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Impact of two different extraction methods on chemical composition and antimicrobial activities of multi-ingredients essential oils and hydrosols

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Abstract

Herbal medicinal oils and hydrosols are of most useful preparations in folk Persian medicine. Till now no comprehensive evaluation has been performed on the impact of simultaneous extraction or mixing the extracted products on chemical composition profile of those preparations. In current study, the impact of two different extraction methods for essential oils (EO) and hydrosols (separated and mixed)on chemical composition and activity is chemically assessed. Samples of *Mentha spicata* L. (MS), *Zataria multiflora* Boiss. (ZM), *Bunium persicum* (Boiss.) B.Fedtsch. (BP), and *Trachyspermum ammi* (L.) Sprague (TA) were subjected to hydrodistillation, either individually or in combination with each other. Hydrosols and EO samples of each plant were mixed to prepare new hydrosol and poly-EO samples mixtures. All samples were injected to GC/MS for analysis. Moreover, anti-microbial activity of EOs and hydrosols were measured by MIC method. ATR-IR spectroscopies were used for recorded finger print from EOs. Carvone, thymol, cuminic aldehyde, and thymol were identified as the major constituents of MS, ZM, BP, TA, and EO samples, respectively. Hydrosol of MS, ZM, BP, and TA revealed to have piperitenone, carvacrol, cuminol and thymol as the main components, respectively. The mixed oil samples, from first part had γ-terpinene and carvacrol as major components and hydrosol samples had thymol as the component, respectively. In mixed oils and hydrosols, the major components were γ-terpinene and thymol in the respective order. This study showed that there were differences between main components, antimicrobial activity, antioxidant, and ATR-IR spectroscopy of mixed samples in both preparation methods.

Keywords: Essential oil, Hydrosol, Traditional dosage form, Traditional pharmacy.

1. Introduction

Traditional Persian medicine (TPM) is used in prevention, elimination, and diagnosis of diseases from ancient times until today (1). This school of medicine is not only a summation of previous medical knowledge, but is a complex of Persian physicians' and scholars' experiences in the field of medicine and pharmacy for thousands years of practice (2). A main part of TPM is about

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the science of pharmacy, which is often spoken as traditional Persian pharmacy. Description, characterization, and medicinal properties of natural medicines as well as medieval or traditional preparation methods, dosage forms, administration routes, and combination of those medicaments as a multi-ingredient formula are fully mentioned in this field (3).

In TPM, herbal medicinal oils and hydrosols are of most useful preparations, which are obtained by hydro or steam distillation (4). Herbal medicinal plants are gone thorough hydro or steam distillation either individually or in combination together.

Essential oils are natural, volatile secondary metabolites, which are known by their antimicrobial (5-9), spasmolytic (10), analgesic, and anti-inflammatory (11, 12) activities. They are synthesized by different organs and stored in secretary cells. They have lower density of water and soluble lipid (13). Essential oils are complex mixtures of monoterpenes, oxygenated terpenes, sesquiterpenes, and oxygenated sesquiterpenes (14). By hydro- or steam distillation, water soluble components are transferred to distilled water and produce hydrosols (15, 16). Hydrosols come from all parts of plants and contain hydrophilic components (17, 18). Generally, there are two types of hydrosols in TPM: single (extraction from one medicinal plant) and polyherbal (extraction from multiple medicinal plants). For polyherbal hydrosols, there are two methods of mixing and extracting: method A and B (Figure 1). In method A, a hydrosol is yielded via mixing the employed medicinal plants prior hydrodistillation procedure. In method B, the hydrosol is a mixture of each individual extraction or hydrosol yielded via distillation of each concerned medicinal plant (19). These two methods have been repeatedly cited in the preparation of various hydrosols in TPM pharmaceutical manuscripts. However, there is no document on the differences of these two methods, either in regard to the components, or biological activities.

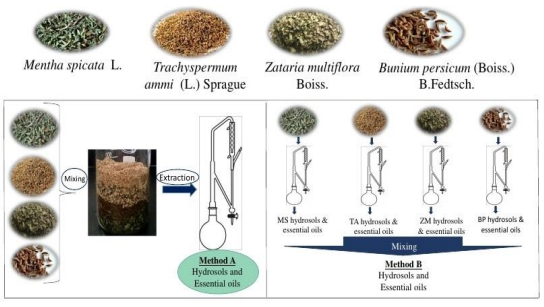

Figure 1. Method A & Method B.

Chāhār Giāh (a hydrosol consisting of four medicinal plant) hydrosol is a popular medicinal beverage in Persian folk and traditional medicine, which is prepared by both aforementioned methods. But the differences in chemical compositions and medicinal properties of these extracted hydrosols are evidently clear. Accordingly, this study aimed to prepare the *Chāhār Giāh* essential oil and respective hydrosol via the two mentioned methods and chemically evaluate the volatile compositions of prepared samples as well as those antimicrobial properties.

2. Materials and methods

2.1. Plant material

The employed hydrosol consists of four medicinal plants as *Mentha spicata* L. (MS) and *Zataria multiflora* Boiss. (ZM) aerial parts, as well as seeds of *Bunium persicum* (Boiss.) B.Fedtsch. (BP), and *Trachyspermum ammi* (L.) Sprague (TA). These herbs were purchased from local medicinal plants market (Shiraz, South of Iran). All plants were authenticated by the botanist of Department of Traditional Pharmacy of Shiraz, School of Pharmacy. Each sample was deposited in Shiraz School of Pharmacy Herbarium with a voucher number.

2.2. Extraction of hydrosols and essential oils

Same weight of each sample (200 g) were subjected to hydrodistillation for 4 h (20) via a Clevenger apparatus, either individually or in combination with each other (a mixture of sample).

2.3. Mixtures

As described in the introduction section, each method was categorized into two separated groups. In the first group, same weight of each sample was mixed together (abbreviated as EW or equal weight for each method). In the second

group, equal volumes of essential oil extracted from each plant sample were mixed together (abbreviated as EV or equal essential oil volume for each method).

2.4. Liquid-liquid extraction

For transforming the components into the organic phase, liquid-liquid extraction was conducted before GC/MS analysis for hydrosols. At first, 500 ml of each extracted hydrosol was mixed with an equal volume of petroleum ether. The solvent was heated for 150 min up to 45 °C. Subsequently, the organic phase enriched with aromatic content was separated. For two consequential steps, 500 ml of fresh solvent was added and heated with the same condition (21). The organic phase from each extraction step was collected together, concentrated, and kept for GC/MS analysis.

2.5. GC/MS analysis

GC/MS analysis was performed using a Hewlett-Packard 6890/5973 operating at 70.1 eV ionization energy, equipped with a HP-5 capillary column (phenyl methyl siloxane, 25 m×0.25 mm i.d.) with He as the carrier gas and split ratio, 1:150. The oven temperature was programmed as follows: 60 °C (2 min) to 260 °C at 2 °C/min; detector temperature, 250 °C; carrier gas, He (0.6 mL/min)(22). N-alkanes were injected with the same chromatographic condition. Identification of components was based on a comparison of retention indices (RI) and mass spectra with the Wiley library or Adams (23) libraries spectra.

2.6. ATR-IR spectroscopy

IR spectroscopy was used to achieve the fingerprint pattern of samples. In this method, ATR apparatus and Bruker vertex-70 instrument was used. IR acquisition range was obtained as 600-3400 cm^{-1}. All data was processed by Standard Normal Variety (SNV) to suppressed fluctuation of baseline, and then entered into MATLAB (Math Works Inc.) for further analysis.

2.6.1. Hierarchical cluster analysis (HCA)

Hierarchical cluster analysis was done by unweighted pair group method (UPGMA) and means of Euclidean distance was used as a measure of similarity. The obtained matrix was subjected to MATLAB software. The resulted dendrograms show distances versus samples and represent data based on their similarities.

2.6.2. Principal component analysis (PCA)

To analyze similarities between samples using their ATR-IR finger prints, PCA was used as a clustering method using an unsupervised approach (24). This method can show correlations between variables in two dimensional spaces. It can also simplify the data matrix (25) by using singular value decomposition algorithm. Accordingly, all principal components (PCs) were extracted from the resulted matrix. The first PC in a data set has the largest variance. Orthogonal features of the second PC with the first one make it possible to visualize the whole data in a 2D space (24).

2.7. Antimicrobial assay

Disc diffusion method was employed to assess the inhibitory activity of each sample against certain microorganisms. Micro dilution broth method was performed to determine the MICs, MBCs, and MFCs for essential oils. All tests were performed in triplicate.

2.7.1. Microbial strains

Two strains of Gram-positive bacteria (*Enterococcus faecalis*, *Staphylococcus aureus*), two strains of Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*), and one strain of fungi (*Candida albicans*) were used for antibacterial assay.

2.7.2. Disc Diffusion Method

The antibacterial activity of each sample was measured by disc diffusion method. The suspension of each microorganism, which was cultured overnight at 37 ºC, was diluted with Muller-Hinton Agar to reach 0.25 McFarland. Petri dishes containing specific media (Muller-Hinton agar for bacterial strains and Sabouraud Dextrose agar for fungi strain) was inoculated with 100 µL of each microorganism. 30 µL of essential oils was loaded on discs then placed on the media. Inoculated media plates were incubated at 37 ºC for bacteria and 28 ºC for fungi overnight. Disc of ampicillin was

Table 1. Yield of essential oils.

Sample	1st	2nd	3rd	Mean%±SD
Mentha spicata	1.50cc/200g	2.00cc/200g	2.00cc/200g	0.91±0.14cc/g
Zataria multiflora	6.80cc/200g	6.00cc/200g	5.90cc/200g	3.45± 0.47 cc/g
Bunium persicum	6.00cc/200g	5.80cc/200g	6.00cc/200g	3.30± 0.52 cc/g
Trachyspermum ammi	9.40cc/200g	9.60cc/200g	9.80cc/200g	4.80± 0.10 cc/g

used as the positive control and disc with no essential oil petri dish used for the control. All experiments were done tree times. Diameter of each resulting zone of growth inhibition was measured.

2.7.3. Microdilution broth method

Serial dilution method could determine minimal inhibitory concentration (MIC), minimal bacterial concentration (MBC), and minimal fungicidal concentration (MFC). For bacterial strains, tests were performed with Muller-Hinton broth then incubated overnight at 37 ºC. For the fungi strain, tests were performed with RPMI media then incubated overnight at 28 ºC. Each microorganism was adjusted to a final density of 0.5 McFarland.

Serial two-fold dilution ranging from 0.35 µL/mL to 11.25 µL/mL were made in 96-well plates. Sample wells contained media+ sample+ (10 µL) bacteria, growth control wells contained media+ (10 µL) bacteria, and sterility control wells contained media. All experiments were done tree times.

2.7.4. Statistical analysis

For statistical analysis, SPSS IBM software was used. The significant level was set at $P<0.05$.

3. Results and discussions

3.1. Medicinal plant essential oil

The four employed samples of medicinal herbs were subjected to hydrodistillation. The yields of those essential oils are represented in Table 1.

3.2. Chemical composition of hydrosols

In all, 97.80, 97.14, 99.35, 100, 99.13, 99.82, and 100% of compounds were identified for MS, BP, ZM, TA, $EW_{Method\text{-}A}$, $EV_{Method\text{-}A}$, and method B hydrosols, respectively. Piperitenone (38.27%), carvone (22.07%) and pulegone (14.75%) were as major compounds in MS hydrosol. BP hydrosol contained cuminol (32.48%), cuminic aldehyde (29.35%) and γ-terpinen-7-al (19.41%) as the main ingredients. Carvacrol (55.94%), thymol (40.37%), and p-cymene (0.50%) were the main ingredients in ZM. TA hydrosol had thymol (90.94%), p-cCymene (4.48%), and γ-terpinene (3.34%) as the major compounds. In $EW_{Method\text{-}A}$ hydrosol, thymol (43.74%), carvacrol (33.23%), and cuminic aldehyde (6.38%) were identified as the major ones. $EV_{Method\text{-}A}$ contained thymol (29.71%), carvacrol (27.41%), and piperitenone (15.07%) as the main ingredients. For method B hydrosol sample, major components were thymol (44.46%), carvacrol (20.54%), and piperitenone (10.23%). Table 2 represents the chemical composition of the essential oil obtained from the studied hydrosols.

3.3. Chemical composition of essential oil

Taken together, 97.44, 99.14, 98.52,

Table 2. Chemical composition of hydrosols

NO.	Component	KI[1]	MS[2]	BP[3]	ZM[4]	TA[5]	$EW_{Method\text{-}B}$[6]	$EV_{Method\text{-}B}$[7]	Mixing Sample
1	β-pinene	980.7	0.00	0.00	0.00	0.14	0.00	0.00	0.00
2	3-Octanol	996	0.38	0.00	0.00	0.00	0.15	0.16	0.00
3	p-Cymene	1026.6	0.00	0.00	0.50	4.48	1.94	0.00	0.00
4	o-Cymene	1026.7	0.00	0.27	0.00	0.00	0.00	0.00	1.94
5	Dl-limonene	1030.9	0.00	0.00	0.00	0.00	0.18	0.00	0.00
6	1,8-cineole	1034	9.69	0.58	0.00	0.00	1.75	4.66	4.29
7	Benzene acetaldehyde	1045.4	0.21	0.00	0.00	0.00	0.00	0.00	0.00

Continued Table 2.

NO.	Component	KI[1]	MS[2]	BP[3]	ZM[4]	TA[5]	$EW_{Method-B}$[6]	$EV_{Method-B}$[7]	Mixing Sample
8	γ-terpinene	1060.5	0.00	0.39	0.00	3.34	2.11	0.00	0.87
9	Cis sabinene hydrate	1069.5	0.70	1.18	0.00	0.00	0.00	0.00	0.00
10	α-Terpinolen	1100.9	0.00	0.00	0.31	0.00	0.43	0.28	0.25
11	p-Menth-2-en-1-ol	1123.9	0.14	0.00	0.00	0.00	0.00	0.00	0.00
12	Verbenol	1147.7	0.11	0.00	0.00	0.00	0.00	0.00	0.00
13	Menthone	1156.6	0.14	0.00	0.00	0.00	0.00	0.00	0.00
14	Borneol	1168.8	2.61	0.00	0.00	0.00	0.37	1.02	0.60
15	4-Terpineol	1179.9	1.49	1.72	0.26	0.24	1.00	1.25	0.59
16	p-Cymene-8-ol	1186.6	0.00	0.70	0.10	0.00	0.00	0.20	0.00
17	α-Terpineol	1193.0	1.33	0.81	0.21	0.00	0.48	0.73	0.37
18	Dihydrocarvone	1201.2	1.16	0.00	0.00	0.00	0.00	0.00	0.00
19	Unknown	1221.5	1.93	0.00	0.00	0.00	0.00	2.60	0.46
20	Cis-carveol	1233.8	0.46	0.00	0.00	0.00	0.00	0.32	0.00
21	Pulegone	1243.7	14.75	0.86	0.00	0.00	0.00	0.00	6.21
22	Cuminic aldehyde	1244.5	0.00	29.36	0.00	0.00	6.38	5.40	0.00
23	Carvone	1249.8	22.08	0.00	0.00	0.00	1.97	7.99	6.88
24	Piperitone	1262.6	0.15	0.00	0.00	0.00	0.00	0.00	0.00
25	Unknown	1278.5	0.78	1.71	0.00	0.00	0.00	0.51	0.00
26	α-Terpinen-7-al	1291.1	0.00	19.41	0.00	0.00	0.00	0.68	1.82
27	Thymol	1297.1	0.00	0.00	40.37	90.94	43.74	29.71	44.46
28	Cuminol (p-cymene-7-ol)	1299.4	0.00	32.48	0.00	0.00	2.51	1.24	0.00
29	Carvacrol	1307.24	0.11	0.51	55.94	0.86	33.23	27.41	20.54
30	1-4-p-Menthadien-7-ol	1332.5	0.00	7.16	0.00	0.00	0.33	0.34	0.48
31	Piperitenone	1346.7	38.27	0.00	0.00	0.00	2.11	15.07	10.23
32	Thymyl acetate	1357.4	0.00	0.00	0.12	0.00	0.00	0.00	0.00
33	Eugenol	1360.8	0.00	0.00	0.00	0.00	0.20	0.25	0.00
34	Piperitenone oxide	1373.1	0.12	0.00	0.00	0.00	0.00	0.00	0.00
35	Carvacryl acetate	1375.6	0.00	0.00	0.16	0.00	0.00	0.00	0.00
36	Cis-jasmone	1403.9	0.34	0.00	0.00	0.00	0.00	0.00	0.00
37	trans-Caryophyllene	1426.5	0.22	0.00	0.42	0.00	0.25	0.00	0.00
38	Unknown	1445.5	0.00	0.00	0.77	0.00	0.00	0.00	0.00
39	Germacrene D	1490.1	0.16	0.00	0.00	0.00	0.00	0.00	0.00
40	Caryophyllene oxide	1591.6	0.47	0.00	0.19	0.00	0.00	0.00	0.00
	Known compounds (%)		97.80	97.14	99.35	100	99.13	99.82	100
	Unknown compounds (%)		2.2	2.86	0.65	-	0.87	0.18	-
	Monoterpene (%)		-	0.66	0.81	7.96	4.66	0.28	3.06
	Oxygenated Monoterpene (%)		92.61	93.59	96.88	92.04	94.07	96.27	96.10
	Sesquiterpenes		0.38	-	0.42	-	0.25	-	-
	Oxygenated Sesquiterpenes		0.47	-	0.19	-	-	-	-
	Other (%)		4.34	2.89	1.05	-	0.15	3.27	0.46

[1]Kovats Index. [2]MS: *Mentha spicata* [3]BP: *Bunium persicum*. [4]ZM: *Zataria multiflora*. [5]TA: *Trachyspermum ammi*. [6]$EW_{Method-B}$: Equal weight of method B. [7]$EV_{Method-B}$: Equal essential oil volume of method B.

99.90, 99.20, 98.78, 99.54, and 99.01% of compounds were identified for MS, BP, ZM, TA, $EW_{Method-A}$, $EV_{Method-A}$, $EW_{Method-B}$, and $EV_{Method-B}$ essential oils, respectively. MS es-

sential oils contained carvone (28.91±13.87%), pulegone (16.35±5.46%), and dl-limonene (9.74±1.64%). Cuminic aldehyde (26.76±2.42%), γ-terpinene (24.03±3.95%), and γ-terpinen-7-al (14.10±1.25%) were as the major compounds in BP. For ZM, the major compounds were thymol (36.36±2.44%), carvacrol (30.69±2.44%), and o-cymene (11.87±1.31%). TA essential oils contained thymol (53.44±2.71%s), o-cymene (21.09±1.37%), and γ-terpinene (20.19±1.37%) as the main ingredients. In $EW_{Method-A}$ essential oils, carvacrol (17.95±1.27%), thymol (16.70±1.64%), and γ-terpinene (16.66±1.32%) were identified as the major ones. $EV_{Method-A}$ essential oils contained carvacrol (14.87±2.96%), γ-terpinene (12.27±0.79%), and thymol (11.95±0.77%) as the major compounds. γ-terpinene(19.75±2.28%), o-cymene (19.45±1.47%), and thymol (15.98±3.73%) were the major components in $EW_{Method-B}$. γ-terpinene (15.30±1.95%), o-cymene (15.15±1.44%), and thymol (12.12±0.83%) were identified as the major compounds in $EV_{Method-B}$.

Table 3 represents the chemical composition of the essential oils.

Table 3. Chemical composition of essential oils.

		KI[1]	MS[2]			Bp[3]			Zm[4]			Ta[5]			CSW[6]		MSW[7]		CSE[8]		MSE[9]	
No.	Component		1	2	3	1	2	3	1	2	3	1	2	3	1	2	1	2	1	2	1	2
1	α-Thujene	908.4	0.02	0.05	0.04	0.45	0.54	0.64	0.08	0.11	0.13	0.57	0.65	0.59	0.51	0.55	0.51	0.40	0.22	0.32	0.38	0.45
2	α-Pinene	913.3	0.38	1.21	0.86	0.96	1.34	1.72	1.72	2.42	2.48	0.35	0.32	0.30	1.41	1.59	1.22	0.85	1.12	1.30	1.27	1.42
3	Camphene	925.8	0.11	0.38	0.32	0.03	0.04	0.04	0.11	0.18	0.18	0.02	0.02	0.02	0.09	0.10	0.09	0.05	0.11	0.14	0.14	0.13
4	Sabinen	949	0.24	1.08	0.82	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	β-Pinene	954.2	0.55	1.95	1.43	12.46	9.96	8.17	0.22	0.26	0.27	1.85	1.74	1.63	3.24	3.75	3.73	4.88	3.95	3.43	3.69	3.33
6	β Myrcene	966.6	0.40	0.84	0.65	0.94	0.90	0.95	0.99	1.21	1.11	0.60	0.56	0.52	0.91	1.05	0.90	0.76	0.86	0.85	0.85	0.88
7	L-Phellandrene	980.8	0.00	0.00	0.00	0.51	0.36	0.23	0.13	0.19	0.16	0.04	0.04	0.03	0.20	0.22	0.15	0.20	0.26	0.20	0.18	0.15
8	δ-3-Carene	986.5	0.00	0.00	0.00	0.07	0.09	0.09	0.07	0.12	0.09	0.08	0.07	0.07	0.12	0.12	0.09	0.08	0.10	0.10	0.09	0.10
9	α-Terpinene	994.8	0.15	0.39	0.3	0.22	0.28	0.32	1.01	1.27	1.24	0.46	0.52	0.49	0.75	0.84	0.73	0.52	0.66	0.65	0.64	0.70
10	O-Cymene	1008.4	0.00	0.00	0.00	8.26	8.5	10.79	10.41	12.95	12.26	22.5	21.01	19.76	12.2	13.94	18.4	14.28	12.31	10.71	14.14	16.18
11	dl-Limonene	1010.0	8.46	11.59	9.17	1.32	2.57	3.27	0.49	0.48	0.47	0.00	0.00	0.00	1.53	1.71	0.97	0.75	2.27	2.89	1.64	2.36
12	β-Phellandrene	1011.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.61	0.86	1.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
13	1,8 Cineole	1012.3	1.11	2.33	2.47	0.00	0.00	0.00	0.65	0.73	0.60	0.00	0.00	0.00	1.41	1.67	0.81	0.63	1.99	2.73	1.36	1.57
14	cis-β-Ocimene	1015.1	0.49	0.49	0.4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
15	trans-β-Ocimene	1025.5	0.11	0.13	0.09	0.00	0.22	0.32	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.14	0.14	0.10	0.00	0.13	0.10	0.14

Continued Table 3.

16	γ-Terpinene	1040.5	0.16	0.52	0.43	20.36	23.53	28.20	2.71	3.12	2.94	21.77	19.32	19.48	15.73	17.60	18.14	15.86	13.63	12.83	13.92	16.68
17	cis Sabinene hydrate	1043.7	0.00	0.52	0.66	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
18	α-Terpinolene	1062.7	0.03	0.11	0.10	0.12	0.3	0.29	0.09	0.10	0.10	0.00	0.00	0.00	0.3	0.15	0.11	0.08	0.17	0.14	0.11	0.14
19	L-Linalool	1080.6	0.03	0.17	0.22	0.23	0.22	0.09	0.91	0.62	0.58	0.00	0.00	0.00	0.54	0.49	0.44	0.21	0.71	0.33	0.42	0.45
20	1-Terpineol	1117.7	0.00	0.00	0.00	0.07	0.06	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
21	Menthone	1127.2	0.08	0.41	0.91	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.08	0.00	0.09	0.22	0.29
22	Isomenthone	1136.0	0.12	0.24	0.38	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
23	4-Terpineol	1152.2	1.68	2.33	2.21	0.13	0.26	0.32	0.73	0.68	0.18	0.38	0.06	0.39	0.64	0.71	0.53	0.4	0.88	1.15	0.61	0.75
24	Unknown	1159.3	0.19	0.39	0.41	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
25	α-Terpineol	1168.1	0.00	0.00	0.00	0.00	0.00	0.00	0.46	0.51	0.44	0.10	0.03	0.10	0.78	0.75	0.62	0.63	0.59	0.90	0.51	0.52
26	cis-Dihydrocarvone	1174.6	2.13	1.79	1.85	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.09	0.00	0.00	0.83	0.14	0.19	0.28
27	Neodihydrocarveol	1186.4	4.34	0.70	0.70	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.49	0.00	0.00	0.00
28	Thymyl methyl ether	1208.0	0.00	0.00	0.00	0.00	0.00	0.00	0.94	1.02	0.99	0.00	0.00	0.00	0.19	0.20	0.36	0.28	0.32	0.10	0.26	0.21
29	Pulegone	1212.5	11.20	22.07	15.77	1.76	1.62	1.37	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.26	0.36	0.64	2.54	4.68	3.30	2.70
30	Carvacrol methyl ether	1217.1	0.00	0.00	0.00	0.00	0.00	0.00	0.97	0.95	0.95	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
31	Cuminic aldehyde	1220.4	0.00	0.00	0.00	28.95	27.19	24.16	0.00	0.00	0.00	0.04	0.00	0.00	9.42	9.83	7.67	7.22	11.28	11.10	8.61	8.30
32	Carvone	1224.1	44.69	18.63	23.42	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.08	3.35	2.85	3.59	3.86	11.31	6.17	5.18	6.19
33	α-Terpinen-7-al	1254.4	0.00	0.00	0.00	6.6	5.74	5.35	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.09	0.43	0.48	0.93	1.02	0.88	0.78
34	Isobornyl acetate	1256.3	0.39	0.53	0.44	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
35	γ-Terpinen-7-al	1275.5	0.00	0.00	0.00	14.85	14.79	12.65	0.00	0.00	0.00	0.00	0.00	0.00	3.34	2.94	4.99	4.44	1.69	0.76	4.55	3.99

Continued Table 3.

36	Thymol	1287.0	0.00	0.00	0.00	0.00	0.00	0.00	39.00	34.17	35.92	50.32	54.75	55.25	17.86	15.55	18.63	13.34	9.85	12.48	12.71	11.53
37	Carvacrol	1296.1	0.00	0.00	0.00	0.00	0.00	0.00	31.16	30.08	30.76	0.00	0.00	0.00	18.85	17.06	11.06	10.74	9.31	12.78	12.09	9.25
38	Dihydrocarvyl acetate	1300.2	1.05	0.20	0.61	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
39	Piperitenone	1321.1	0.52	16.2	10.89	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.05	1.82	1.37	1.04	0.00	6.09	2.93	2.92
40	Thymyl acetate	1332.8	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.10	0.74	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
41	Carvacryl acetate	1347.5	0.00	0.00	0.00	0.00	0.00	0.00	0.69	0.74	0.79	0.00	0.00	0.00	0.49	0.58	0.51	0.36	0.00	0.00	0.85	1.00
42	β Bourbonene	1348.9	2.61	1.11	1.77	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.41	0.44	0.85	0.84
43	β Elemene	1357.8	0.27	0.76	0.91	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.36	0.18	0.29	0.23
44	α Gurjunene	1371.0	0.40	0.16	0.18	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
45	trans Caryophyllene	1382.7	8.10	2.95	5.05	0.21	0.04	0.13	2.52	2.53	2.52	0.10	0.00	0.00	1.53	1.48	1.31	1.14	4.05	1.68	1.92	1.64
46	trans α Bergamotene	1399.0	0.00	0.00	0.00	0.07	0.04	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
47	Aromadendrene	1399.2	0.40	0.24	0.36	0.00	0.00	0.00	1.22	1.21	1.23	0.00	0.00	0.00	0.21	0.2	0.40	0.29	0.44	0.15	0.32	0.32
48	cis-Muurola-3,5 diene	1404.7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.14	0.04	0.00	0.00
49	trans-Muurola-3,5 diene	1408.1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.16	0.03	0.00	0.00
50	α Humulene	1413.7	1.30	0.36	0.64	0.00	0.00	0.00	0.20	0.20	0.18	0.00	0.00	0.00	0.10	0.09	0.12	0.09	0.56	0.13	0.20	0.15
51	Allo-Aromadendrene	1420.3	1.30	0.45	1.01	0.00	0.00	0.00	0.17	0.18	0.16	0.00	0.00	0.00	0.13	0.09	0.04	0.04	0.61	0.22	0.04	0.04
52	Caryophyllene(9-epi-E)	1423.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.15	0.11	0.00	0.00	0.29	0.19
53	trans β Farnesene	1425.8	0.00	0.00	0.00	0.20	0.16	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
54	γ Muurolene	1431.6	0.00	0.00	0.00	0.21	0.14	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Continued Table 3.

55	Germacrene D	1441.9	2.00	2.15	3.74	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.40	0.32	0.44	0.35	0.69	0.87	1.01	0.78
56	γ-Amorphene	1447.7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.04	0.00	0.00	0.00	0.00
57	Bicyclogermacrene	1456.3	0.53	0.61	1.11	0.00	0.00	0.00	0.71	0.74	0.81	0.00	0.00	0.00	0.27	0.24	0.30	0.29	0.46	0.31	0.47	0.41
58	Germacrene A	1465.5	0.03	0.37	0.57	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.02	0.00	0.11	0.13	0.09
59	β Bisabolene	1472.6	0.00	0.00	0.00	0.05	0.06	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00
60	γ Cadinene	1475.5	0.20	0.19	0.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.09	0.06	0.09	0.05
61	1s,cis-Calamenene	1484.9	0.73	0.20	0.54	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.04	0.07	0.01	0.22	0.1	0.14	0.11
62	α Cadinene	1499.3	0.18	0.04	0.16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
63	Caryophyllene oxide	1544.5	1.27	3.68	4.06	0.00	0.00	0.00	0.52	1.24	1.02	0.00	0.00	0.00	0.11	0.06	0.20	0.32	0.08	0.19	1.52	0.65
	Known compounds		97.86	98.51	95.93	99.03	98.99	99.40	99.35	97.89	98.30	99.81	99.94	99.97	99.21	99.19	99.67	99.41	98.84	98.72	99.10	98.91
	Unknown compounds		2.13	1.48	4.06	0.96	1.00	0.59	0.64	2.10	1.69	0.19	0.06	0.3	0.78	0.80	0.32	0.59	1.16	1.28	0.89	1.08
	Monoterpene		11.08	18.73	14.59	45.69	48.62	55.03	18.02	22.42	21.44	48.84	45.10	44.14	37.18	41.76	45.13	51.28	31.13	33.68	37.14	42.67
	Oxygenated Monoterpene		65.83	64.86	58.8	52.60	49.87	43.97	73.65	67.82	69.47	50.86	54.85	55.83	58.73	54.30	50.93	45.06	62.07	60.52	53.82	49.73
	Sesquiterpene		18.04	9.58	16.36	0.74	0.49	0.39	4.82	4.86	4.90	0.10	-	-	2.70	2.48	2.89	2.37	5.10	4.33	5.76	4.86
	Oxygenated Sesquiterpene		1.26	3.68	4.06	-	-	-	0.52	1.24	1.01	-	-	-	0.11	0.06	0.20	0.32	0.53	0.19	1.52	0.65
	Other		1.24	1.11	1.68	-	-	-	2.66	2.79	2.48	-	-	-	0.48	0.58	0.51	0.36	-	-	0.84	1.00

[1]Koats Index. [2]MS: *Mentha spicata*. [3]BP: *Bunium persicum*. [4]ZM: *Zataria multiflora*. [5]TA: *Trachyspermum ammi*. [6]S1:$EW_{Method-B}$ (Equal weight of method). [7]S2:$EW_{Method-A}$ (Equal weight of method A). [8]S3: $EV_{Method-B}$ (Equal essential oil volume of method B). [9]S4: $EV_{Method-A}$ (Equal essential oil volume of method A).

3.4. Data analysis with MatLab software

To investigate similarities between the samples, the matrices of data are classified with HCA and PCA methods.

3.4.1. HCA and PCA dendrogram of GC/MS data analysis

HCA and PCA dendrograms of GC /MS data analysis are showsn in Figure 2 and 3, respectively.

Figure 2 shows HCA dendrogram of sam-

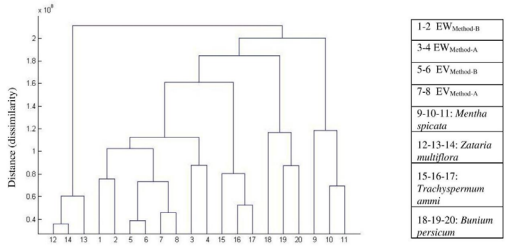

Figure 2. HCA dendrogram of GC/MS data analysis

ples. It investigates the similarities between the same samples showing the reproducibility of the extraction methods.

As shown in Figure 3, the same samples

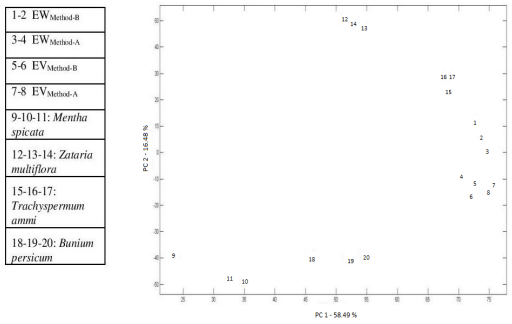

Figure 3. PCA dendrogram of GC/MS data analysis.

are located near each other in 2 dimensional spaces. It shows reproducibility of the extraction procedure.

3.4.2. HCA and PCA dendrogram of GC/MS mean data analysis

HCA and PCA dendrogram of GC/MS mean data analysis are shown in Figure 4 and 5,

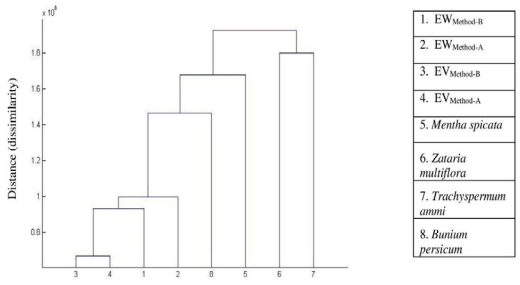

Figure 4. HCA dendrogram of GC/MS mean data analysis.

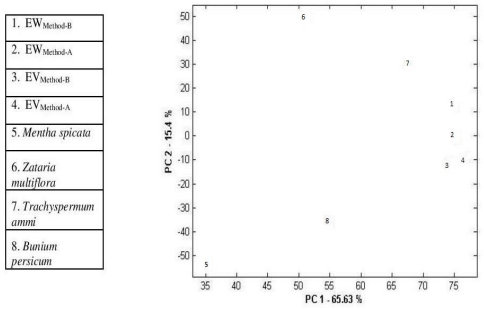

Figure 5. PCA dendrogram of GC/MS mean data analysis.

respectively. To study about similarities between the extraction methods, the mean data of GC/MS analysis were analyzed with MatLab software.

$EV_{Method-B}$ and $EV_{Method-A}$ samples showed the most similarities to each other (Figure 4). PCA dendrogram of GC /MS mean data analysis is shown in Figure 5.

3.4.3. HCA and PCA dendrogram of ATR-IR spectroscopy data analysis

In order to study about the reproducibility of extraction methods, data of ATR-IR spectroscopy was analyzed with MatLab software. HCA and PCA dendrograms of ATR-IR spectroscopy are shown in Figure 6 and 7, respectively.

As shown in Figure 6, the repeated samples represent the most similarities with each other, which shows the reproducibility of extraction methods.

As shown in Figure 7, the repeated samples are located near each other in 2D space, which shows the reproducibility of extraction procedure.

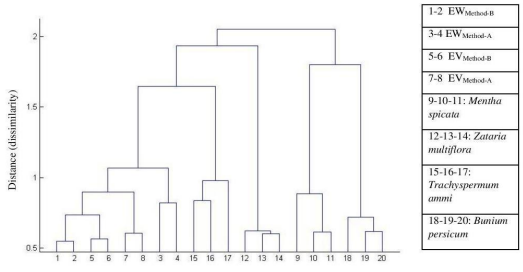

Figure 6. HCA dendrogram of ATR-IR spectroscopy data analysis.

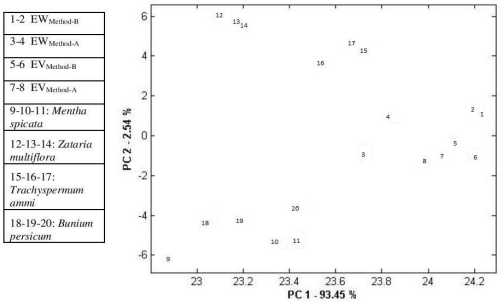

Figure 7. PCA dendrogram of ATR-IR spectroscopy data analysis.

3.4.4. HCA and PCA dendrogram of ATR-IR spectroscopy mean data analysis

HCA and PCA dendrograms of ATR-IR spectroscopy mean data analysis are shown in Figure 8 and 9, respectively. To study about similarities between the extraction methods, the mean data of ATR-IR spectroscopy were analyzed with MatLab software.

In HCA, as displayed in Figure 8,

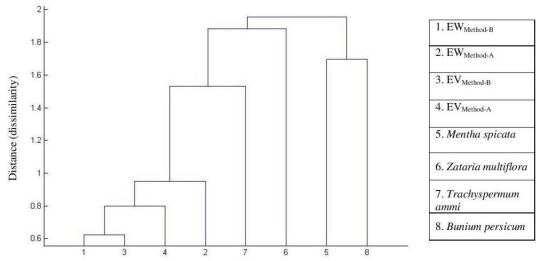

Figure 8. HCA dendrogram of ATR-IR spectroscopy mean data analysis.

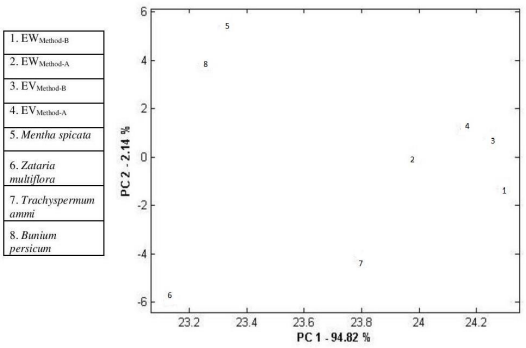

Figure 9. PCA dendrogram of ATR-IR spectroscopy mean data analysis.

$EW_{Method-B}$ and $EV_{Method-B}$ samples show the most similarities to each other. PCA dendrogram ATR-IR spectroscopy mean data analysis is shown in Figure 9.

3.5. Antimicrobial activity

Antimicrobial assays including disc diffusion method for measuring the diameter of inhibition zone and microdilution broth method to determine MIC, MBC, and MFC were done.

3.5.1. Disc Diffusion assay for hydrosols

Disc diffusion assay for hydrosols showed no significant results compared to the control disc.

3.5.2. Disc diffusion assay for essential oils

The diameter of inhibition zone of essential oils, are represented (Inhibition diameter (mm)±SD (mm)) in Figure 10. For *E. coli*, the best result was in $EW_{Method-B}$ (10.33±0.58

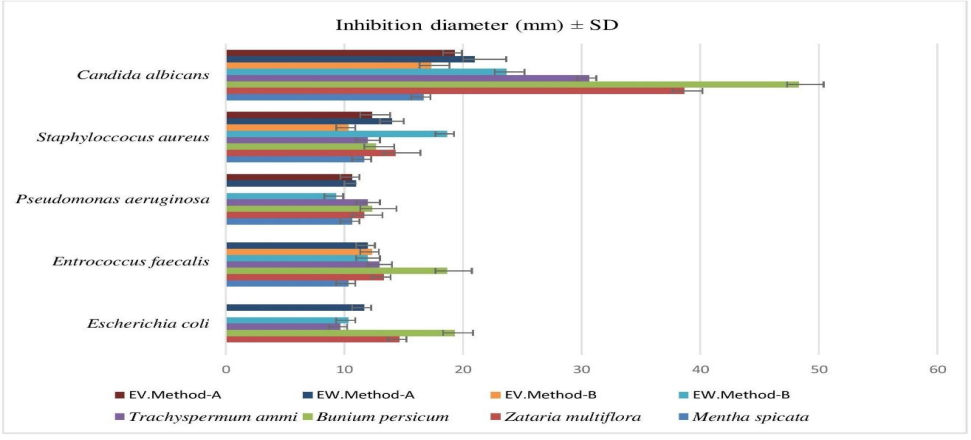

Figure 10. Disc diffusion results for essential oils (Inhibition diameter (mm)±SD).

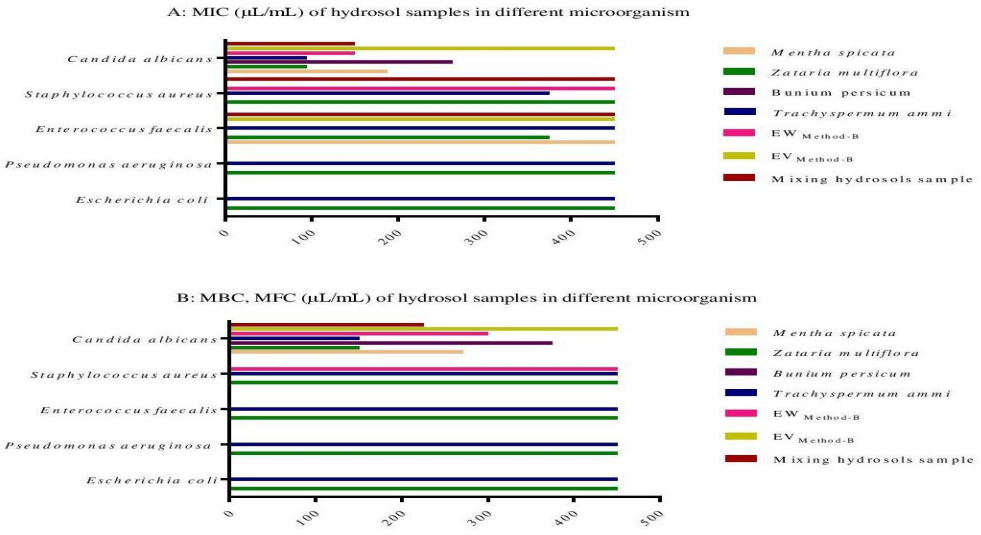

Figure 11. MIC (part A), MBC and MFC (part B) hydrosol samples in different microorganism.

mm), but no significant antimicrobial activity was shown in $EV_{Method-B}$ and $EV_{Method-A}$ samples compared to the control group. The best result of *E. faecalis* was for $EV_{Method-B}$ (12.33±0.58 mm), while $EV_{Method-A}$ had no significant antimicrobial activity. $EW_{Method-A}$ with 11.00±0.00 mm showed the best result for *P. aeruginosa*, but no significant antimicrobial activity was shown in $EV_{Method-B}$. The best result of *S. aureus* was for $EW_{Method-B}$ with 18.67±0.58 mm and the lowest antimicrobial activity, (10.33±0.58 mm), was for $EV_{Method-B}$. For *C. albicans*, $EW_{Method-B}$ had the best result (23.67±1.15 mm), whereas $EV_{Method-B}$ had the lowest antimicrobial activity (17.33±1.53 mm).

3.5.3. Microdilution broth for hydrosols

Results of microdilution broth assay for hydrosols sample are shown in Figure 11. For *E. coli* and *P. aeruginosa*, there was no significant MIC and MBC compared to the control group. For *E. faecalis*, the MIC of $EV_{Method-A}$ and also method B samples were 450 µL/mL. For *S. aureus*, the MIC of $EW_{Method-A}$ and method B were 450 µL/mL; and $EV_{Method-A}$ sample did not show a significant effect compared to the control. For *C. albicans*, the best MFC was 150 µL/mL, which was for $EW_{Method-A}$ and method B sample. Tthe best MBC was 225 µL/mL, which was observed for method B sample.

3.5.4. Microdilution broth for essential oils

Figure 12 shows results of microdilution Broth broth assay of essential oils. For *E. coli*, the best antimicrobial activity was MBC=2.81±0.00 µL/mL and MIC=3.28±2.14 µL/mL, for $EW_{Method-B}$ sample. For *P. aeruginosa*, two samples had the same and the best MBC, method B samples for both group with MBC=7.50±3.25 µL/mL and the best MIC=4.68±1.62 µL/mL was for $EW_{Method-B}$ sample. For *E. faecalis*, the best MBC was for $EV_{Method-A}$ sample with MBC=3.74±1.62µL/mL and the best MIC was for $EW_{Method-B}$ sample with MIC=1.64±1.07 µL/mL. In *S. aureus*, the sample of $EW_{Method-B}$ had the best and the same MIC and MBC, which was 1.40±1.21 µL/mL. For *C. albicans*, the sample of $EW_{Method-A}$ had the best MBC and MFC, which were 1.64±1.07 µL/mL and 1.06±0.61 µL/mL, respectively.

4. Conclusion

Essential oils and hydrosols are the most useful preparations of TPM. The goal of this study was to find out the appropriate method for extracting mixture samples. For this purpose, the major components and pharmacological effects were considered as evaluation criteria.

In Table 2, the major components of mixture hydrosols are shown. For $EW_{Method-A}$ sample, the major components were thymol, carvacrol, and cuminic aldehyde. For $EV_{Method-A}$, the major

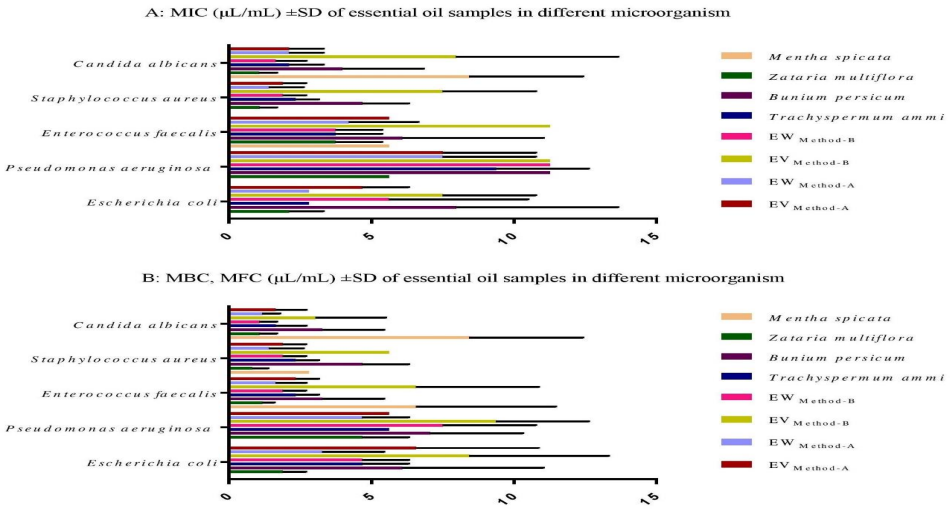

Figure 12. MIC (part A), MBC and MFC (part B) of essential oil samples in different microorganism.

components were thymol, carvacrol, and piperitenone. For method B sample, the major components were thymol, carvacrol, and piperitenone. The first and the second major components were already the same. But the percentage of these components was different.

The major components of mixing essential oils are shown in Table 3. For $EW_{Method\text{-}A}$ sample, the major components were carvacrol, thymol, and γ-terpinene. For $EV_{Method\text{-}A}$ sample, the major components were carvacrol, γ-terpinene, and thymol. For $EW_{Method\text{-}B}$, the major components were γ-terpinene, o-cymene, and thymol. For $EV_{Method\text{-}B}$, the major components were γ-terpinene, o-cymene, and thymol. For both methods, the first major components were the same, and the order or the percentage of the two other major components were different. It means that the different processes for mixing samples can affect the profile of major components.

HCA and PCA dendrograms of GC/MS mean data analysis, which are shown in Figure 4 and 5, respectively, indicate that $EV_{Method\text{-}A}$ and $EV_{Method\text{-}B}$ have the most similarity with each other, then $EW_{Method\text{-}A}$ is similar to them, and the least similar is $EW_{Method\text{-}B}$.

The main difference (between HCA and PCA dendrograms) of ATR-IR spectroscopy and GC/MS is that GC/MS shows known components but ATR-IR is like a finger print. Therfore, if there is an unknown component, ATR-IR spectroscopy can show it.

Figure 2, 3 and also 6, 7, and HCA and PCA dendograms of hydrosols and essential oils indicated the reproducibility of methods and analysis procedure.

The result of HCA and PCA dendrograms (Figure 8 and 9) of ATR-IR spectroscopy mean data analysis showed that both samples of method A had the most similarity to each other. This result is almost shown in the major component analysis as well.

Despite the increasing use of medicinal plants and the important role of dosage form in traditional treatment process, no comprehensive evaluation has been done about different extraction methods till now. To investigate the best method for extraction of mixing dosage forms, two popular dosage forms, hydrosol and essential oil, were considered. Besides, for studying about different extraction methods two criteria were evaluated: chemical composition and pharmacological effects.

To consider the different extraction methods of mixing samples, which could be extracted together or extracted individually then mixed together, differences in the profile of chemical composition and pharmacological activities were observed. Therefore, it is better to study about the

specific pharmacological effect that is expected from the special dosage form and standards of the specific method for extraction of that dosage form.

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Conflict of Interest

None declared.

5. References

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