method. In order to determine extract and essence MIC, bacterial suspension inoculation to Broth Moller Hinton culture medium consisted of 25 µl of Alcoholic extracts inhibitory concentrations (50, 100, 200, 400, 800) and essence inhibitory concentrations were used (100, 50, 25, 12.5). After incubation for 24 hours in 37 °C and investigation of tubes for turbidity resulted from bacterial growth, the least dilution of extract and essence with no turbidity (lack of growth) was reported as minimum inhibitory concentration (MIC).

It was cultured on Mueller-Hilton agar to determine MBC of essence and extraction from tubes with lack of bacterial growth, and after incubation, the plate related to the tube with minimum extract and essence concentration (lack of bacterial growth) was reported as MBC of the essence and extract. Positive control tubes contained culture medium with bacteria, without extract and essence. Negative control tubes contained a culture medium without bacteria.

Separation and identification of *A. officinalis* essence oil constituents: Constituents were separated and identified by chromatofigurey method and mass spectrometer (GC/MS). One microliter volume of each extracted essences was injected into GC/MS system in the laboratory complex. This System Properties are as follows:

GC system; System model: Hewlett-Packard 6890 (HP). Injection gate temperature: 250 °C. Column type: HP-5MS. Heating planning: 220-60 °C. Carrier gas: Helium. Gas flow rate: 1 ml/min. temperature rise rate: 6 °C/min. column Length: 30 m. internal diameter: 250micron.

MS system; Model: HP-5973. Ionization energy: 70 eV.

RESULTS

Statistical analysis: In this study, one-tailed variance analysis, Duncan test, and SAS software were used for data analysis and comparison for lack of growth Halo diameter Average and $P< 0.05$ was considered significant. Excel software 2016 was used for drawing charts.

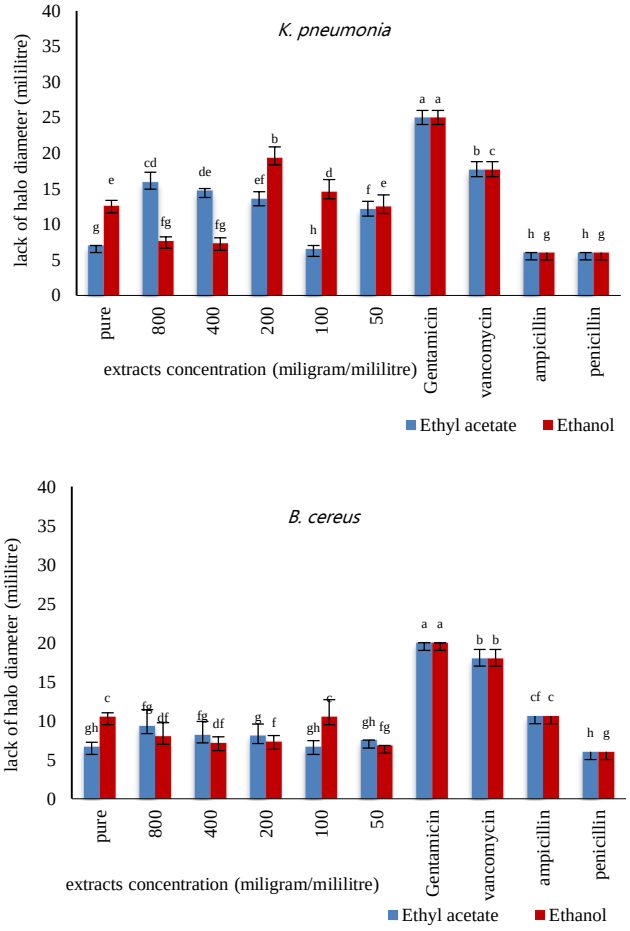

Fig. 1 Comparison of *A. officinalis* alcoholic extracts lack of growth halo diameters with antibiotics over *B. cereus* and *K. pneumonia*. Common letters in each column show significant differences in the $P< 0.05$ level and uncommon letters in each column show significant differences in the $P> 0.05$ level.

The diameter and lack of growth halo of the essence and alcohol extracts are reported as average and standard deviation. By reducing the percentage of

extract, the different treatments of ethanol and ethyl acetate extracts will affect the reduction of growth halo diameter in the two bacteria (Fig. 1-5).

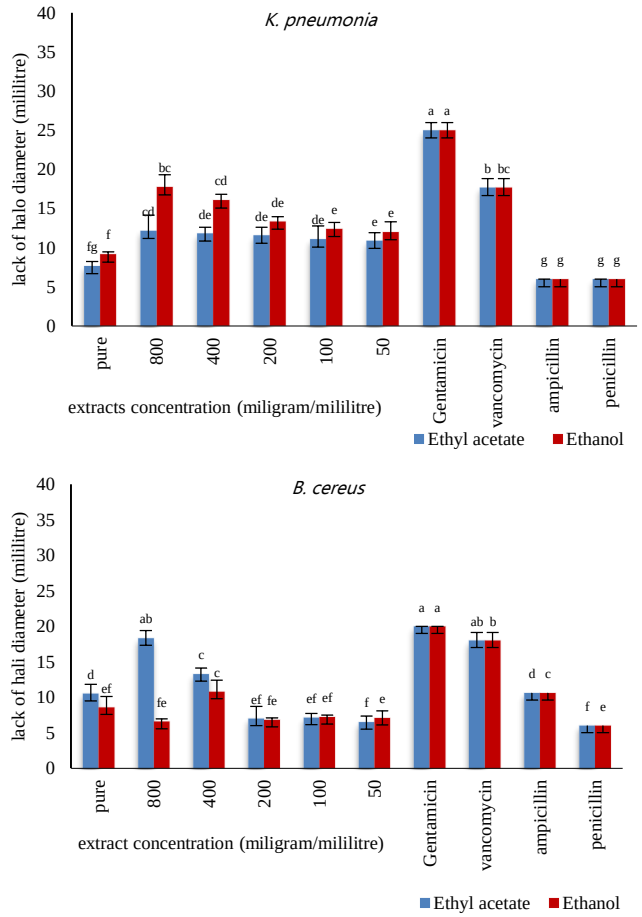

Fig. 2 Comparison of *A. officinalis* L. leaf alcoholic extracts lack of growth halo diameters with antibiotics over *B. cereus* and *K. pneumonia.* Common letters in each column show significant differences in $P< 0.05$ level and uncommon letters in each column show significant differences in $P> 0.05$ level.

Among all investigated *A. officinalis* components, total ethanolic extract over *K. pneumoniae* had the greatest microbial properties. Ethyl acetate extract in 100 mg/ml concentration over *k. pneumoniae* and the leaf ethyl acetate extract in 50 mg/ml concentration over *B. cereus* bacteria have the least anti-microbial properties (Fig. 1-5).

Lack of growth halo diameter in negative controls (Ethanol and Ethyl acetate solvents in alcoholic extracts and DMSO solvents in essences were equal 0 mm (Fig. 1-5).

Flower essence has the greatest Halo diameter of lack of growth and inhibitory effect against bacteria, while 100 µl/ml over *K. pneumoniae* had more anti-bacterial properties against *B. cereus* and 25 µl/ml over *B. cereus* had the least anti-bacterial effect that showed more strength of *B. cereus* to the flower essence (Fig. 5).

Gentamicin Anti-biotic, as a positive control comparing other antibiotics, had a good inhibitory effect against bacteria, while in alcoholic extracts, Gentamicin was effective for both bacteria and in the essence by a slightly significant difference over *K. pneumoniae* had a more inhibitory effect on *B. cereus*. Anti-biotic, vancomycin, ampicillin and penicillin, had less inhibitory strength (Fig. 5).

General results of inhibitory and bactericidal effects of essence and extract over bacteria

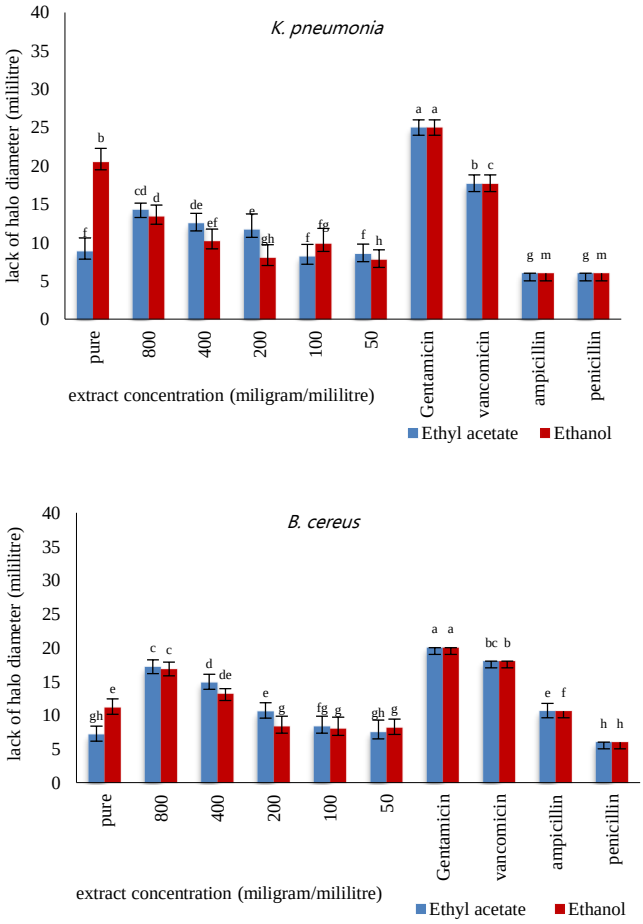

Fig. 3 Comparison of *A. officinalis* L. stem alcoholic extracts lack of growth halo diameters with an antibiotic over *B. cereus* and *K. pneumonia.* Common letters in each column show significant differences in $P< 0.05$ level and uncommon letters in each column show a significant difference in $P> 0.05$ level.

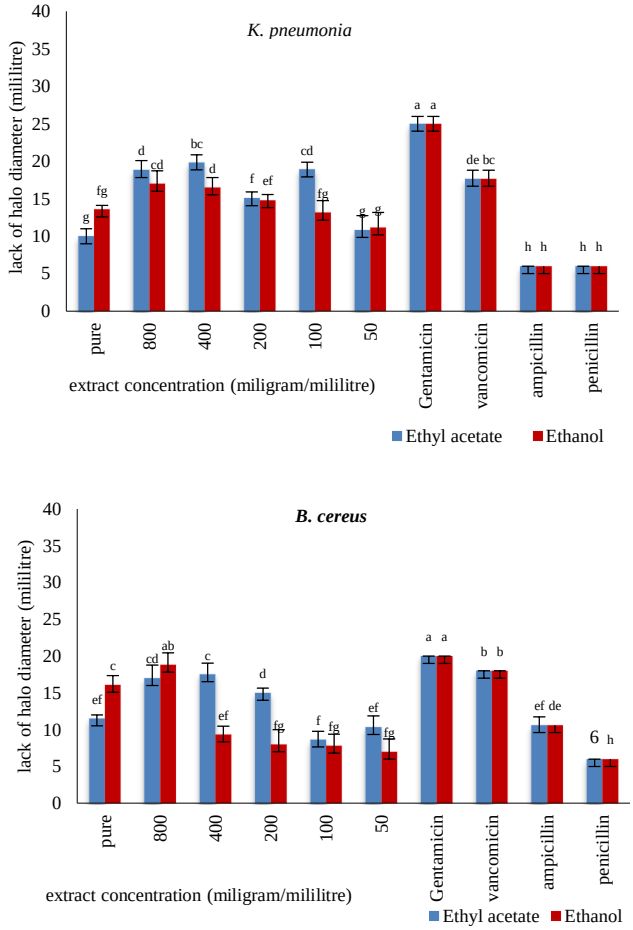

Fig. 4 Comparison of *A. officinalis* L. roots alcoholic extracts lack of growth halo diameters with antibiotics over *B. cereus* and *K. pneumonia*. Common letters in each column show significant differences in $P< 0.05$ level and uncommon letters in each column show significant differences in $P> 0.05$ level.

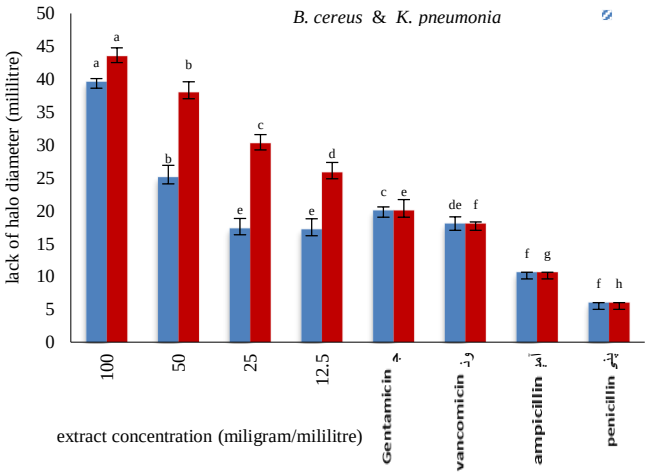

Fig. 5 Comparison of *A. officinalis* L. essence lack of growth halo diameters with antibiotics over *B. cereus* and *K. pneumonia*. Common letters in each column show significant differences in $P< 0.05$ level and uncommon letters in each column show significant differences in $P> 0.05$ level.

Table 1 MIC and MBC of *Althaea officinalis* alcoholic extracts over *Bacillus cereus* and *Klebsiella pneumonia*.

Extract concentration	Ethanolic extract		Ethylacetatic extract	
	B. cereus	*K. pneumonia*	*B. cereus*	*K. pneumonia*
800	-	-	+	-
300	+	-	+	+
200	+	+	+	+
100	+	+	+	+
50	+	+	+	+
MIC	800	400	0	800
MBC	0	800	0	0

Where (-) observation of microorganism lack of growth (+) microorganism growth

Table 2 MIC and MBC of *A. officinalis* L. alcoholic extract over *B. cereus* and *K. pneumonia*.

Extract concentration	Ethanolic extract		Ethylacetatic extract	
	B. cereus	*K. pneumonia*	*B. cereus*	*K. pneumonia*
800	-	-	+	-
400	+	-	+	-
200	+	+	+	+
100	+	+	+	+
50	+	+	+	+
MIC	800	400	0	400
MBC	0	800	0	0

Where (-) observation of microorganism lack of growth (+) microorganism growth

Table 3 MIC and MBC of *A. officinalis* L. stem alcoholic extract over *B. cereus* and *K. pneumonia*.

Extract concentration	Ethanolic extract		Ethylacetatic extract	
	B. cereus	*K. pneumonia*	*B. cereus*	*K. pneumonia*
800	-	-	+	-
400	-	-	+	-
200	+	+	+	+
100	+	+	+	+
50	+	+	+	+
MIC	400	400	0	400
MBC	800	800	0	800

Where (-) observation of microorganism lack of growth (+) microorganism growth

The greatest concentration of *A. officinalis* essence was 12.5 µl/ml over both types of inhibitory and bactericidal (Tables 1-5). Regarding normal Alkanes exit pattern, inhibitory index, quartz index and their adaptation with librarian patterns related to spectrums of anybody interpreted and essences general combinations were determined.

Table 4 MIC and MBC of *A. officinalis* L. root alcoholic extracts over *B. cereus* and *K. pneumonia*.

Ethylacetatic extract	Ethanolic extract		Ethylacetatic extract	
	B. cereus	*K. pneumonia*	*B. cereus*	*K. pneumonia*
800	-	-	+	-
400	-	-	+	+
200	+	+	+	+
100	+	+	+	+
50	+	+	+	+
MIC	400	400	0	800
MBC	800	800	0	0

Where (-) observation of microorganism lack of growth (+) microorganism growth

Table 5 MIC and MBC *of A. officinalis* L. essence over *B. cereus* and *K. pneumonia*.

Essence concentration	*B. cereus*	*K. pneumonia*
100	-	-
50	-	-
25	-	-
12.5	-	-
MIC	12.5	12.5
MBC	12.5	12.5

Where (-) observation of microorganism lack of growth (+) microorganism growth

Table 6 *A. officinalis* L. essence identified combination by GC/MS

PK	Essence combination	Area%	Essence combination	RT	KI
1	Thymol	58.91	Thymol	20.379	1318
				20.596	1325
2	Persimmon	15.12	Persimmon	10.310	1026
3	Gama terpenin	14.67	Gama terpenin	11.550	1062
				16.227	1159
4	Beta pinene	2.34	Beta pinene	8.455	969
				16.227	1195
5	Terpineol	1.01	Terpineol	16.913	1215
				20.659	1327
6	Carvacrol	0.88	Carvacrol	37.04	1902
				39.487	2003

The greatest inhibitory and bactericidal effect was related to ethanoic extract of flower and leaf over *K. pneumoniae*. Both types of extracts from the stem were effective over *K. pneumonia*, but only the stem ethanolic extract over *B. cereus* had the greatest effect. The root ethanolic extract had the greatest effect on both types of bacteria. 56 combinations were extracted by anti-microbial effects which allocated more than 93% including Thymol (58.91), p-Cymene (15.12), γ-Terpinene (14.67), β-pinene (2.34), Terpineol (1.01) and combinations with low percent such as Carvacrol (0.88) and etc. (Table 6). Chromato Figureic spectrum of essence constituting chemical combinations was presented by any component frequency percent (Fig. 6).

DISCUSSION

The increase of stable bacterial strains outbreak to antimicrobial drugs and multi-drugs strong strains, caused new strategies for suppressing bacterial infections [14]. *A. officinalis* is investigated due to species diversity and the presence of different effective combinations in extract and essence for their anti-microbial effects [15].

Results showed that *A. officinalis* extracts have considerable effects on *B. Cereus* and *K. pneumoniae* bacteria which their antibacterial and inhibitory properties increased by rising concentration. There are reports about *A. officinalis* extracts on fungus and anti-microbial effects [15-17].

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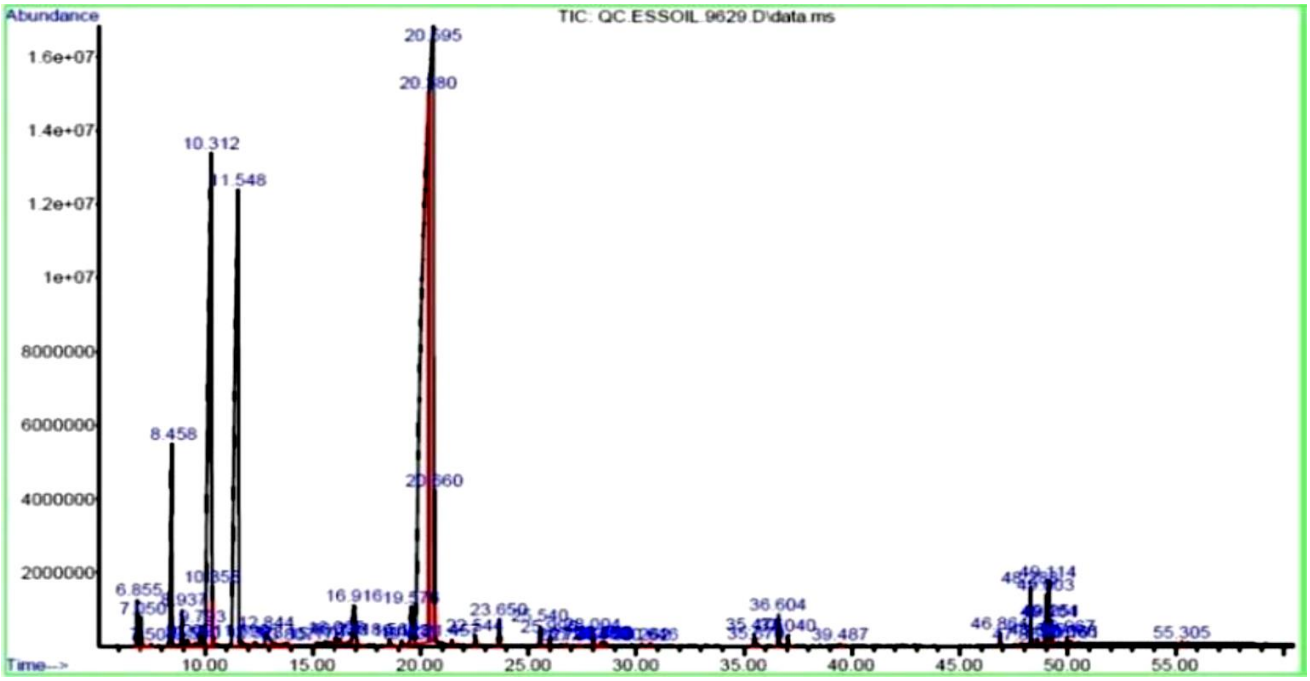

Fig. 6 chromatofigureic spectrum GC/MS of *A. officinalis* L.

In the present studies, identifying chemical combinations by high percentage were considered as rational, reasons for proving *A. officinalis* essence anti-bacterial effects. Suleiman et.al (2010) considered combinations including thymol, carvacrol, linalool, eugenol, camphor, phenol, which adapted with obtained combinations of GC-MS results in this study [18]. In these reports, the presence of Siporin was implied in *A. officinalis* alcoholic extract which is its anti-bacterial reason [19, 20].

Lack of growth halo diameter is decreased by reducing concentration percentage which is due to an increase of effective materials concentration rate and is increased by the rise of concentration. Zareii *et al.* (2014) showed a decreasing trend of halo diameter over *k. pneumoniae* in low concentration [16].

Among all investigated components; stem total ethanolic extract over *K. pneumoniae* negative gram bacteria, has the greatest anti-microbial properties, while in Zareii et.al study, stem ethanolic extract has anti-bacterial effects over *k. pneumoniae*, any more anti-microbial effects over positive gram bacteria [16]. Somewhat inconsistent results in these studies showed that *A. officinalis* had the least anti-bacterial effect on negative-gram bacteria [21].

Gentamicin antibiotic, as positive control comparing with ethanolic, ethyl acetate diluted extracts effects and other antibiotics had a good inhibitory effect against bacteria which has a less significant difference with gentamicin antibiotic effect against *K. pneumoniae* [16].

A. officinalis essence had the greatest Lack of growth Halo diameter and inhibitory effect to the other concentrations and anti-biotic and *A. officinalis* essence anti-bacterial properties are more than *A. officinalis* extracts and other components which implied different herbal potentials in their anti-bacterial properties discussion. Gautam *et al.* (2015), found that in their study about antimicrobial effects of *A. officinalis* essence oil and seed extract over some respiratory pathogens, antifungals and antibacterial effects of essential oil to the extract were higher which were in line with the present results [22].

In this study, *A. officinalis* essence with a low significant difference had more anti-bacterial properties over *K. pneumoniae* than positive gram bacteria *B. cereus*, while in Gautam *et al*. (2015) study, the highest lack of growth Halo diameter and *A. officinalis* essence inhibitory was over positive gram bacteria [22].

Also in this study, the highest amount of inhibitory effect compared to total extracts and bacteria over *k. pneumoniae* was 400 and 800 mg/ml, respectively, which in Zareii *et al.* (2014) study, minimum inhibitory concentration was 200 mg/ml and minimum bactericidal concentration was 800 mg/ml and the results corresponded in MBC level [16].

The highest inhibitory and bactericidal effect was considered for ethanoic extract of flower, leaf and also alcoholic extracts of stem over *K. pneumoniae* but stem acetate ethyl extract was effective only over *B. cereus*. The roots ethanoic extracts and flower essence were effective on both bacteria. There is a consistent result in Zareii *et al*. (2014) study with the highest *A. officinalis* ethanolic extract MBC and MIC effect showed over *k. pneumonia* [16].

The combinations with anti-bacterial properties are separable by using a special solvent. According to studies, among both alcoholic extracts, ethanol was the more appropriate solvent for extracting effective combinations of *A. officinalis* flower. In Zareii *et al* study, the effect of *A. officinalis* ethanolic extract is considerable [16].

CONCLUSION

The study results showed that *A. officinalis* alcoholic extracts and essence have anti-microbial properties that regarding this plant frequency, could be more investigated as a material with anti-bacterial, anti-septic and anti-oxidant combination with a natural origin for pharmacologic consumptions in pharmacology, food and agricultural industry.

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