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The Effect Of Different Essential Oil Extraction Methods On The Efficiency And Antibacterial Properties Of Myrtus Communis L. Leaves

Abdollah Dadazadeh[1], Hassan Nourafcan[1]*

[1] *Department of medicinal plants, Miyaneh Branch Islamic Azad University, Miyaneh, Iran*
First author:e-mail: a.dadazadeh@gmail.com
**Corresponding author:e-mail: nourafcan@m-iau.ac.ir,*

Abstract

The effect of different essential oil extraction methods on the efficiency, composition and antibacterial properties of *M. communis* L. leaf essential oil was studied. *M. communis* L. essential oil was prepared by three methods: hydro distillation, steam distillation, and hydro-steam distillation. The highest essential oil efficiency was related to hydro-steam distillation, which was significantly different from the other two methods. 29 essential components were identified in GC/MS analysis, of which 7 essential components, α-pinene**,** 1,8 -cineol**,** linalool, decane, linalyl acetate, α-terpineol, camphene, were the most predominant compounds and showed significant differences in the three different essential oil extraction methods. The antibacterial effect of essential oils obtained from the three distillation methods in different concentrations on three Gram-positive bacteria (*Staphylococcus aureus*, *Streptococcus mutants*, *Staphylococcus epidermidis*) and three Gram-negative bacteria (*E .coli*, *Pseudomonas aeruginosa*, *Salmonella typhimuriu*) was investigated, which revealed significant differents among them. The important result was that the essential oil obtained by steam distillation at a concentration of 80µL/mL had a more active antibacterial effect on Staphylococcus aureus and *Streptococcus mutans*, to the extent that it had the same effect as antibiotics, and in some cases, it was even more active than antibiotics.

Keywords; Antibacterial, Anti-infection, Essential oil, Herbal medicine, *Myrtus communis* L.

Introduction

Myrtus communis L. is an aromatic tree of the Myrtaceae family with an average height of 5 meters. Regarding ethnobotany, this plant has a lot of branches which are straw like, and it is flowers are singular with white petals that are covered with fine hairs. It has blackish-blue egg-shaped fruits with the same size as the chickpeas ranging from 1 cm to 3 cm (1). This plant has life duration of 300 years (2), is adaptable to various kinds of soil, and can grow in silica soil or calcareous soil. It can also endure extreme cold and hot weather (2).

M. communis L. has a vast geographical distribution over the Mediterranean (3) and South European regions (2), Asian and Middle Eastern countries such as Afghanistan, Saudi Arabia, Iraq (4) and Iran. It has been reported that 16 species of *M. communis* L. are distributed over Middle East and Asia (3). This plant has various applications in pharmaceutical industry (5), cosmetics and aromatherapy (6), and food industry (7). It is a rich source of natural and antimicrobial compounds, and it is used as an organic preservative in the food industry (3). It has also been used as food flavor in the production of sausages (8). Regarding ethnopharmacology, this plant is used as a herbal medicine due to it is phytochemical compounds and different Bioactive materials(4). The leaf of this plant constitutes phytochemical compounds, these include essences (1,8-cineole, α-pinene, geranyl acetate, linalool, etc.), flavonoids, caffeine, coumarin, galloyl glucoside, glycolic acid, ellagic acid, fatty acids (palmitic acid, linoleic acid, oleic acid and stearic acid), etc. (9). *M. communis* L. leaves have many application in traditional medicine and phytotherapy to treat vascular diseases such as; hemorrhoids (9, 10), hematoma (2), diarrhea and Diabetes (4), bronchitis (8), tuberculosis (2), whooping cough (9), indigestion (2), infections such as urinary tract infection (8, 10), otitis (5), stomatitis (9) and herpes simplex (2), psoriasis (2, 10), eczema (4), abscess (8), hair loss (2), wound healing (9), menorrhagia and vaginal prolapse (5), eye diseases (10), prostatitis (5), menstrual disorder (11) and hydatid cyst(12). It has also been used as a contraceptive (11), as an antiparasitic drug for treating plasmodium resistant to chloroquine treatments (9), as a repellent of Anopheles (Marsh Mosquito) which is the cause of Malaria (12), and as an antidote for spider and scorpion bites (9). Intriguing studies has been done on the different aspects of *M. communis* L. For instance, WHO has verified the effectiveness of *M. communis* L. in repelling the anopheles in the case of fighting malaria (12). It has also been approved that *M. communis* L. can deactivate the hydatid cyst, which can prevent patients from going under

surgery and using drugs with severe side effects such as toxic hepatitis, leukopenia, thrombocytopenia (13). In addition, the essential oil of *M. communis* L. has antibacterial, antiviral and antifungal properties (6), which is why it is of great importance in the pharmaceutical industry. It can also be an intriguing food preservative because of it is natural properties when compared to the synthetic preservatives such as benzoic acid, sorbic acid, nitrates, sulfites, nitrites and propionic acid. Due to the research development and improvement of the general knowledge of the people, synthetic preservatives have lost their popularity, and demand for organic preservatives is increasing. Not only do organic preservatives prolong the lifespan of food, they can also provide food safety because of their natural components. The essential oil of *M. communis* L. is one of the options to be used as an organic preservative which can prolong the lifespan of foods especially meat (8). One of the topics discussed is the effect of *M. communis* L. leaf essential oil on *Staphylococcus aureus*. *Staphylococcus aureus* is one of the five most common causes of nosocomial infections, especially in postoperative wounds. About 500,000 people in US hospitals get staph infections each year, ranging from simple skin infections to deadly infections such as meningitis, heart infections, pneumonia, bone and joint infections, blood infections, and septicemia (14). After the discovery of penicillin, this antibiotic was used to treat the diseases caused by staphylococcal infections, but the strains of this bacterium became more and more resistant to this drug to the extent that in 1950, the resistance of

Fig **1.** Myrtus communis L.

Complete *M. communis* L shrub
Source: https://plantsexpress.com

M. communis L fruit
Source: https://antropocene.it

Dried *M. communis* L e leaves
Source: authors

these strains in the hospital reached 40% and increased to 80% by the year 1960, a trend which seems to become extremely worrying in the long run (15). These events indicate the importance this study holds, as microbial resistance to antibiotics is becoming a crucial issue. This incident has led to naming the year 2011 "Antimicrobial resistance, a dire threat to life" by World Health Organization (WHO). The antimicrobial resistance not only is dangerous but also escalates the expenses for treating the infections and diseases. For instance, the EU has spent 1.5 billion euros of treating infectious diseases of over 25000 patients. Also, this problem has cost 35 billion dollars for the USA (16, 17), though there are no statistics of the whole world at hand. Consequently, the identification of natural compounds that have desirable antimicrobial effects can be an alternative to using antibiotics and preservatives of food and cosmetics.

1. Materials and Methods

1.1. Materials

1.1.1. Plant material

M. communis L. plant leaves were purchased from Tabriz medicinal plants market (East Azerbaijan province, Iran) and it was registered and verified with the voucher number 7250 in East Azerbaijan's Agricultural Research Education and Extension Organization herbarium. The plant list website's record number for this plant is 132410. The leaves of this plant were gathered and chopped into pieces, and their essential oil was extracted (Fig. 1).

1.1.2. Chemical material

Hexane, DSMO solvent and Sodium sulfate were purchased form Merck (Germany), and %96 ethanol were purchased from Kimia Alcohol Zanjan (Iran).

1.1.3. Microbial material

The standard microbial susceptibility strains of *Staphylococcus aureus* (American Type Culture Collection (ATCC) 25923), *Streptococcus mutans* (ATCC 35668), *Staphylococcus epidermidis* (ATCC 12228), *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 14028) and *Pseudomonas aeruginosa* (ATCC 27853) were purchased from Bahar Afshan company. Blank discs and 12 antibiogram antibiotics were purchased from Padtan Teb company. Culture medium of Brain Heart Infusion Broth (BHI Broth), of Ibresco brand and culture medium of Mueller Hinton Agar (MHA) and Tryptone Soya Broth (TSB), Spain's Scharlau brand and Serum ringer tablet of Merck brand were purchased.

1.2. Methods

1.2.1. **Hydro distillation method (HD)**

Hydro distillation was performed according to the method by Khalil and Li (18) with slight modifications. 200 g of dried *M. communis* L. was added to 2000 mL distilled water (weight to volume ratio: 1 to 10). Afterwards, the mixture was poured into a Clevenger particular for hydro distillation and was heated for 6 h. The obtained essential oil was dehydrated using Sodium sulfate (2, 19). It was then poured into dark glass jars and kept at 4℃ until the experiment time (20, 21).

2.2.2. Steam distillation method (SD)

Steam distillation was performed according to the method by Khalil and Li (18) with slight modifications. 200 g of dried *M. communis* L. was added to 2000 mL distilled water (weight to volume ratio: 1 to 10). Afterwards, the mixture was poured into a Clevenger particular for steam distillation and was heated for 6 h. The obtained essential oil was dehydrated using sodium sulfate (2, 19). It was then poured into dark glass jars and kept at 4℃ until the experiment time (20, 21).

2.2.3. Hydro-Steam distillation method (HSD)

Hydro-steam distillation was performed according to the method by Khalil and Li (18) with slight modifications. 200 g of dried *M. communis* L. was added to 2000 mL distilled water (weight to volume ratio: 1 to 10). Afterwards, the mixture was poured into a Clevenger particular for hydro-steam distillation and was heated for 6 h. The obtained essential oil was dehydrated using sodium sulfate (2, 19). It was then poured into dark glass jars and kept at 4℃ until the experiment time (20, 21).

1.3. GC/MS analysis

GC/MS (GC Agilent USA 6890N, MS Agilent USA 5973N) with HP-5MS 19091S-433 column ($0.25 \times 30\ m$ in 0.25 μm) was used to determine the extracted essential oil compounds according to the method described by Khalil & Li (18). The helium was used as the mobile phase and the carrier was used with incremental rate of $1\ \mathrm{mL/min}$ (19). The essential oil was diluted ten times with hexane (2), and then $1\ \mu\mathrm{L}$ of the solution was injected to the device. GC/MS was programmed to execute the following procedure (19):

a) Keep the temperature at $60℃$ for one minute.

b) Raise the temperature to $140℃$ at the rate of $2.3\ ℃/\mathrm{min}$.

c) Raise the temperature to $240℃$ at the rate of $25\ ℃/\mathrm{min}$.

d) Keep the temperature at 240℃ for one minute.

The voltage applied for ionization was 70 eV, and Chemstation plus Wiley 7.1 software was used to analyze the data (18). In order to determine the essential oil sample retention index (RI^b), sigma retention standard was used. This standard consists of a mixture of aliphatic hydrocarbons ranging from C8 to C32, solved in hexane. The literature retention index (RI^a) was obtained from NIST website, Babushak et. al. (22) and Goodner (23) in accordance with the column type of the used GC/MS device (22, 23).

1.4. Antibacterial activities

Disk diffusion method: For determining the antibacterial effect, disk diffusion test was applied in the following manner (Based on the CLSI 2011 standards). Bacterial suspensions and a 0.5 McFarland solution were prepared. In order to prepare the bacterial suspensions, 2 mL sterilized BHI Broth was injected into a bacteria stub culture which was in a lyophilized ampoule. Afterwards, the bacteria were incubated for 4 hours at 37℃. Then they were cultivated in BHI Agar. Later on, 3-5 colonies were picked up and solved in ringer's solution in a way that its turbidity was equal to that of 0.5 McFarland ($1 - 2 \times 10^8$ CFU/mL). For better accuracy, the solutions were poured into test tubes and compared in front of a white paper with black stripes (24).

Bacterial inoculation in MHA culture medium: For the inoculation and cultivation of the bacteria, a sterile cotton was swap dipped into the test tubes containing 0.5 McFarland bacterial suspensions, and then applied on the culture medium in a zig-zag pattern. In order to remove the excess moisture, the plates' lid was left half open for a few minutes.

Disc placement, incubation and recording results: DMSO solvent was used as the diluter of the essential oils. DMSO was passed through bacteriological filter for sterilization. The essential oils were diluted with DMSO in the orders of $5, 10, 20, 40, 80$ µL/mL. Blank sterile disks were impregnated with the aforementioned concentrations and inoculated into the culture medium. To prevent the evaporation of the essential oils, the plates were kept in a refrigerator at 4℃. After the stabilization of the essential oils and distribution of them through the culture medium, they were transferred to the incubator and were kept there for 24 h at 35 ± 2℃. Eventually, the formed inhibition zones were measured and reported (18).

2.5. Determination of Minimum Inhibitory Concentration (MIC)

In order to determine the MIC of the bacteria based on the CLSI 2012 guidelines, the () method was applied with some modifications (25). The 96-well microtiter plate was used in the experiment. 100 µL of MHB was poured into the wells. The Two-fold serial dilution was applied to achieve the desired concentrations from 2000 µL/mL to 7/8 µL/mL. The dilution was processed as follows: 4 ml of the essential oil was solved in 1 ml %10 DMSO. 100 µL of the solution was poured into the first well. After homogenizing the liquid, 100 µL of the first well was drawn up and transferred to the second well. This process continued to the ninth well. The ninth drawn up solution was discarded. 5 µL of the bacterial suspension with a turbidity equal to that of 0.5 McFarland ($1 - 2 \times 10^8$ CFU/mL) was poured into each well except the ninth, tenth and eleventh wells. These wells were negative control wells. The tenth well contained only culture medium, and the eleventh well contained culture medium with DMSO solvent, while the twelfth well-acted as a positive control and contained culture medium with bacterial suspension. Afterwards, the 96-well microtiter plate was incubated for 18 h at 36℃. Subsequently, the turbidity of the wells was measured. The clear wells were recorded as the MIC results, and the turbid wells were neglected (25, 26). It is worth noting that for %10 DMSO preparation, normal Saline was used (27).

2.6. Determination of Minimum Bactericidal Concentration (MBC)

The aforementioned clear wells selected in the MIC test underwent the MBC test. 100 µL of the clear wells were cultivated separately on MHA. These plates were incubated for 24 h at 37℃. The plates containing no bacteria colonies and with the least essential oil concentrations were reported as the MBC results (26, 28, 29).

2.7. Statistical analysis

The factorial experiment was performed in a Completely Randomized Design using SPSS statistics software. The data (efficiency of distillation methods, value of the components in the essential oil and antibacterial effect)

were gathered through 3 replications, and their mean values and deviations were calculated. For the comparison of the mean values of the data, the Duncan's new multiple range test was applied ($p \leq 0.05$).

2. Results and discussion

2.1. Efficiency of different distillation methods

In this study, the leaves of *M. communis* L. were distilled by three different methods (Hydro, steam and hydro-steam), and the essential oil yields of these methods were compared with each other. The compounds of each essential oil were analyzed with GC/MS and compared with each other. Afterwards, the antibacterial effects of these three essential oils were investigated on three Gram-positive bacteria (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus mutans*) and three Gram-negative bacteria (*Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*), and then were compared with the antibiogram results of 12 types of antibiotics (Tobramycin, Cefalexin, Gentamicin, Ceftriaxone, Ampicillin, Ciprofloxacin, Tetracycline, Vancomycin, Cloxacillin, Penicillin, Erythromycin, Amikacin).

2.2. Essential oil yields of different extraction methods

Different extraction methods have different essential oil yields. Some of these methods have qualitative values and some have quantitative values, and one of these methods has both qualitative and quantitative values. The hydro distillation, steam distillation and hydro-steam distillation were performed to extract the essential oils of *M. communis* L., and the essential oil yields were obtained (Table 1).

No.	Extract method	Dry Plant (g)	Essential Oil Content (mL)	Essential Oil Yield (%)
1	Hydro Distillation	200	1.9^{a*}	0.95
2	Steam Distillation	200	1.15^{b}	0.88
3	Hydro-Steam Distillation	200	1.17^{b}	1.58

Table 1. Efficiency of *Myrtus communis L.* essential oil extraction via different distillation methods.

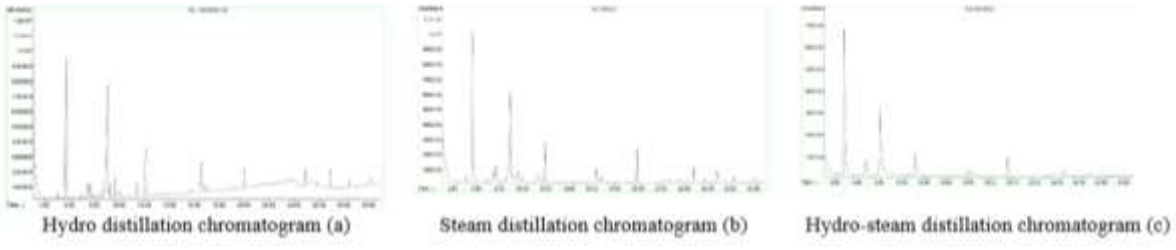
	Sum of squares	df	Mean square	F	Sig.
Between Groups	1.096	2	.548	342.438	0.00
Within Groups	.010	6	.002		
Total	1.105	8			

*Different small letters show significant differences ($p<0.05$) between data obtained with different distillation methods.

The essential oil yield obtained by HD was %0.95. The essential oil yield obtained through HSD was %0.88, and the essential oil yield obtained by SD was %0.58. Therefore, HD method was the most efficient method of them all.

According to the previous reports in "PDR Herbal medicine", the essential oil yield of *M. communis* L. is between %0.1 to %0.5 (1). In Brada's study, the fruits and leaves of *M. communis* L. were distilled using HD

Fig 2. Chromatograms of essential oils obtained by various distillation methods



method, and it was reported that the essential oil yield of the fruits was %0.1 (w/w) and the essential oil yield of the leaves was %0.3 (30).

Another study showed that the MSD method not only lessened the duration of distillation but also had

higher essential oil yield than that of SD method (6).

2.3. GC/MS analysis of the essential oils

(Fig. 2) shows the chromatograms related to the three extraction methods, which 29 compounds were identified through analyzing *M. communis* L. essential oil, among which the most dominant

components in all the three methods were α-pinene, 1,8-cineole, linalool L, decan, linalyl acetate, α-terpineol and camphene. The sum of these seven compounds (Fig. 3) made up %71.18 of the essential oil from HD, %60.74 of the essential oil from SD, and %71.07 of the essential oil from HSD (Table 2)

No.	Component	RI[a]	RI[b]	HD%	SD%	HSD%
1	Propanoic acid	740	743	0^{c**}	0.58^{a}	0.45^{b}
2	*α*-Thujene	927	929	0^{b}	0^{b}	0.12^{a}
3	***α*-Pinene**	**936**	**339**	**27.87^{b}**	**22.70^{c}**	**28.85^{a}**
4	**Camphene**	950	951	**2.68^{a}**	**2.35^{b}**	**1.89^{c}**
5	*β*-Pinene	977	**980**	0^{a}	0.34^{a}	0.24^{b}
6	*β*-Myrcene	989	987	0^{b}	2.20^{a}	0^{b}
7	**Decane**	1000	1002	**3.84^{b}**	**2.34^{c}**	**5.19^{a}**
8	*δ* – 3- Caren	1011	1012	0^{b}	0^{b}	1.39^{a}
9	*β*-Terpinene	1017	1016	0^{b}	0^{b}	0.42^{a}
10	*p* – Cymene	1024	1027	0.53^{b}	0.75^{a}	0.32^{c}
11	**1,8 -Cineol**	**1031**	**1034**	**20.20^{b}**	**19.03^{c}**	**21.76^{a}**
12	*β* -Ocimene	1038	1039	0^{b}	2.16^{a}	0^{b}
13	*α* -Ocimene	1041	1040	0^{c}	2.58^{a}	2.21^{b}
14	*γ*-Terpinene	1059	1062	0^{b}	1.04a	0b
15	**Linalool L**	1099	1101	**9.27a**	**6.54^{b}**	**6.55^{b}**
16	Veratal	1112	1115	0^{b}	0.91^{a}	0^{b}
17	Benzoic acid	1159	1160	0^{c}	0.58^{a}	0.45^{b}
18	α-Terpinolene	1186	1185	0.34^{c}	2.34^{a}	1.51^{b}
19	***α*-Terpineol**	**1189**	**1190**	**3.07^{a}**	**2.84^{a}**	**2.45^{b}**
20	Dodecane	1200	1204	1.71^{a}	1.24^{b}	2.03^{a}
21	Nerol	1228	1230	0^{b}	0^{b}	0.7^{a}
22	**Linalyl acetate**	**1255**	**1258**	**4.25^{b}**	**4.94^{a}**	**4.38^{b}**

23	Carvacrol	1300	1303	0^{c}	0.74^{a}	0.11^{b}
24	Neryl acetate	1309	1311	0.48^{a}	0.50^{a}	0^{b}
25	Geranyl acetate	1361	1360	1.63^{a}	0^{c}	1.11^{b}
26	Tetradecane	1416	1416	0.8^{a}	0^{c}	0.33^{b}
27	Caryophyllene (E)	1419	1421	1.15^{a}	0.85^{b}	0.61^{c}
28	α-Humulene	1458	1455	1.35^{a}	1.23^{a}	0.91^{b}
29	Geranyl propionate	1476	1479	0^{b}	2.23^{a}	0^{b}
+	Total	-	-	79.17	80.43	83.53

Table 2. Components of *Myrtus communis L.* via various distillation methods

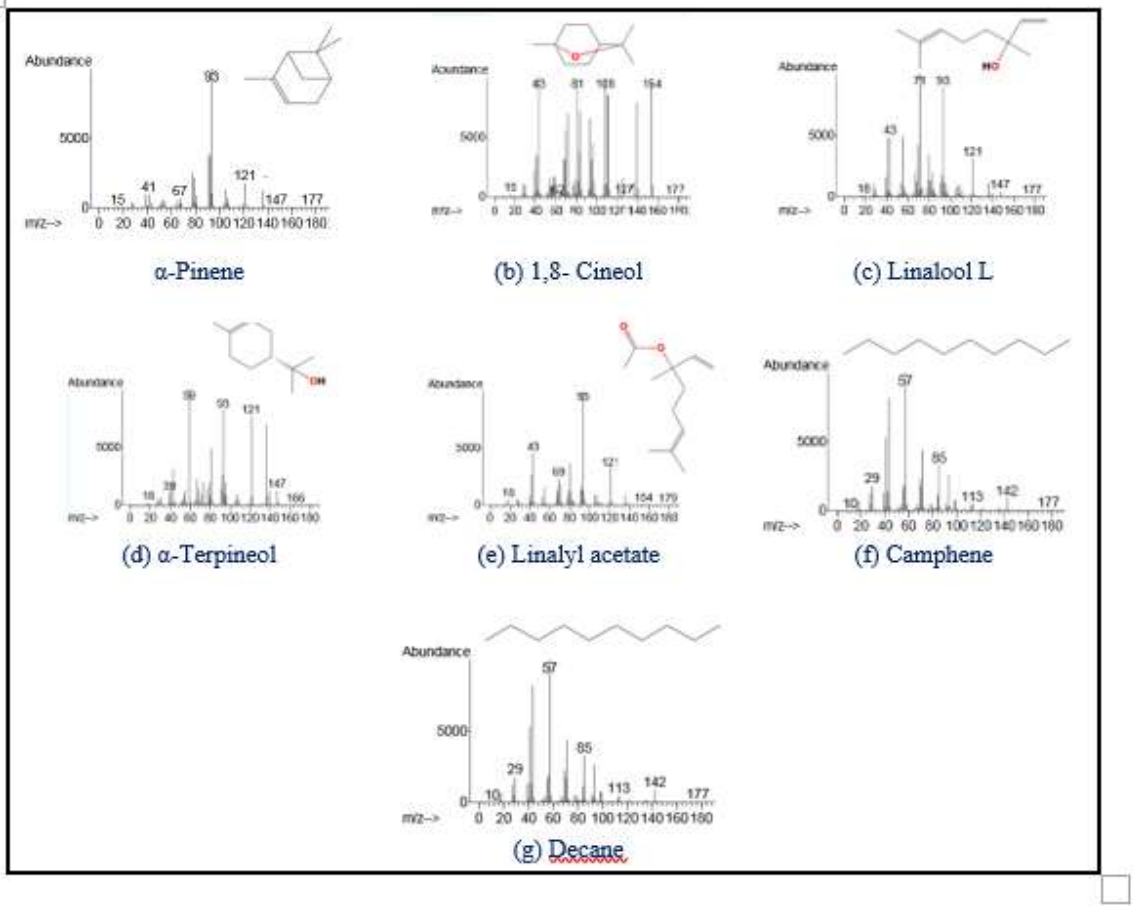
*The bold items are the dominant compounds found in each method.

** Different small letters show significant differences (p<0.05) between data obtained with different distillation methods.

RT, Retention time

RI[a], sample Retention indices

Fig 3. Spectrum and molecular structure (Courtesy of NIST Chemistry WebBook) of essential oil components



RI[b], literature retention index reported in the literature on HP-5MS column taken from NIST and of the computer mass libraries Adams

HD: Hydro distillation method, **SD**: Steam distillation method, **HSD**: Hydro-Steam distillation meth

In a previous study, 32 compounds of *M. communis* L. essential oil were identified, of which the most dominant ones were α-pinene, limonene, cineol, Linalool, α-terpineol and linalyl acetate in sequence (7), which accords with the results of the present study. Also, another study reported α-pinene, limonene, 1,8-cineole, linalool, α-terpineol, linalyl acetate, α-terpineol acetate and geranyl acetate as the dominant compounds of *M. communis* L. essential oil (31), which relatively matches results of this study. α-pinene, as the first dominant compound of *communis* L. essential oil in all the three methods, composed %28.85 of the essential oil in HSD method, %22.7 of the essential oil in SD method and %27.87 of the essential oil in HD method. These results had significant differences from one another. The second dominant compound was 1,8-cineole, which composed %21.76 of the essential oil in HSD method, %19.03 of the essential oil in SD method and %20.2 of the essential oil in HD method. Other studies have also reported α-Pinene as the most dominant compound and 1,8-cineole as the second dominant compound in *M.*

communis L. essential oil. The values for α-pinene in these studies were %29.1 (7), %31.31 (31), %15.59 (32) and %23.7 (33), and the values for 1,8-

cineole were %25.2 (30), %16.55 (32) and %17.9(7). These studies indicate relatively similar results to those of the present study. Other dominant compounds of *M. communis* L. essential oil viz. linalool, decane, linalyl acetate, α-terpineol, camphene, etc. showed significant differences in distillation methods, which indicated that distillation methods affect the quality of the extracted essential oil. Also, other dominant compounds, namely β-myrcene, o-cymene and geranyl propionate were only reported in the essential oil extracted through HSD, which can be a distinguishing factor in identifying the distillation method. Identifying the method in which α-pinene and 1,8-cineole have higher values in the essential oil is important due to their antibacterial property and their application in the food industry, cosmetics and pharmaceutical industry.

2.4. Agar disk diffusion test

The essential oils obtained from the leaves of *M. communis* L. had different antibacterial effects based on their distillation methods (Table 3).

Table 3. Antibiogram test results (mm) of *Myrtus communis* L .essential oil extracted by different methods

		Concentration of the essential oils (µL/mL) obtained by different methods														
		Hydro Distillation					Steam Distillation					Hydro-Steam Distillation				
		5	10	20	40	80	5	10	20	40	80	5	10	20	40	80
Gram-Positive	*Staphylococcus aureus*	0^{f*}	0^{f}	$8.3^{e}\pm0.4$	$13.3^{c}\pm0.4$	$18^{b}\pm0.8$	0^{f}	0^{f}	0^{f}	$7.3^{e}\pm0.4$	$11.3^{d}\pm0.4$	0^{f}	$8.3^{e}\pm1.2$	$10.6^{d}\pm0.4$	$17.6^{b}\pm1.2$	$28.3^{a}\pm0.9$
	Staphylococcus epidermidis	0^{j}	$7.3^{i}\pm0.4$	$14.3^{gh}\pm0.4$	$16.6^{de}\pm0.4$	$21.6^{b}\pm1.2$	0^{j}	0^{j}	$13.6^{h}\pm0.4$	$15.6^{ef}\pm0.4$	$19.6^{c}\pm0.4$	0^{j}	$7.3^{i}\pm0.4$	$15^{fg}\pm0.8$	$17.6^{d}\pm0.9$	$27.6^{a}\pm0.4$
	Streptococcus mutans	0^{g}	$7.3^{f}\pm0.4$	$9.6^{de}\pm0.5$	$14.6^{b}\pm0.4$	$16.3^{a}\pm0.4$	0^{g}	0^{g}	$7.3^{f}\pm0.4$	$10.6^{d}\pm0.9$	$12^{c}\pm0$	$0^{g}.2$	$7.3^{f}\pm0.9$	$9.3^{e}\pm0.5$	$13.6^{b}\pm0.9$	$16.3^{a}\pm0.9$
Gram-Negative	*Pseudomonas aeruginosa*	0^{e}	0^{e}	0^{e}	$8.3^{cd}\pm0.4$	$9.6^{b}\pm0.4$	0^{e}	0^{e}	0^{e}	$7.6^{d}\pm0.4$	8^{cd}	0^{e}	0^{e}	0^{e}	$7.3^{c}\pm0.4$	$9.3^{a}\pm0.5$
	E. coli	0^{d}	0^{d}	0^{d}	0^{d}	$8^{b}\pm0.8$	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}	0^{g}	$7.3^{f}\pm0.2$

Salmonella typhi	0^{c}	0^{c}	0^{c}	0^{d}	$7.6^{b}\pm0.4$	0^{c}	0^{c}	0^{c}	0^{c}	0^{c}	0^{c}	0^{c}	0^{c}	$7.3^{b}\pm0.4$	$8.3^{a}\pm0.4$

* Different small letters show significant differences (p<0.05) between data obtained with different distillation methods.

Other studies have related the antibacterial property of *M. communis* L. essential oil to α-pinene, 1,8-cineole (3, 31) and linalool (34). These biomaterial compounds target the membrane of the bacterium and interrupt its enzymes' activity, which results in the blockage in the bacterium membrane, so nothing can either enter or exit (4, 35). α-pinene, a monoterpene, is often found in some of the essential oils of medicinal plants. The aforementioned compound is a transparent organic liquid soluble in alcohol and ethanol but not in water. This compound alone has an antibacterial effect on some bacteria, namely *S. aureus*, *E. coli* and *Bacillus cereus* (36). Furthermore, α-pinene can act as a modulator for antibiotics and improve the effectiveness of the drugs (36, 37). While 1,8-cineole, the second dominant compound, has an antibacterial effect, it is not poisonous for mammals (37). linalool, the third dominant compound, has a synergistic effect on the properties of the other compounds (34). The existence of a significant difference between these compounds that are extracted through various methods, shows that the essential oil which is mostly composed of the aforementioned compounds has a stronger antibacterial effect. In addition, the results of the antibiogram test verify these statements. Other studies have also proven the applications of *M. communis* L. essential oil in treating some of the antibiotic-resistant infections (4).

3.4. Antibiogram results of the essential oils on various bacteria

3.4.1. Staphylococcus aureus

The essential oils obtained from of all the three methods had no or neglectable antibacterial effect on *S. aureus* at the concentrations of 5, 10 and 20 µL/mL. The essential oil obtained by HSD method had the strongest antibacterial effect on *S. aureus*, and the essential oil obtained by SD method had the weakest antibacterial effect, while the essential oil obtained by HD method had a moderate antibacterial effect. The strongest antibacterial effect reported was related to the 80 µL/mLl concentration of the essential oil from HSD method with the Inhibition Zone Diameter (IZD) equal to 28.3 mm. When compared to the IZDs of 12 types of antibiotics (Table 4), *M. communis* L. essential oil obtained through HSD method had a stronger antibacterial effect than Cephalexin (IZD: 27 mm), Vancomycin (IZD: 27.8 mm) and Amikacin (IZD: 27.3 mm) while having a relatively weaker antibacterial effect than Ceftriaxone (IZD: 29.7 *mm*) and Penicillin (IZD: 29.1 mm). It also had a weaker antibacterial effect than Tobramycin (IZD: 39.8 mm), Gentamycin (IZD: 33.5 mm), Ampicillin (IZD: 40 mm), Ciprofloxacin (IZD: 31.3 mm) Tetracycline (IZD: 37.5 mm), Cloxacillin (IZD: 34.8 mm) and Erythromycin (IZD: 37.5 mm). In other studies, the IZD reported for the antibacterial effect of *M. communis* L. essential oil on *S. aureus* was 32.66 *mm* (31). It is shown that *M. communis* L. essential oil is effective against *Streptococcus pneumonia* and *Moraxella Catarrhalis* as well as haemophilus influenzae in an in vitro environment (12). In addition, the IZD reported for the antibacterial effect of the ethanolic extraction of *M. communis* L.' leaves at a concentration of 0.4 g/mL on *S. aureus* was 25 mm (4). In another study, the extract of *M. communis* L. at a concentration of 50 µL/mL was obtained using different solvents, and its antibacterial effect was investigated on *S. aureus* (CECT 110T). The IZDs reported for ethanolic extract, methanolic extract and ethyl acetate extract were 15 mm, 22 mm and 8 mm, respectively (8). Ben Hsouna had reported the IZD of the *M. communis L.* essential oil on *S. aureus* (ATCC25923) at a concentration of 50 µL/well equal to 25 mm (32).

Table 4. Antibiogram test results (mm) of different antibiotics

Bacterium	**Antibiotics**											
	Tobramycin	**Cefalexin**	**Gentamicin**	**Ceftriaxone**	**Ampicillin**	**Ciprofloxacin**	**Tetracycline**	**Vancomycin**	**Cloxacillin**	**Penicillin**	**Erythromycin**	**Amikacin**

Gram-Positive	Staphylococcus aureus	39.8^{a}±0.2	27^{e}±0.4	33.5^{c}±0.4	29.7^{d}±0.5	40.8^{a}±1.3	31.3^{d}±06	37.5^{b}±0.4	27.8^{e}±0.2	34.8^{c}±1.3	29.1^{d}±0.6	37.5^{b}±0.4	27.5^{e}±0.4
	Staphylococcus epidermidis	34.7^{a}±0.2	35.8^{a}±0.2	35^{a}±0.4	29.7^{c}±0.8	29.7^{c}±0.5	34.5^{a}±0.4	31.3^{b}±0.9	24.3^{d}±0.2	23.7^{d}±1.2	23.7^{d}±1	29.3^{c}±0.6	29.8^{bc}±0.6
	Streptococcus mutans	21.7^{f}±0.7	30.2^{c}±0.6	31.2^{bc}±0.8	21.7^{f}±0.8	31.3^{bc}±0.6	31.8^{b}±0.2	26^{e}±0.4	19^{g}±0.8	0^{h}	34.5^{a}±0.4	20.2^{fg}±1	28^{d}±0.4
Gram-Negative	Pseudomonas aeruginosa	23.2^{b}±0.2	0^{g}	20.8^{c}±0.6	20^{c}±0.4	10.2^{e}±0.6	26.8^{a}±1	10^{e}±0.4	7.2^{f}±0.2	0^{g}	0^{g}	12^{d}±0.4	20.8^{c}±0.2
	E. coli	15^{d}±0.4	20^{c}±0.4	19.7^{c}±1.2	29.7^{a}±0.5	13.5^{d}±0.4	24.2^{b}±0.6	23.8^{b}±0.2	0^{f}	0^{f}	8.2^{e}±0.2	0^{f}	19.8^{c}±0.6
	Salmonella typhi	10.7^{g}±0.2	16.2^{d}±0.2	12^{f}±1	22.8^{a}±0.6	16.5^{d}±0.4	13.8^{e}±0.2	17.8^{c}±0.6	19.5^{b}±0.4	0^{h}	20^{b}±0.4	0^{h}	22.3^{a}±0.5

3.4.2. Staphylococcus epidermidis

The essential oils obtained through of all the three methods had no or neglectable antibacterial effects on *S. epidermidis* at the concentrations of 5 and 10 µL/mL while they had antibacterial effects at concentrations of 20, 40 and 80 µl/ml. The essential oil obtained through HSD method at the 80 µL/mL concentration had the strongest antibacterial effect with the IZD equal to 27.6 mm. When compared to the 12 types of antibiotics, it had a stronger antibacterial effect than Vancomycin (IZD: 24.3 mm) and Cloxacillin (IZD: 23.7 mm), and a similar effect to Ceftriaxone (IZD: 29.7 mm), Erythromycin (IZD: 29.3 mm), and Amikacin (IZD: 29.8 mm). However, it had a weaker antibacterial effect than Tobramycin (IZD: 34.7 *mm*), Cefalexin (IZD: 35.8 mm), Gentamycin (IZD: 35 mm), Ciprofloxacin (IZD: 34.5 mm) and Tetracycline (IZD: 31.3 mm). In a study, the ethanolic extract of *M. communis* L. at a concentration of 0.4 g/mL had created an inhibition zone of 15 mm(4). Ben Hsouna had reported the IZD of the *M. communis* L. essential oil on *S. epidermidis* (ATCC12228) at a concentration of 50 µL/well equal to 15 mm (32).

3.4.3. Streptococcus mutans

The study showed that the essential oil at a concentration of 5 µL/mL had no antibacterial effect, and at the concentrations of 10 and 20 µl/ml, it had a neglectable effect. The only essential oils effective on *S. mutans* were the ones obtained through HD and HSD method at a concentration of 80 µL/mL with IZDs equal to 16 mm. The aforementioned concentrations were weaker than the antibiotic, and their antibacterial effect might improve should the essential oils become more potent.

3.4.4. Pseudomonas aeruginosa

The essential oils obtained by of all the three methods had no antibacterial effect on *P. aeruginosa* at the concentrations of 5, 10 and 20 µL/mL. While the 40 µL/mL concentration of the essential oil had a neglectable effect, the 80 µL/mL concentration had an antibacterial effect on *P. aeruginosa* with the IZD equal to 10.6 *mm*. When compared to the 12 types of antibiotics, it had a stronger antibacterial effect than Cefalexin (IZD: 0 mm), Vancomycin (IZD: 7.2 mm), Cloxacillin (IZD: 0 mm) and Penicillin (IZD: 0 mm), while it had a weaker antibacterial effect than Tetracycline (IZD: 30.2 mm), Gentamycin (IZD: 31.2 mm), Ceftriaxone (IZD: 20 mm), Ciprofloxacin (IZD: 26.8 mm), Erythromycin (IZD: 12 mm) and Amikacin (IZD: 20.8 mm). It also had a relatively similar antibacterial effect to that of Ampicillin (IZD: 10.2 mm). In a similar study, the antibacterial effect of the essential oil of *M. communis* L.' leaves on *P. aeruginosa* (ATCC9027) at a concentration of 50 µL/well was investigated and the IZD was reported 20 *mm* (32). Also, the antibacterial effect of the extract of *M. communis* L' leaves on *P. aeruginosa* (CECT 110T) at a concentration of 50 µL/ml was investigated. The ethanolic, ethanolic and ethyl acetate extracts had IZDs of 16, 22 and 8mm, respectively (8). Furthermore, the antibacterial effect of hydroalcoholic extract of *M. communis* L.' leaves on *P. aeruginosa* at a concentration of 40 mg/mL was investigated, and the IZD was reported 22.83 mm (11).

3.4.5. Escherichia coli

The essential oils obtained by of all the three methods had no antibacterial effect on *E. coil* at the concentrations of 5, 10 and 20 µL/mL. Also, concentrations of 40 and 80 µL/mL had a weak antibacterial effect. The strongest effect achieved was related to the essential oil obtained by HSD method at the 80 µL/mLl concentration with the IZD equal to 10.6 mm. When compared to the 12 types of antibiotics, it had a stronger antibacterial effect than Vancomycin (IZD: 0 mm), Cloxacillin (IZD: 0 mm) and Penicillin (IZD: 8.2 mm), while it had a weaker

antibacterial effect than Tobramycin (IZD: 15 mm), Cefalexin (IZD: 20 mm), Gentamycin (IZD: 19.7 mm), Ceftriaxone (IZD: 29.7 mm), Ampicillin (IZD: 13.5 mm), Ciprofloxacin (IZD: 24.2 mm), Tetracycline (IZD: 23.8 mm) and Amikacin (IZD: 19.1 mm).In other studies, the antibacterial effect of the essential oil of *M. communis* L.' leaves on *E. coil* (ATCC25922 and ATCC 8739) at a concentration of 50 µL/well was investigated and the IZD was reported 19 mm (32). The hydroalcoholic extract of *M. communis* L.' leaves at 40 mg/mL concentration had created an inhibition zone of 19.16 mm on *E. coli* (11). Also, the water extract of *M. communis* L.' leaves at a 50 µL/well concentration had created an inhibition zone of 22 mm on *E. coli* (ATCC 25922) (10).

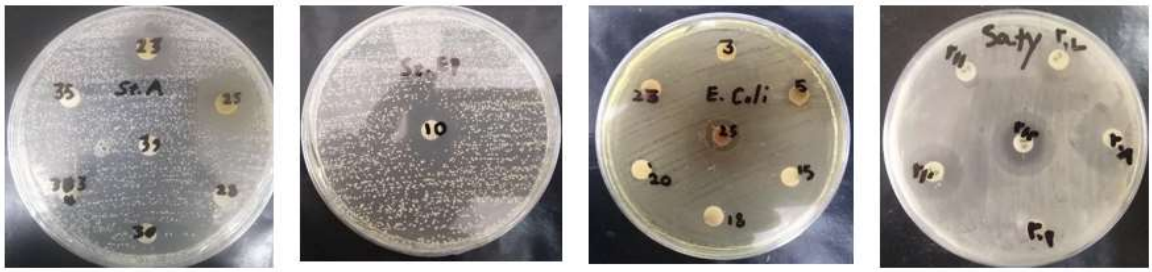
3.4.6. Salmonella typhimurium

The essential oils obtained through all the three methods had no or neglectable antibacterial effects on *S. typhimurium* at any concentration. The essential oil obtained through HSD method at a 80 µL/mL created an inhibition zone of 8.3 mm on *S. typhimurium*, which had a stronger effect than Cloxacillin (IZD: 0 mm) and Erythromycin (IZD: 0 mm), and it was weaker than any other antibiotic. Due to the fact that weak antibacterial effects of the essential oil of *M. communis* L.' leaves at lower concentrations have been reported, there is a possibility that higher concentrations might have a better antibacterial effect on *S. typhimurium* (4). (Fig. 4)

3.5. The MIC and MBC

The MIC and MBC results of the essential oils obtained through all the three distillation methods are shown in Table 5. Considering *S. aureus*, the essential oil obtained by HSD method had MIC and MBC equal to 125 µL/mL and 500 µL/mL, respectively. In another study, the MBC of the extraction of *M. communis* L.' leaves was reported 500 µL/mL (11). Also, the MIC of the essential oil of *M. communis* L.' leaves was reported 7/8 µL/mL (32).

Fig 4. Antibiogram test of antibiotics and Myrtus communis L. essential oil on various bacteria



(a) Antibacterial effect of different concentrations of *Myrtus communis* L. essential oil on *Staphylococcus aureus*

(b) Antibacterial effect of *Myrtus communis* L essential oil on *Staphylococcus epidermidis*

(c) Antibacterial effect of *Myrtus communis* L essential oil on E. coli

(d) Antibacterial effect of several different antibiotics on S. typhimurium

Table 5. The MIC and MBC results (µL/mL) of essential oils of *Myrtus communis* L. obtained by different extraction methods

	Bacterium	HD		SD		HSD	
		MIC	MBC	MIC	MBC	MIC	MBC
Gram-Positive	*Staphylococcus aureus*	500	1000	1000	2000	125	500
	Staphylococcus epidermidis	250	500	500	1000	500	500
	Streptococcus mutans	31	2000	125	500	250	1000
Gram-Negative	*Pseudomonas aeruginosa*	-	-	-	-	-	-

E. coli	-	-	-	-	2000	-
Salmonella typhi	1000	-	2000	-	1000	-

Considering *S. epidermidis*, the essential oil obtained by HD method had MIC and MBC equal to 250 µL/mL and 500 µL/mL, respectively. Considering *S. mutans*, the essential oil obtained through HD method had MIC and MBC equal to 31 µL/mL and 500 µL/mL, respectively. Considering *S. mutans*, the essential oil obtained by HD method had MIC and MBC equal to 31 µL/mL and 500 µL/mL, respectively. The essential oils obtained through all the three methods had no MIC and MBC results for *S. aeruginosa*, even at 2000 µL/mL concentration. Considering *E. coli*, the essential oil obtained through HSD method only had MIC equal to 2000 µL/mL. Considering *S. typhimurium*, the essential oils obtained through all the three methods had no MBC results and the MIC was related to the essential oil obtained by HD and HSD method, which was equal to 1000 µL/mL

4. Conclusion

Different distillation methods have significant effects on the essential oil yield. The essential oil yield was 1.19 mL in HD method, 1.15 mL in SD method and 1.17 mL in HSD method, which shows that the HD method has the highest essential oil yield. 28 compounds of the essential oil were identified in which α-pinene, 1,8-cineole, cineol, linalool L, decane, linalyl acetate, α-terpineol and camphene were the dominant compounds found in all of the three essential oils extracted through different methods but their values differed significantly. The investigation of the antibacterial effect of *M. communis* L. essential oil showed that the essential oil obtained through HSD at 80 µL/mL concentration had the strongest antibacterial effect on S. aureus with the IZD equal to 28.3 mm, and it had a similar antibacterial effect on *S. epidermidis* with the IZD equal to 27.6 mm when compared to those of the antibiotics.

References

1. Staff TP. PDR for herbal medicines: Physician's Desk Reference (PDR); (2004).
2. Berka-Zougali B, Ferhat M-A, Hassani A, Chemat F, Allaf KS. Comparative study of essential oils extracted from Algerian Myrtus communis L. leaves using microwaves and hydrodistillation. International journal of molecular sciences,13(4):4673-95 (2012).
3. Aleksic V, Knezevic P. Antimicrobial and antioxidative activity of extracts and essential oils of Myrtus communis L. Microbiological Research,169(4):240-54 (2014).
4. Mir MA, Bashir N, Alfaify A, Oteef MD. GC-MS analysis of Myrtus communis extract and its antibacterial activity against Gram-positive bacteria. BMC Complementary Medicine and Therapies,20(1):1-9 (2020).
5. Mahboubi M. Effectiveness of Myrtus communis in the treatment of hemorrhoids. Journal of Integrative Medicine,15(5):351-8 (2017).
6. Lingan K. A review on major constituents of various essential oils and its application. Translational Medicine,8:2161-1025.1000201 (2018).
7. Yadegarinia D, Gachkar L, Rezaei MB, Taghizadeh M, Astaneh SA, Rasooli I. Biochemical activities of Iranian Mentha piperita L. and Myrtus communis L. essential oils. Phytochemistry,67(12):1249-55 (2006).
8. Amensour M, Bouhdid S, Fernández-López J, Idaomar M, Senhaji NS, Abrini J. Antibacterial activity of extracts of Myrtus communis against food-borne pathogenic and spoilage bacteria. International Journal of Food Properties,13(6):1215-24 (2010).
9. Hennia A, Nemmiche S, Dandlen S, Miguel MG. Myrtus communis essential oils: insecticidal, antioxidant and antimicrobial activities: a review. Journal of Essential Oil Research.;31(6):487-545. (2019)
10. Messaoud C, Laabidi A, Boussaid M. Myrtus communis L. infusions: the effect of infusion time on phytochemical composition, antioxidant, and antimicrobial activities. Journal of food science,77(9):C941-C7 (2012).
11. Sisay M, Bussa N, Gashaw T, Mengistu G. Investigating In Vitro Antibacterial Activities of Medicinal Plants Having Folkloric Repute in Ethiopian Traditional Medicine. Journal of evidence-based integrative medicine,24:2515690X19886276 (2019).

12. Tavassoli M, Shayeghi M, Abai MR, Vatandoost H, Khoobdel M, Salari M, et al. Repellency effects of essential oils of Myrtle (Myrtus communis), Marigold (Calendula officinalis) compared with DEET against Anopheles stephensi on human volunteers. Iranian journal of arthropod-borne diseases,5(2):10 (2011).
13. Mahmoudvand H, Fallahi S, Mahmoudvand H, Shakibaie M, Harandi MF, Dezaki ES. Efficacy of Myrtus communis L. to inactivate the hydatid cyst protoscoleces. Journal of Investigative Surgery,29(3):137-43 (2016).
14. Bowersox J. Experimental staph vaccine broadly protective in animal studies. NIH News. 1999;27.
15. Chambers HF. The changing epidemiology of Staphylococcus aureus? Emerging infectious diseases,7(2):178 (2001).
16. Shomali T, Mosleh N. Zataria multiflora, broiler health and performance: a review. Iranian journal of veterinary research,20(2):81 (2019).
17. Leung E, Weil DE, Raviglione M, Nakatani H. The WHO policy package to combat antimicrobial resistance. Bulletin of the World Health Organization. 2011;89:390-2.
18. Khalil R, Li Z-G. Antimicrobial activity of essential oil of Salvia officinalis L. collected in Syria. African Journal of Biotechnology,10(42):8397-402 (2011).
19. Mahboubi M, Bidgoli FG. Antistaphylococcal activity of Zataria multiflora essential oil and its synergy with vancomycin. Phytomedicine,17(7):548-50. (2010).
20. Roktim Gogoib, Rikraj Loyingb , Neelav Sarmab , Sunita Mundaa,b , Sudin Kumar Pandeya,b , Mohan Lala. A comparative study on antioxidant, anti-inflammatory, genotoxicity, antimicrobial activities and chemical composition of fruit and leaf essential oils of Litsea cubeba Pers from North-east India. Journal of Industrial Crops & Products, 125-131-139(2018).
21. Ghasemi Pirbalouti A, Nourafcan H, Solyamani-Babadi E. Variation in chemical composition and antibacterial activity of essential oils from Bakhtiari Savory (Satureja bachtiarica Bunge.). Journal of Essential Oil Bearing Plants,20(2):474-84 (2017).
22. Babushok V, Linstrom P, Zenkevich I. Retention indices for frequently reported compounds of plant essential oils. Journal of Physical and Chemical Reference Data,40(4):043101 (2011).
23. Goodner K. Practical retention index models of OV-101, DB-1, DB-5, and DB-Wax for flavor and fragrance compounds. LWT-Food Science and Technology,41(6):951-8 (2008).
24. Sagun E, Durmaz H, Tarakci Z, Sagdic O. Antibacterial activities of the extracts of some herbs used in Turkish herby cheese against Listeria monocytogenes serovars. International Journal of Food Properties,9(2):255-60 (2006).
25. Valgas C, Souza SMd, Smânia EF, Smânia Jr A. Screening methods to determine antibacterial activity of natural products. Brazilian journal of microbiology,38(2):369-80 (2007).
26. Waites KB, Bade DJ, Bébéar C, Brown SD, Davidson MK, Duffy LB, et al. Methods for antimicrobial susceptibility testing for human mycoplasmas, Approved guideline (2011).
27. Cavas M, Beltrán D, Navarro JF. Behavioural effects of dimethyl sulfoxide (DMSO): changes in sleep architecture in rats. Toxicology letters,157(3):221-32 (2005).
28. Owuama CI. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using a novel dilution tube method. African Journal of Microbiology Research,11(23):977-80 (2017).
29. Salama HM, Marraiki N. Antimicrobial activity and phytochemical analyses of Polygonum aviculare L.(Polygonaceae), naturally growing in Egypt. Saudi journal of biological sciences,17(1):57-63 (2010).
30. Brada M, Tabti N, Boutoumi H, Wathelet J-P, Lognay G. Composition of the essential oil of leaves and berries of Algerian myrtle (Myrtus communis L.). Journal of essential oil Research,24(1):1-3 (2012).
31. Bajalan I, Pirbalouti AG. Variation in antibacterial activity and chemical compositions of essential oil from different populations of myrtle. Industrial Crops and Products,61:303-7 (2014).
32. Ben Hsouna A, Hamdi N, Miladi R, Abdelkafi S. Myrtus communis essential oil: chemical composition and antimicrobial activities against food spoilage pathogens. Chemistry & biodiversity,11(4):571-80 (2014).
33. Bouzouita N, Kachouri F, Hamdi M, Chaabouni MM. Antimicrobial activity of essential oils from Tunisian aromatic plants. Flavour and fragrance journal,18(5):380-3 (2003).
34. Herman A, Tambor K, Herman A. Linalool affects the antimicrobial efficacy of essential oils. Current microbiology,72(2):165-72 (2016).
35. Pirbalouti AG, Mirbagheri H, Hamedi B, Rahimi E. Antibacterial activity of the essential oils of myrtle leaves against Erysipelothrix rhusiopathiae. Asian Pacific journal of tropical biomedicine,4:S505-S9 (2014).
36. Salehi B, Upadhyay S, Erdogan Orhan I, Kumar Jugran A, LD Jayaweera S, A Dias D, et al. Therapeutic

potential of α-and β-pinene: A miracle gift of nature. Biomolecules,9(11):738 (2019).
37. Pegalajar-Jurado A, Easton CD, Styan KE, McArthur SL. Antibacterial activity studies of plasma polymerised cineole films. Journal of Materials Chemistry B,2(31):4993-5002 (2014).