



Chemical Composition, Antimicrobial and Analgesic Properties of *Rosmarinus officinalis* L. from North of Iran

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Abstract

Medicinal plants can be used in food, pharmaceutical and cosmetic products due to their biological and nutritional properties. The objective of this research was to examine the chemical composition, antimicrobial and analgesic activity of *Rosmarinus officinalis* L. plant originating Amol, north of Iran for the first time. The essential oil was obtained by hydrodistillation. Gas chromatography-mass spectrometry study was used for oil analysis. *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enteritidis*, *Bacillus subtilis* and *Candida albicans* were used to examine the antimicrobial activity of the essential oils using agar disk diffusion assay. GC-MS analysis revealed that the oil was dominated by 1,8-cineole (20.20%), α -Pinene (17.29%) and borneol (7.59%). Antimicrobial assays of different dilutions of essential oil showed significant antimicrobial activity against gram-positive bacteria. Furthermore, the water and ethanolic extracts prevented the pain resulting from hot-plate test. The results suggested *Rosmarinus officinalis* from Amol as an alternative natural compound which has antimicrobial and analgesic effects to use in pharmaceutical industries.

Keywords: Analgesic, Antimicrobial Activity, Chemical Composition, Essential Oil, Extract, *Rosmarinus officinalis*

Introduction

Rosmarinus officinalis L. belongs to the *Labiatae* family is one of the most widely used shrub aromatic plant in traditional medicine, remedy and as antifungal and antibacterial [1]. It is cultivated mostly in Mediterranean region and in Asia, Europe and Africa [2]. Since, *Rosmarinus officinalis* contains different bioactive components including carnosol, carnosic acid, betulinic acid, ursolic acid, rosmanol, rosmarinic acid, oleanolic acid, and micromeric acid methyl ester [3,4], its oil and extract are used in various industries such as food, pharmaceutical, perfumes and cosmetics [5]. Extensive study on this plant has revealed many its pharmacological activities such as anti-inflammatory [6,7], antibacterial [8, 9], antioxidant [10], antiviral, antidepressant, hypoglycemic,

hypolipidemic [11, 12] and hepatoprotective [13]. Diaz-Maroto *et al.*, 2007 [14] showed that rosemary essential oil contains monoterpenes (95–98%) and sesquiterpenes (2–5%) compounds. In another study, 1,8-cineole, borneol, verbenone, α -pinene and camphene were the main components of rosemary essential oils [2]. A previous report by Ladan Moghadam, A.R. 2015, indicated that only 20 components were present in essential oil of *Rosmarinus officinalis* originating from Kermanshah, Iran. The major components of the essential oils were α -pinene (43.12%), camphene (10.5%), 1,8-cineole (10.02%), camphor (8.07 %), linalool (8.09%) and limonene (6.12%) [15]. The chemical compositions of 10 Sicilian *Rosmarinus officinalis* were studied by Tuttolomondo, T. *et al.* 2015. Eighty-two compounds have been identified which represent 96.7–99.9% of the essential oil with the yield

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varied from 0.8 to 2.3 (v/w). The main compounds in the essential oils included 1,8-cineole, α -terpineol, linalool, verbenone, limonene, α -pinene, bornyl acetate and terpinolene [16]. Other studies reported 1,8-Cineole, camphor, α -pinene and borneolas the main components of *Rosmarinus officinalis* originated from Tunisian [17] and β -Myrcene and 1,8-cineole as the major constituents of *Rosmarinus officinalis* from Argentina [18]. These quantitative and qualitative differences between the present results and other researches could be ascribed to the harvest time, geographical origins and environmental factors [19].

In addition, several attempts have been made to describe the antibacterial activities of *Rosmarinus officinalis* essential oils and extracts [20, 21]. In spite of several studies undertaken on this herb, there is rare information on antifungal and analgesic properties of *Rosmarinus officinalis*. As far as we know, it is a first report on chemical composition, antimicrobial and analgesic activities of *Rosmarinus officinalis* aerial parts from Amol to evaluate its potential use as an antimicrobial and anti-pain agent.

Material and Methods

Material

The aerial parts of *Rosmarinus officinalis* L. were collected in June 2016 from Amol, Mazandaran province (north of Iran). Voucher sample was deposited at the herbarium center of Ayatollah Amoli Branch, Islamic Azad University, Amol, Iran. Samples were air-dried in the shade for 4 days.

Plant Essential Oil and Extract Preparation

The dried samples were ground and 100 g of obtained powders (mesh<25) in 1 L of water was subjected to hydrodistillation for 3 h in Clevenger-type apparatus. The collected essential oils were dried over anhydrous sodium sulphate and after filtration, weighed and stored in a sealed dark vials at 4 °C until tested and used. The yield was determined based on dry weight of the sample. For preparation of plant extract, 100 g of powdered dry parts of plant were extracted with ethanol and water separately by maceration. The extract was collected and filtered by Whatman filter papers. The yielded extract was concentrated under vacuum and kept in a dark bottle in a refrigerator at 4 °C until use. The

percent yields of the extract were 13.4% and 9.1% for ethanol and water extract, respectively.

Gas Chromatography - Mass Spectrophotometer Analysis

Separation and identification of essential oil components were carried out using gas chromatography-mass spectrophotometer (GC-MS) analysis. The analysis was performed on an Agilent 6890A gas chromatograph equipped with a Flame Ionization Detector (FID), using HP-5MS capillary column (30 m \times 0.25 mm \times 0.25 μ m) coupled to a Mass Selective Detector MSD 5975C in EI mode (ionization energy voltage 70 eV). Helium was used as carrier gas at a flow rate of 1.0 ml/min. Initial column temperature was 60°C with 4 min hold and was raised at the rate of 6 °C/min to 250 °C. Injector and detector temperatures were set at 280 °C. Essential oil samples (1 μ l) in hexane (HPLC grade) were autoinjected. All components were identified by comparison of their spectra and retention time with those of the Wiley and NIST Libraries in the computer library and literature [22, 23].

Microbial Strains and Antimicrobial Test

Staphylococcus aureus ATCC 25923, *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633 and *Salmonella enteritidis* ATCC 10708 were used in this study. The bacteria species were maintained in Mueller Hinton Agar. Fungal strain was *Candida albicans* (ATCC 10231) which was maintained on Sabour and Dextrose Agar.

The antimicrobial activity of the essential oil was tested by using agar disc diffusion method. In this method, microorganisms were cultured overnight at 37 °C in Mueller Hinton agar and then adjusted with sterile saline to concentration of 1.5×10^8 CFU/ml. Then, a suspension of the tested microorganism was spread on nutrient agar. Different dilutions of essential oils were prepared with DMSO at dilutions of 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128. Filter paper discs of 6 mm in diameter were individually impregnated with 10 μ l of tested oil aliquots and then placed on the prepared agar plates. The Petri dishes incubated at 37°C (24 and 72h for bacteria and fungi, respectively). Gentamicin, Streptomycin and Fluconazole (each of 10 μ g) were used as positive controls. Negative controls were prepared using DMSO. The inhibition zone diameters (mm) were considered as antimicrobial potential. All tests were done in

triplicate and reported as means \pm standard deviation.

Animals

The tests were performed on Swiss male mice with mean weight of 18–25 g. Animals were maintained over a 12 h light-dark cycle and humidity of 50–60% at an environment temperature of $23 \pm 2^\circ\text{C}$, in the animal room of the School of Pharmacy, Mazandaran University of Medical Sciences, Iran. They were fed a standard diet and water ad libitum before use. In each group, six mice were studied. The experiments were done in a quiet room and in the light period of 9 am to 5 pm [24].

Analgesic activity of the Extract and hot-plate test

Five groups of six mice were prepared for injection as follows: group I – saline solution with 10% Tween 80 (negative control), groups II, III, and IV – different dose of water or ethanolic extract (100, 200 and 400 mg/kg), and group V –10 mg/kg morphine (positive control). Ethanolic extract solutions were prepared in saline solution with 10% Tween 80. In each experiment, mice were injected with 2ml of solution into the subplantar space of the right hind paw. Then, 15, 30, 45, 60, 75 and 90 min after injection, the animals were placed individually on a hot plate (Pars AZMA Co., Isfahan, Iran), maintained at $52 \pm 5^\circ\text{C}$ and the time between the placement of the animal on the hot plate and the occurrence of either the licking of the hind paws, shaking or jumping off from the surface was recorded as there sponselatency [25]. A cut-off time of 40s was considered if the animal did not show any reaction to the painful stimulus.

Result and Discussion

Chemical Composition of Essential Oil

The essential oil of *Rosmarinus officinalis* was in high yield of 0.82%. The GC-MS chromatogram of compounds detected in essential oil is shown in Fig. 1. The identified constituents, retention times, chemical structures and their percentages in the oil composition are summarized in Table 1. The GC/MS analysis of oil showed a total of 45 volatile compounds representing 98.17% of total oil. The most abundant components of the oil were 1,8-cineole (20.20%), α -Pinene (17.29%), borneol (7.59%), 1,4-Dimethylbicyclo[3.3.0] oct-3-en-2-one

(7.2%), camphor (5.50%), Linalool L (5.06%), verbenone (5.21%), Geraniol (4.29%) and camphene (3.01%). The results show that oil consists of 11monoterenes, 21 oxygenated monoterenes, 4 sesquiterpenes and 2 oxygenated sesquiterpene. Oxygenated monoterpene represented the main fraction of the oil. Esters, alcohols, ketone and ether were other components which exist in the *Rosmarinus officinalis* L. oil (Tables 1).

Antimicrobial Potential of *Rosmarinus officinalis* Essential Oils

In vitro antimicrobial activities of *Rosmarinus officinalis* essential oils were assessed against four gram-positive and gram-negative bacteria and *Candida albicans* using disk diffusion method. Table 2 represents the inhibition zone of undiluted and diluted essential oils determined against tested microorganisms. The result showed that the essential oils exerted different inhibiting activity. The gram-positive *Staphylococcus aureus* was the most susceptible strain examined to the undiluted oil of *Rosmarinus officinalis* with the strongest inhibition zone (26 ± 1.30 mm). On the contrary, the antimicrobial activity of the essential oils against the gram-negative *salmonella enteritidis* was the lowest for *Rosmarinus officinalis*.

According to Table 2, it can be concluded that all undiluted essential oils of *Rosmarinus officinalis* showed considerable activity against all tested microorganism compared with gentamicin and streptomycin antibiotics and Fluconazole antifungal(10 μg). some oxygenated monoterpenes, such as 1,8-cineol, linalool, camphor and borneol were documented to have different amounts of antimicrobial potential against various microorganism [26]. Therefore, the significant antimicrobial characteristics of the essential oil of *Rosmarinus officinalis* are correlated to the components present in this essential oil. Our results demonstrated that gram-positive bacteria were more sensitive to the compounds of the essential oils than gram-negative bacteria. The higher susceptibility of gram-positive bacteria may be related to their impermeable cell membrane which limits diffusion of hydrophobic compounds through its outer wall due to Lipopolysaccharide layers [27].

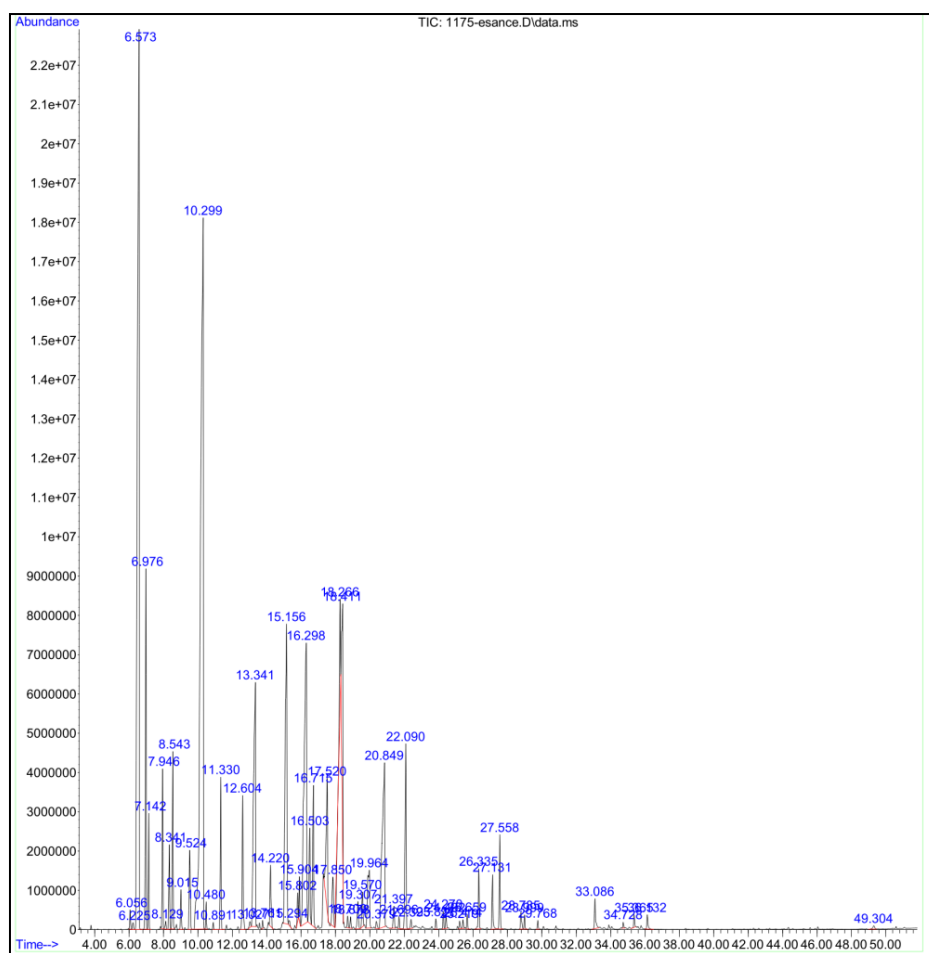


Fig. 1 GC-MS chromatogram of the essential oil of *Rosmarinus officinalis* L.

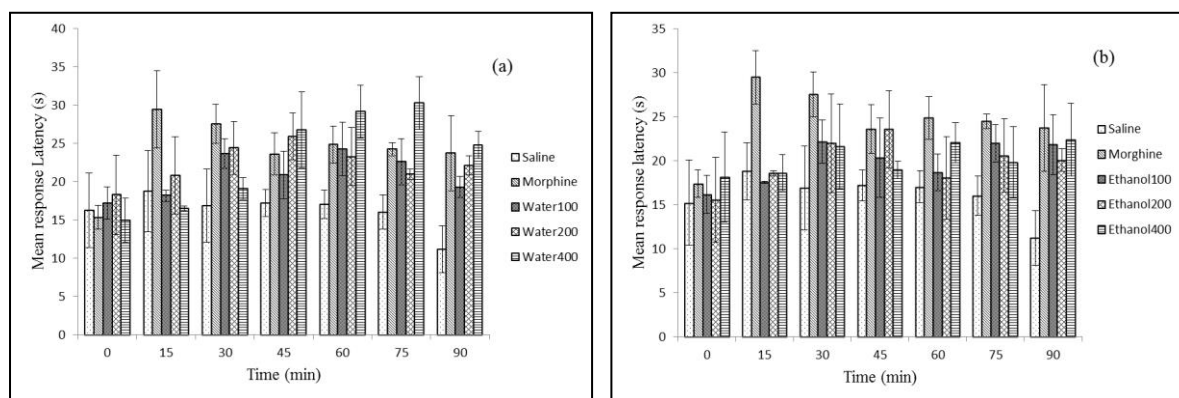


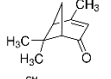

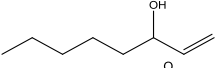
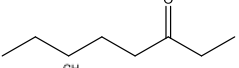
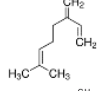
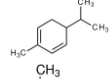
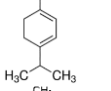
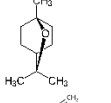
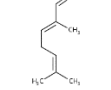
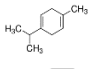
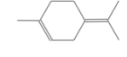
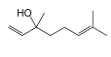
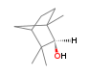
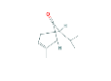
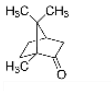
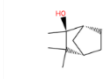
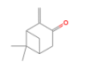
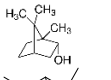
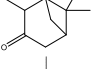
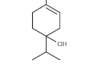
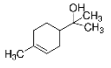
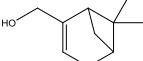
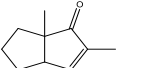
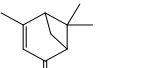
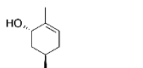
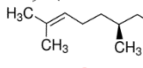
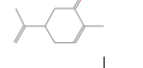
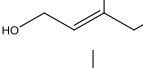
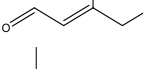
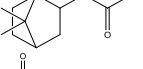
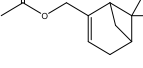
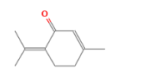
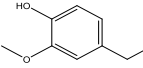
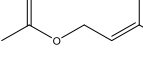
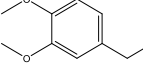
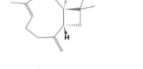

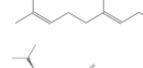
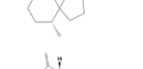

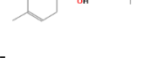


Fig. 2 The effect of *Rosmarinus officinalis* L. (a) water and (b) ethanol extract on the pain induced by hot-plate test. The results were shown by (mean \pm SEM) the time of pain in 6 animals.

Table 1 Chemical composition of *Rosmarinus officinalis* L. essential oil identified by GC/MS analysis

Serial No.	Component	RT(min)	%Area	Exact mass (g/mol)	Chemical structure	MS Fragment-ions	Formula
1	Tricyclene	6.055	0.16	136.1		55, 67, 79, 93, 105, 121, 136	C ₁₀ H ₁₆
2	α -Thujene	6.224	0.08	136.1		65, 93, 119, 136, 354	C ₁₀ H ₁₆

3	α -Pinene	6.574	17.29	136.1		55, 77, 93, 121, 138, 354	C ₁₀ H ₁₆
4	Camphene	6.974	3.010	136.1		55, 61, 67, 73, 79, 86, 93, 107, 121, 136	C ₁₀ H ₁₆
5	Verbenene	7.144	0.760	150.1		56, 65, 77, 91, 105, 119, 134	C ₁₀ H ₁₄ O
6	β -Pinene	7.945	1.260	136.1		55, 63, 69, 79, 93, 107, 121, 136	C ₁₀ H ₁₆
7	1-Octen-3-ol	8.130	0.080	128.1		57, 72, 85, 99, 110, 121, 141	C ₈ H ₁₆ O
8	3-Octanone	8.340	0.740	128.1		57, 72, 85, 99, 121, 128	C ₈ H ₁₆ O
9	Myrcene	8.546	1.540	136.1		55, 69, 79, 87, 93, 107, 121, 136	C ₁₀ H ₁₆
10	α -Phellandrene	9.013	0.360	136.1		58, 65, 77, 93, 105, 119, 136	C ₁₀ H ₁₆
11	α -Terpinene	9.526	0.890	136.1		55, 65, 77, 84, 93, 105, 121, 136	C ₁₀ H ₁₆
12	1,8-Cineole	10.297	20.20	154.2		55, 71, 81, 93, 108, 125, 139, 154	C ₁₀ H ₁₈ O
13	β -trans-Ocimene	10.482	0.200	136.1		55, 67, 79, 93, 105, 121, 136, 154	C ₁₀ H ₁₆
14	γ -Terpinene	11.329	1.340	136.1		55, 65, 77, 93, 105, 121, 136	C ₁₀ H ₁₆
15	Terpinolene	12.602	1.370	136.1		55, 77, 93, 121, 137, 354	C ₁₀ H ₁₆
16	Linalool L	13.742	5.060	154.2		55, 71, 80, 93, 107, 121, 136, 154	C ₁₀ H ₁₈ O
17	β -Fenchyl alcohol	13.763	0.070	154.2		55, 69, 81, 93, 111, 121, 139, 154	C ₁₀ H ₁₈ O
18	Chrysanthenone	14.22	0.690	150.2		56, 65, 72, 79, 91, 107, 122, 135, 150	C ₁₀ H ₁₄ O
19	Camphor	15.155	5.500	152.1		55, 69, 81, 95, 108, 127, 128, 152	C ₁₀ H ₁₆ O
20	exo-methyl-camphenilol	15.293	0.060	154.2		59, 71, 86, 96, 111, 121, 136, 152	C ₁₀ H ₁₈ O
21	Pinocarvone	15.904	0.360	150.2		62, 69, 81, 91, 108, 122, 135, 150	C ₁₀ H ₁₄ O
22	Borneol L	16.30	7.590	154.2		67, 95, 119, 139, 446	C ₁₀ H ₁₈ O
23	Isopinocampnone	16.50	1.120	152.1		55, 69, 83, 95, 110, 139, 152	C ₁₀ H ₁₆ O
24	Terpinen-4-ol	16.716	1.580	154.2		71, 79, 86, 93, 111, 136, 154	C ₁₀ H ₁₈ O
25	α -Terpineol	17.522	1.950	154.2		59, 67, 81, 93, 121, 136, 152	C ₁₀ H ₁₈ O

26	Myrtenol	17.851	0.730	152.1		67, 79, 91, 108, 119, 152	C ₁₀ H ₁₆ O
27	1,3-Dimethylbicyclo[3.3.0]oct-3-en-2-one	18.267	7.200	150.2		67, 95, 135, 155, 446	C ₁₀ H ₁₄ O
28	Verbenone	18.421	5.210	150.2		67, 80, 91, 107, 122, 135, 150	C ₁₀ H ₁₄ O
29	Trans (-+)-Carveol	18.708	0.120	152.1		56, 69, 77, 84, 91, 109, 119, 137, 152	C ₆ H ₁₀ O
30	Citronellol	19.309	0.340	156.2		69, 95, 123, 156, 446	C ₁₀ H ₂₀ O
31	Carvone	19.966	1.330	150.2		58, 82, 108, 150, 446	C ₁₀ H ₁₄ O
32	Geraniol	20.850	4.290	154.2		69, 93, 123, 154, 446	C ₁₀ H ₁₈ O
33	3,7-dimethyl-2,6-Octadienal	21.399	0.260	152.1		56, 69, 84, 94, 109, 123, 137, 152	C ₁₀ H ₁₆ O
34	Bornyl acetate	22.087	2.230	196.1		55, 67, 80, 95, 108, 121, 136, 154, 196	C ₁₂ H ₂₀ O ₂
35	Myrtenyl acetate	23.839	0.090	194.1		69, 91, 119, 152, 354	C ₁₂ H ₁₈ O ₂
36	Piperitenone	24.434	0.130	150.2		67, 91, 107, 150, 354	C ₁₀ H ₁₄ O
37	Eugenol	25.220	0.080	164.1		77, 91, 103, 121, 131, 149, 164, 197	C ₁₀ H ₁₂ O ₂
38	Geranyl acetate	26.334	0.560	196.1		69, 93, 121, 154, 196	C ₁₂ H ₂₀ O ₂
39	Methyleugenol	27.130	0.550	178.1		65, 91, 107, 131, 147, 178, 354	C ₁₁ H ₁₄ O ₂
40	trans-Caryophyllene	27.556	0.880	204.4		55, 69, 79, 93, 105, 120, 133, 147, 161, 175, 189, 204	C ₁₅ H ₂₄
41	α-Humulene	28.784	0.200	204.4		67, 93, 121, 147, 175, 204	C ₁₅ H ₂₄
42	Trans-β-Farnesene	28.999	0.110	204.4		55, 69, 79, 93, 107, 120, 133, 148, 161	C ₁₅ H ₂₄
43	β-Cubebene	29.770	0.08	204.4		57, 67, 81, 91, 105, 119, 133, 161, 204	C ₁₅ H ₂₄
44	Caryophyllene oxide	33.087	0.350	220.4		55, 69, 79, 93, 109, 121, 135, 149, 161, 177, 187, 205, 220	C ₁₅ H ₂₄ O
45	α-Bisabolol	36.132	0.170	222.4		58, 69, 79, 93, 109, 119, 134, 161, 189, 204	C ₁₅ H ₂₆ O
	Monoterpene hydrocarbons	27.50	-	-	-	-	-
	Oxygenated monoterpenes	64.51	-	-	-	-	-
	Sesquiterpene hydrocarbons	1.27	-	-	-	-	-
	Oxygenated sesquiterpens	0.52	-	-	-	-	-
	Other	4.37	-	-	-	-	-
	Total identified	98.17	-	-	-	-	-

RT; retention time. The components are listed in order of elution from the HP-5MS column

Table 2 Diameter of microbial inhibition zones (mm) determined by disk diffusion assay

Plant name and standard	Oil dilutions and antibiotics weight	Gram positive bacteria		Gram negative bacteria		Fungi
		<i>Staphylococcus aureus</i>	<i>Bacillus subtilis</i>	<i>Escherichia coli</i>	<i>Salmonella enteritidis</i>	<i>Candida albicans</i>
<i>Rosmarinus officinalis</i>	1	26±1.30	23±1.40	23±1.09	23±0.90	27±1.50
	½	22±1.20	23±1.30	19±1.30	14±0.90	17±1.20
	¼	14±1.02	22±0.80	16±1.10	13±0.30	16±0.90
	1/8	11±0.94	16±1.05	10±0.85	10±0.30	12±0.70
	1/16	10±1.02	13±0.90	10±0.90	9±0.10	11±0.34
	1/32	10±1.20	13±0.65	10±0.50	9±0.05	8±0.10
	1/64	10±0.85	13±0.30	10±0.30	-	8±0.55
Gentamicin	10µg	24±1.10	18±0.50	18±1.45	16±1.05	-
Streptomycin	10µg	21±0.80	16±0.35	15±0.90	14±0.85	-
Fluconazole	10µg	-	-	-	-	22±1.20

All tests were performed in triplicate. Values are given as mean ± SD (n=3)

Analgesic Potential of *Rosmarinus officinalis* Extract

The mean response latency to thermal stimulus in hot plate test is shown in Fig. 2. Water and ethanolic extract of *Rosmarinus officinalis* (100, 200 and 400 mg/kg), normal saline and morphine exerted anti-pain effects in mice. The results revealed that the effect of both extracts was dose dependent. It is proposed that different doses of extracts may exhibit different effects on the pain system. The mean response latency reached to a maximum of 30.28 s for dose of 400 mg/kg water extract in the case of placement of the mice on the hot plate at 75 min after injection. In addition, the pain latency time of 400 mg/kg water extract was more than morphine for times ranging 45 to 90 min. However, the effect of morphine (10 mg/kg) was more than the effect of ethanol extract at each tested time of injection.

According to our results, the injection of *Rosmarinus officinalis* extract to mice prevented pain in the hot-plate test. Since, the analgesic effect of 1,8-cineole and Linalool was confirmed in some study [28, 29], the extracts may reach in 1,8-cineole and Linalool which inhibit the pain resulting from hot-plate test.

Conclusion

The antimicrobial and analgesic properties of *Rosmarinus officinalis* plant from Amol was reported for the first time. Twenty constituents were identified in the extracted oil which the main components were mostly oxygenated monoterpenes. Antimicrobial assay showed that the essential oils at various dilutions ranged from

1/2 to 1/64 were active against examined microorganism especially gram-positive strains. The injection of water and ethanolic extracts to mice showed inhibition effect on pain induced by hot plate test. Thanks to high amounts of oxygenated monoterpenes and monoterpenes compounds of oils which show therapeutic and nutritional properties, the studied *Rosmarinus officinalis* is potent for its possible use as natural alternatives to chemical-based antimicrobial and anti-pain agents in food, pharmaceutical and cosmetic industries. For supplementary studies, it is suggested to evaluate other pharmacological potential of the mentioned *Rosmarinus officinalis* such as antioxidant and anti-inflammatory effects.

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