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Antibacterial activity and analysis of the volatile compounds in *Artemisia annua* L. using improved HS-SPME-GC-MS and comparison with hydrodistillation method

Ameneh Porgham Daryasari*[1], Mojtaba Soleimani[1], Azam Ghorbani[2]

*[1] Assisted Professor, Department of Chemistry, La.C., Islamic Azad University, Lahijan, Iran.

[1] Assisted Professor, Department of Chemistry, La.C., Islamic Azad University, Lahijan, Iran

[2] Assisted Professor, Department of Chemistry, Sav.C., Islamic Azad University, Saveh, Iran.

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*Corresponding Author E-Mail:

Ameneh.Porgham@iau.ir

ABSTRACT

In this study, gas chromatography-mass spectrometry (GC–MS) following microwave distillation and Microwave-assisted solid-phase microextraction (MW–SPME) was developed for the analysis of volatile constituents from the aerial parts *Artemisia annua* L. In order to improved headspace method, microwave powers; irradiation times and SPME fibers coating were studied. The optimal experiment parameters obtained were: 65µm PDMS/DVB SPME fiber, a microwave power of 400 W and an irradiation time of 5 min. MW–SPME was compared with headspace SPME (HS-SPME) and conventional hydrodistillation for the extraction of volatile compounds in *A. annua L.* A comparative qualitative and quantitative study on the composition of volatile compounds was carried out. α–pinene, 1,8-cineol, artemisia ketone, and camphor were identified as major constituents of this plant in all methods. The relative standard deviation (R.S.D) values of less than 9% show that the MW–SPME method has good repeatability as new extraction method. It has been shown the extraction of volatile compounds from *A. annua L.* with MW–SPME was better in terms of energy saving, extraction time, plant material, oxygenated fractions and product quality. Antibacterial activity of essential oil was tested using agar dilution method and minimum inhibitory concentration (MIC) on the Escherichia coli and Bacillus subtilis were determined 0.5, 0.25 mg/ml respectively.

1-INTRODUCTION

There are thirty-four species of the genus *Artemisia* L. in Iran, among which majority are endemic [1]. *Artemisia annua* L. is a member of the *Asteraceae* family that has antibacterial and antifungal effects [2-6]. The chemical composition of the oil of different species of *Artemisia* indicated that terpenes and sesquiterpenes, were constituents of essential oils. In recent years, several studies have been performed on the chemical composition of *Artemisia* species oil of different origins [7-11]. It was reported that the oil of *A. dracunculus* collected in Turkey, contained thirty compounds representing 99.5% of total oil were identified. The predominant components in the oil were (Z)-anethole (81.0%), (Z)-β-ocimene (6.5%), (E)- β-ocimene (3.1%), Limonene (3.1%) and methylleugenol (1.8%) [4]. The essential oil of *A. anuua* grown under the semi-arid tropical climate of Hyderabad, South India, and distilled by hydro-distillation techniques produced fifty-four constituents that major compounds of the hydro-distilled oil were: 1,8-cineole (11.1%), camphor (36.6%), β-caryophyllene (5.7%) and germacrene D (5.9%) [12]. Hydrodistilled volatile oil obtained from the aerial parts of *A. annua* L., cultivated near Sarajevo, Bosnia, was analyzed by GC–MS. More than one hundred compounds were identified, representing 95.5% of the total oil. The major constituents of essential oil were oxygenated monoterpenes, artemisia ketone (30.7%) and camphor (15.8%) [13]. The volatile constituents of A annua L. plants, grown in the Netherlands were investigated using GC and GC-MS (EI, NICI) analysis and more than forty compounds were identified.The principlal component was Artemisia ketone (63.9%) ; other major constituents included Artemisia alcohol (7.5%), myrcene (5.1%), α-guaiene (4.7%) and camphor (3.3%) [14. In Iran, variation in the quantity and quality of the essential oil of *Artemisia annua* L. at different developmental growth stages including pre-flowering, flowering and post-flowering, are reported. The oils were obtained by hydrodistillation of the air-dried samples and yields of oils (w/w %) in different stages were in the order of pre-flowering (0.97%), flowering (1.23%) and post-flowering (0.87%). In total, 32, 35 and 33 constituents were identified and quantified in the oil of pre-flowering, flowering and post-flowering plants, representing 97.67%, 92% and 92.4% of the oils, respectively. Camphor, 1,8-cineole, camphene, spathunelol, α–pinene and artemisia ketone were the main compounds in all samples. Monoterpenes were the main group of compounds in pre-flowering (69.96%), flowering (72.44%) and post-flowering (70.96%) stages [15]. In term of *A. annua* L., chemical constituents of essential oil obtained from hydrodistillation method was identified [16-17]. However, hydrodistillation is a time-consuming and laborious process and needs large amounts of sample. Solid phase microextraction (SPME) is a unique sample preparation technique, which eliminates most drawbacks to extracting organics, including high cost and excessive preparation time, in particular, SPME is a simple and fast modern tool used to characterize the volatile fraction of aromatic medicinal plants and offers a valid alternative to hydrodistillation for gas chromatographic analysis of essential oil from different sources [18]. Recently, microwave extraction coupled to SPME has widely been applied to environmental analysis. Microwave followed by HS-SPME has been demonstrated to be a reliable method for quantitative analysis of volatile compounds in solid samples [25-40]. In this work, Microwave-assisted solid phase microextraction (MW-SPME) was developed for the analysis of volatile compounds in *A.annua* L. and compared with HS-SPME and conventional hydrodistillation technique.

2-Materials and methods

Plant material: The aerial parts of *A.annua* L. Were collected from the Rudsar, located in height of Rahimabad Road, province of Guilan.Voucher specimens have been deposited at the Herbarium of the Research Institute of Forest and Rangelands (TARI), Tehran, Iran.

Hydrodistillation method

In hydrodistillation method, 100 g the air-dried aerial parts in a 2 l flask were subjected to hydrodistillation for 3.5 h using a Clevenger type according to the standard procedure described in the European pharmacopeia [30]. After trapping the oil with n-hexane (Merck) decanting and drying of the oil over anhydrous Na_2SO_4 (Merck), the - corresponding pale-yellow oil isolated and was stored at 4°C until used.

MW-SPME method

Due to the importance of the extracting fiber, the type of extracting fiber was initially selected based on the nature of the essential oil compounds and fibers available in the laboratory. The used fiber for SPME were 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) and 100 µm polydimethylsiloxane (PDMS) that were purchased from Supelco (Bellefonte, PA, USA). The Microwave apparatus was milestone "Microsynth" microwave oven that had a multimode microwave reactor 2.45 GHz with a maximum delivered power of 1500W variable in 1W increments. A cooling system outside the microwave oven condenses the distillate continuously. Excess water was refluxed to the extraction vessel in order to restore the water to the plant materials. The dimensions of the PTFE coated cavity are 55cm×55cm×55cm. During experiments; time, temperature, pressure and power were

controlled with the "easy-control" software package. Temperature was monitored by a shielded thermocouple (ATC-FO) inserted directly into the sample container [19-20]. The laboratory- made apparatus of MW-SPME is shown in Figure 1.

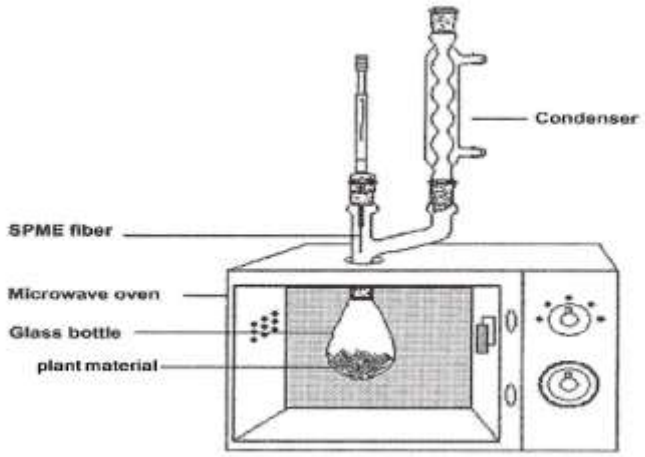

Figure1. Scientific Microwave apparatus applied for Microwave assisted solid-phase microextraction (MW–SPME)

After a suitable extraction time, the fiber was retracted and removed from microwave oven and was immediately thermally desorbed in the GC injector (250°C for 3 min) and then analyzed by GC–MS.

HS-SPME Method

To demonstrate of method, 0.5 g plant was ground to fine powder, and then put into a 10 ml glass bottle sealed with a septum-type cap, without microwave irradiation was performed for conventional HS–SPME with PDMS/DVB fiber. Dry plant was continuously agitated with a 1.0 cm magnet on a stirrer plate. Headspace extraction was performed at 60°C for 20 min and the analytes on the fiber were determined by GC–MS. In this method a double-walled glass device was used to adjust the desired upper space temperature, and hot water at e specific temperature was sent to the walls that enclosed the plant vial. [21-24].

GC and GC-MS analysis

Analysis was carried out on a Hewlett-Packard-6890 gas chromatograph equipped with a split/splitless (20:1) injector (250 °C) and a flame ionization detector (250 °C). N_2 was used carrier gas (1 ml.min). The capillary column used was DB-5 (30 m × 0.25 mm, 0.32 µm film thickness). The oven temperature was held at 60 °C for 3 min, then heated to 220 °C with a 5°C rate and kept constant at 220°C for 5 min. Quantitative data were obtained from GC (FID) area percent without using correction factor. GC-MS analysis were performed using a Hewlett-Packard 6890-5973 GC-MS equipment whit a 30m × 0.25 mm, film thickness 0.32 µm with a HP-5MS column. He (99.999%) was used as carrier gas (1.0 ml/ min). The temperature program was as GC, the injection temperature and ion source temperature were 250°C and 240 °C, respectively. MS spectra were taken at 70 eV. All data were obtained by collecting the full scan mass spectra within the scan range 40-450 amu. The GC-MS was equipped with chemstation software and Wiley 275 library. Identification of constituents of the oil was made by comparison of their mass spectra and retention indices (RI) with those given in the literature and authentic samples [36].

The precision of MW-SPME

The method precision was studied by three replicate analyses of the volatile compound in *A.annua* L. by MW-SPME at the optimum conditions, and the obtained peak areas of the volatile compound were used for the calculation of relative standard deviation (R.S.D) values.

Results and discussion

The aim of this work was to investigate the composition of volatile compounds from aerial parts of *A.annua* L. obtained by MW-SPME and compared with HS-SPME and conventional HD. The
identified constituents in three methods can be seen in Table 1.

Table 1. Chemical composition of essential oils obtained by hydrodistillation, Headspace solid-phase microextraction and Microwave-assisted solid-phase microextraction.

Compound Name	KI[a]	HD[b] (%)	SPME (%)	MW-SPME (%)	R.S.D of MD-SPME (%)
α- thujene	929.6	0.21	-	-	-
α- pinene	940.4	13.04	8.27	11.43	5.2
Comphene	957.1	4.22	3.58	5.62	5.9
β- pinene	985.0	1.32	0.61	1.53	3.2
p-cymene	1031.3	1.31	1.02	4.78	6.3
1, 8- cineol	1040.8	8.36	8.32	20.55	8.7
artemisia ketone	1061.8	9.01	7.29	15.83	5.7
artemisia alcohol	1084.9	0.51	0.32	0.64	6.3
Linalool	1101.0	-	0.96	0.45	7.8
6-champhenol	1137.5	0.29	-	-	-
trans-pinocarveol	1157.9	5.99	3.96	3.38	8.4
Camphor	1167.4	19.06	15.72	23.34	6.5
Pinocarvone	1181.8	5.36	5.00	6.44	4.5
Borneol	1183.9	1.15	-	-	-
4-terpineol	1193.0	0.51	0.26	0.32	6.4
Mytenol	1213.1	-	0.30	-	-
Mytenal	1216.8	0.27	0.56	0.47	3.2
trans-carveol	1231.9	-	0.86	-	-
Thymol	1309.6	1.86	0.90	2.23	4.3
4-hydroxy cryptone	1356.2	-	18.44	-	-
Nepetalactone	1389.8	-	0.64	-	-
β-cubebene	1403.9	-	1.11	-	-
cis-jasmone	1419.9	-	0.56	-	-
β-caryophyllene	1455.4	0.45	0.54	-	-
trans-β-farnesene	1467.1	-	0.65	-	-
allo-aromadendrene	1507.2	1.41	2.37	-	-
dehydro-aromadendrene	1520.6	-	0.65	-	-
β-selinene	1526.4	10.40	10.33	1.73	8.6
caryophyllene oxide	1627.0	7.76	0.26	-	-
Total peak area (%)		**92.49**	**93.48**	**98.74**	
Total oxygenated fraction (%)		**60.13**	**64.35**	**73.65**	
Total extraction time (min)		**210**	**20**	**5**	
Sample amount (gr)		**100**	**0.5**	**5**	

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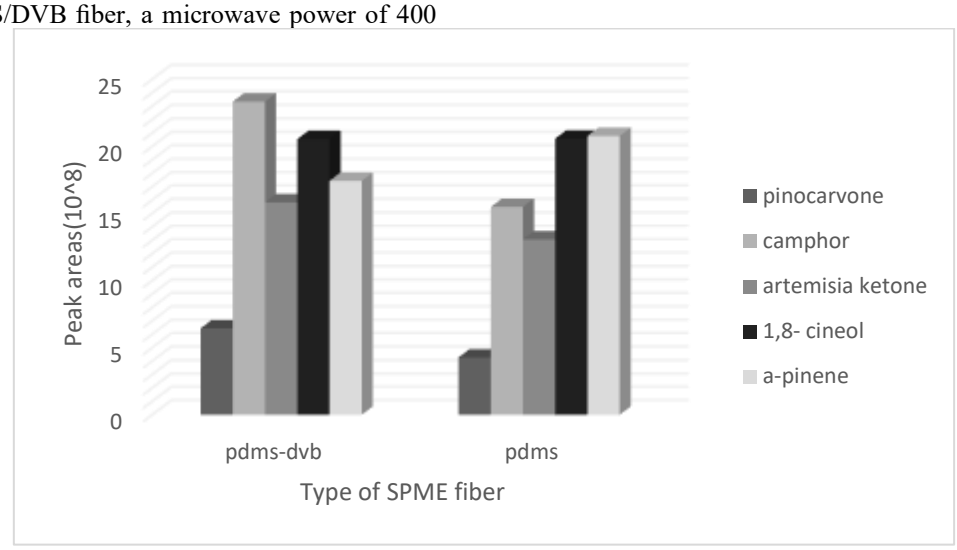
[a] Compounds listed in order of elution.

[b]RI, Kovats indices as determined on a DB-5 column using the homologous series of *n*-hydrocarbons(C9–C19).

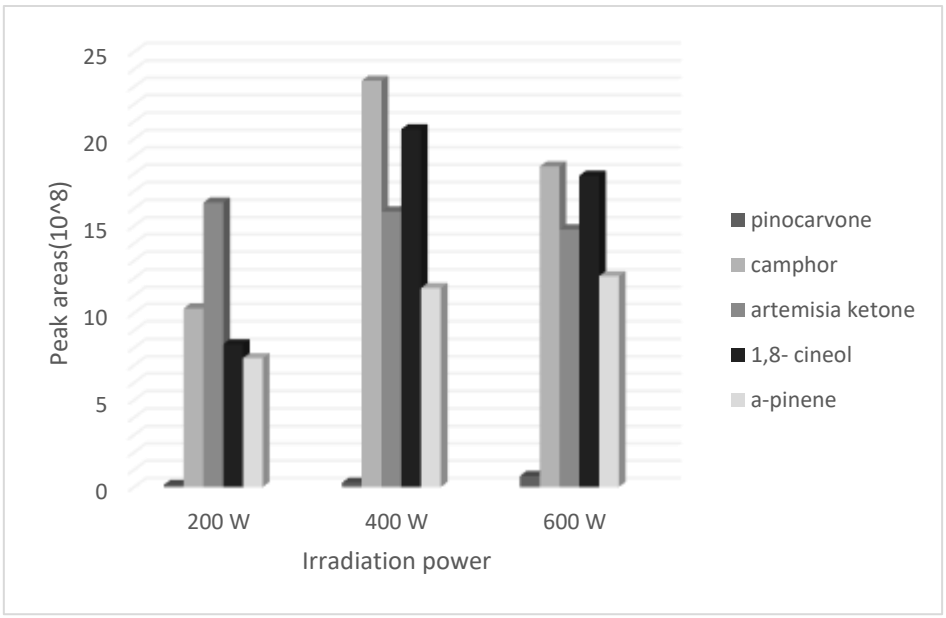
Optimization of the MW–SPME parameters

Three MW–SPME parameters, including SPME fiber coating, microwave power and irradiation time,

were studied. The optimal MW–SPME conditions are: PDMS/DVB fiber, a microwave power of 400 W and an irradiation time of 5 min.



(A)



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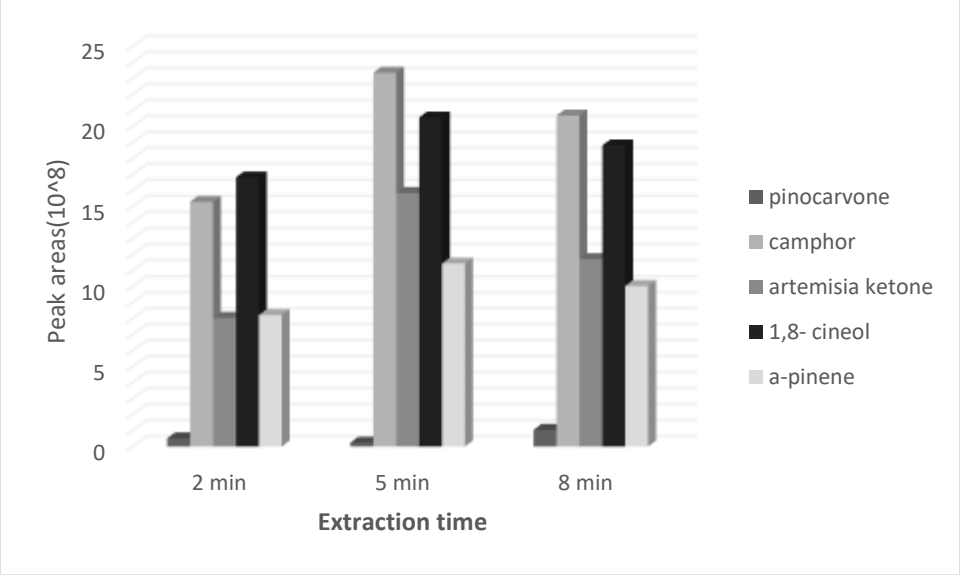

(C)

Figure 2. Effect of different parameters on the peak areas of some volatile compounds in *A.annua* L. aerial parts: (A): Effect of different fiber types ;(B): Effect of microwave power in MW-SPME;(C): Effect of the irradiation time in MW-SPME.

The variation of the concentrations of monoterpene and sesquiterpene hydrocarbons and oxygenated compounds for the *A.annua* L. aerial parts essential oils obtained by each method. The properties of the PDMS/DVB fiber are suitable for extraction of volatile and non-polar and semi-polar compounds, and hence the percentages of monoterpenes obtained by HS-SPME method are higher than conventional hydro-distillation method. As the MW-SPME method more efficient extraction of oxygenated compounds, the percentages of oxygenated compounds extracted by MW-SPME are higher than that of HD and HS-SPME (Figure 2).

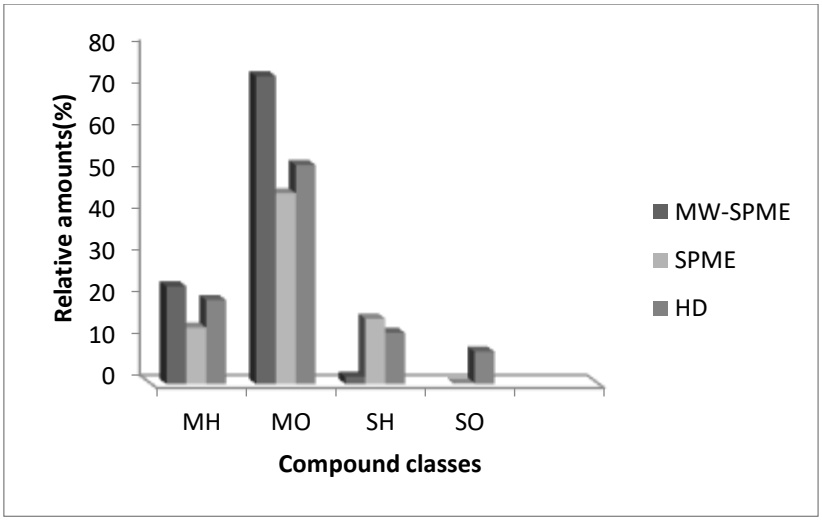

Figure 2. Variations in the composition of volatile compounds in *A.annua* L. aerial parts with respect to different extraction methods. (MH: Monoterpene hydrocarbons, MO: Monoterpene oxygenated compounds, SH: Sesquiterpene hydrocarbons, SO: Sesquiterpene oxygenated compounds)

Composition of the volatile compound in *A.annua* L. by MD-SPME and comparison with conventional methods

The optimized MW–SPME conditions were applied to the extraction of volatile compound in *A.annua* L. The volatile compounds were identified by matching mass spectra with spectra of reference compound in Adams and Wiley mass spectral libraries. Twenty-

five components were identified, and are listed in Table 1. Hydrodistillation method, mainly included camphor (19.06), α –pinene (13.04), β –selinene (10.40), Artemisia ketone (9.01), 1,8-cineol (8.36 %). From Table 1, the proposed SPME/GC–MS method identified the larger percent of oxygenated compounds in *A.annua* L. that has been demonstrated that microwaves can significantly improve the extraction efficiencies of plant volatile compounds. The oxygenated compounds are highly odoriferous and, hence, the most valuable [37]. Moreover, rapidity is another important feature of the proposed MW–SPME extraction method. Conventional HS–SPME required more than 20 min to isolate the volatile compounds to perform further extraction. In the case of HD, a time period of at least 3.5 h was necessary for separation of essential oil. Whereas, MW-SPME procedure performed with very small amount of aromatic plant about 3 min. Furthermore, the isolated products can be directly used for GC and GC-MS analysis without further preparation. All the techniques applied to analysis of the volatile compound in the *A.annua* L. showed that camphor, α –pinene, β –selinene, Artemisia ketone and 1,8-cineol were the major components. The changes in the composition volatile compound might have arisen from several differences (climatical, seasonal, geographical and geological) [38]. In Fig. 3, the chromatograms obtained with different methods for areal part of *A.annua* L. shown.

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Title     :
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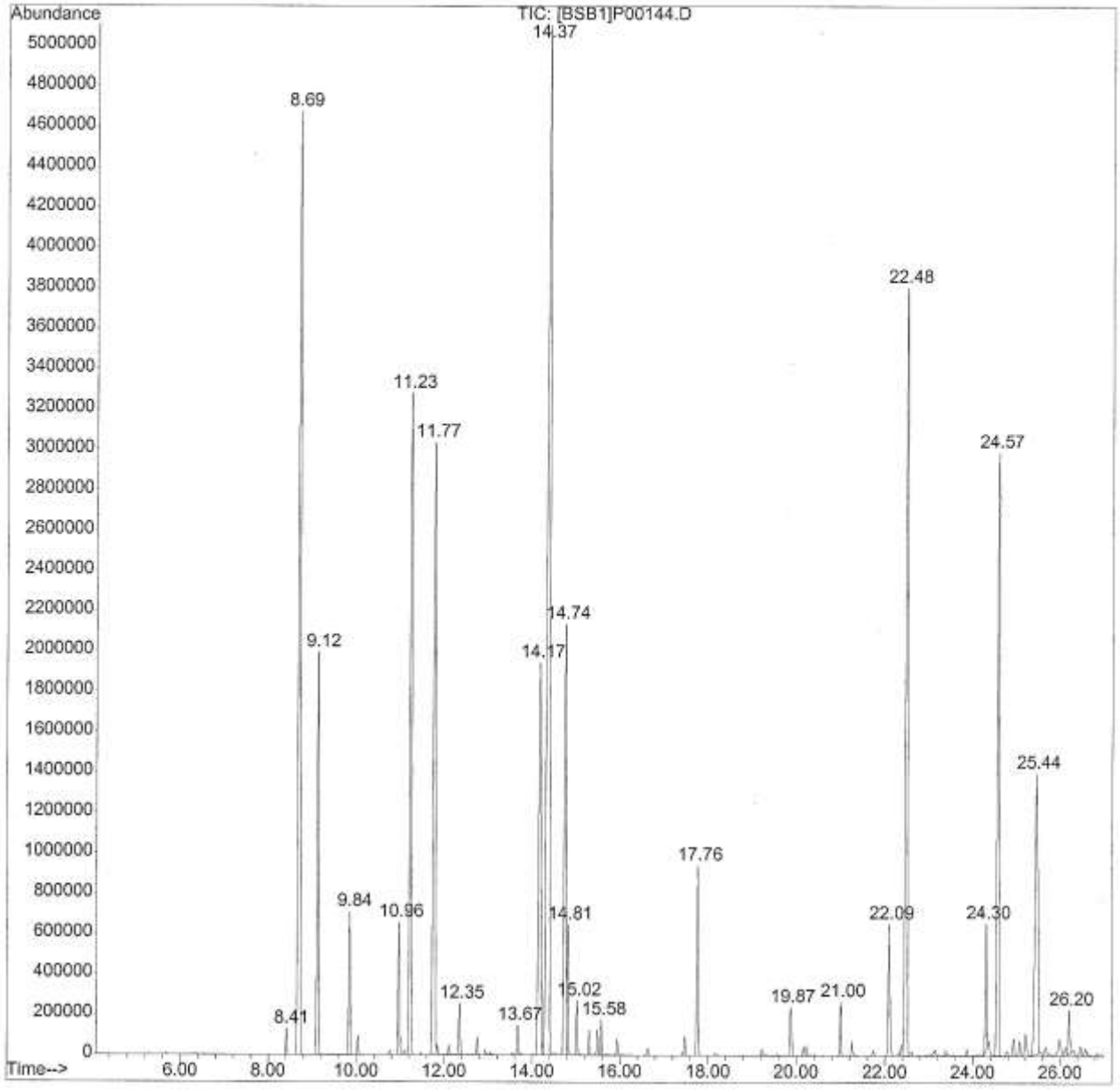
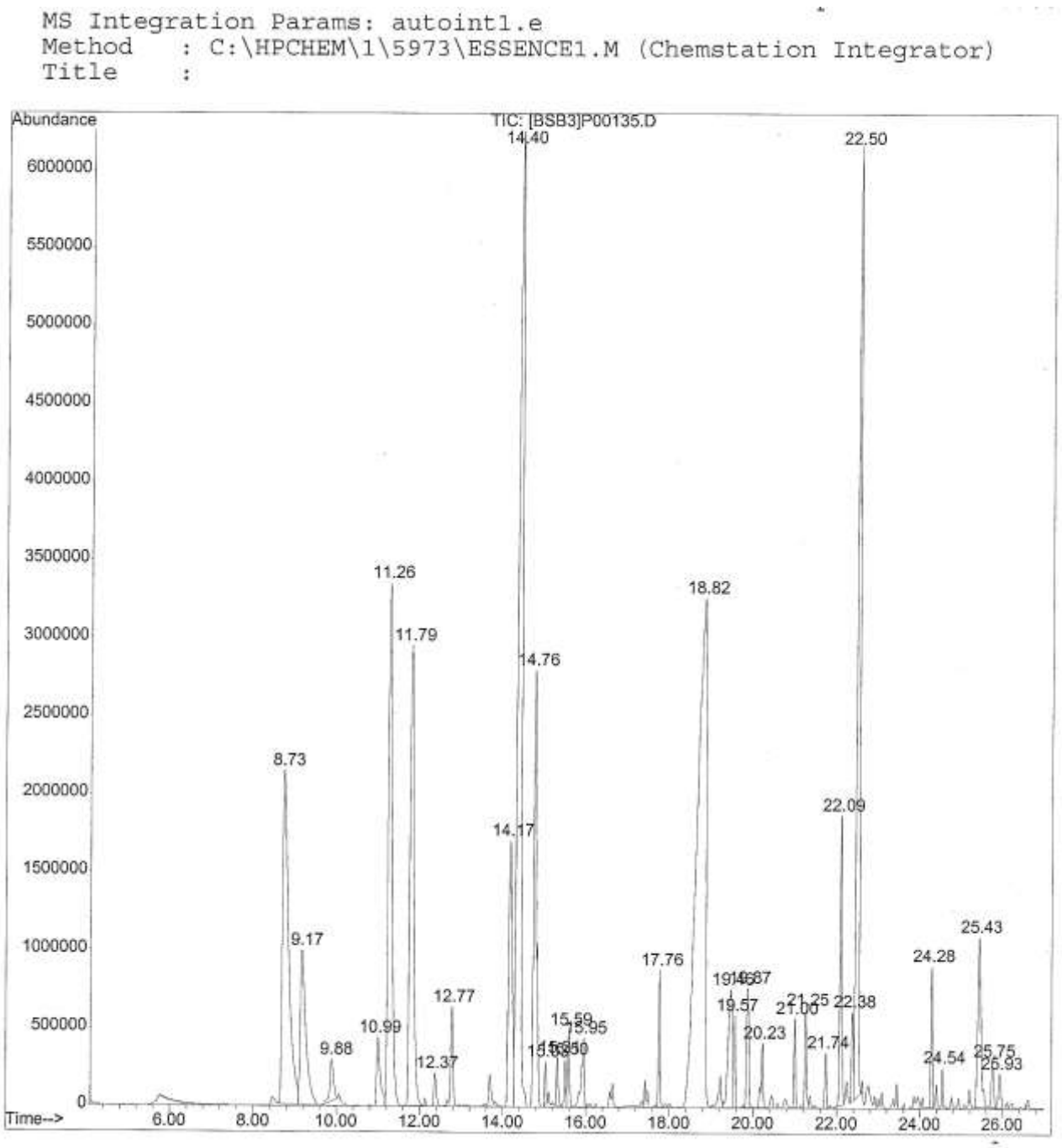


(A)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11.79
14.76
8.73
22.09
14.17
9.17
25.43
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21.74
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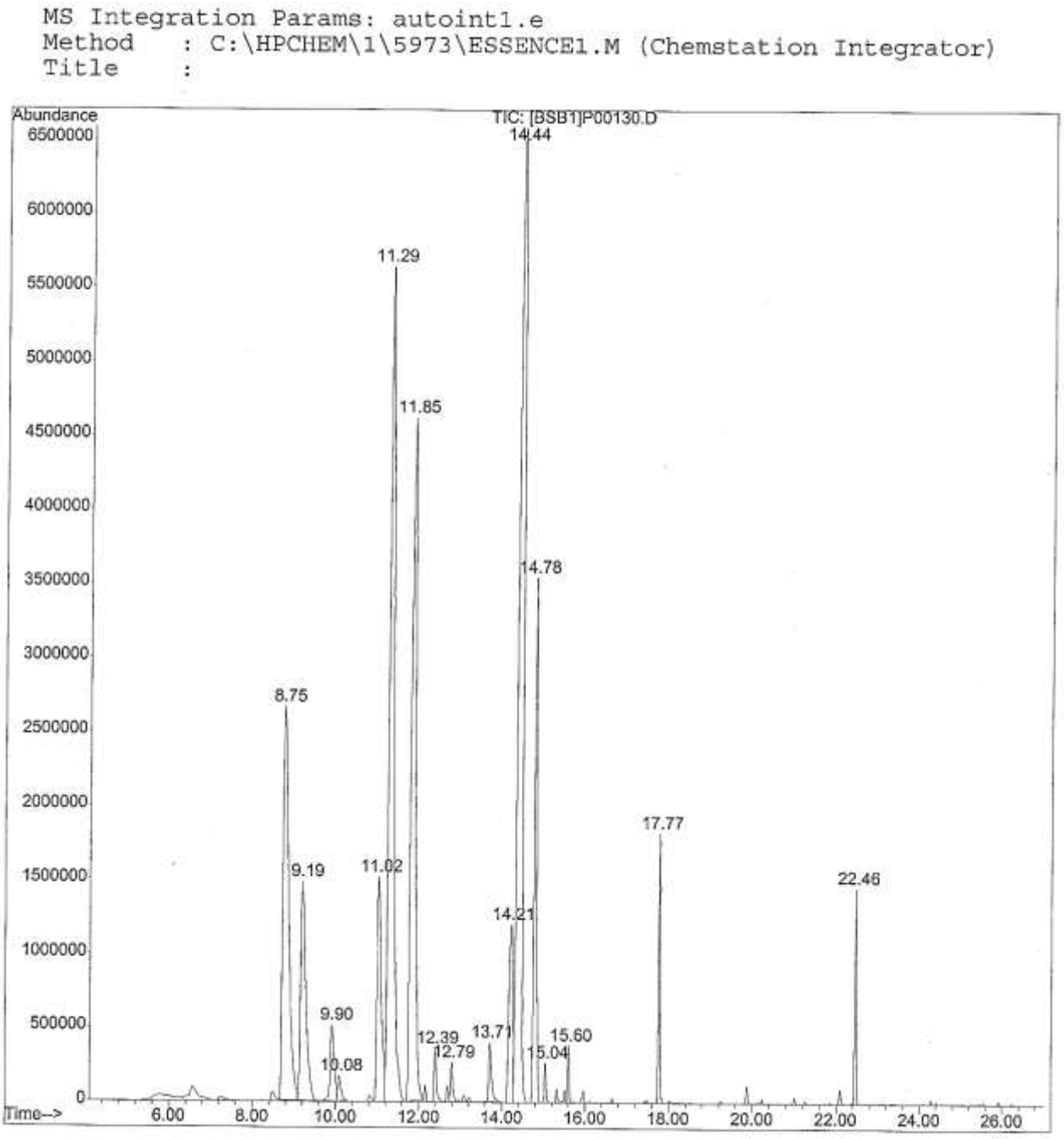
25.75
25.93

(B)



(C)

Figure 3. The GC–MS chromatograms of volatile compounds in *A.annua* L. aerial parts obtained by (a): HD; (b) HS-SPME and (C): MW-SPME.

Antimicrobial activity

Minimum inhibitory concentrations (MICs) of the essential oil was determined by agar dilution method (EUCAST,2000) with respected to different test microorganisms including gram-positive (Bacillus subtilis ATCC 6633) and gram-negative (Escherichia coli PTCC 1330) bacteria. A series of seven dilutions of the oil was prepared in ethanol. Each dilute was added to molten Muller Hinton (MH) agar at 50 ºC to give the final concentrations of 0.025, 0.05, 0.1, 0.15, 0.25, 0.5, 1 mg/ml. The bacteria inocula were prepared by suspending overnight colonies from MH agar media in sterile saline. The inocula were adjusted photometrically at 600 nm to a cell density equivalent to approximately 10^7 CFU/ml. The antibacterial activity of the essential oil is summarized in table.2. Both gram-positive and gram-negative bacteria were sensitive to this oil, Bacillus subtilis with the MIC of 0.25mg/ml and E. coli with the MIC of 0.5 mg/ml. The antibacterial activity showed the essential oil could be attributed to the presence of oxygenated monoterpenes, which are

known to exhibit antibacterial activity [39-40]. However, synergistic and antagonistic effect of compounds in the oil should also be taken in consideration. It appears that the presence of camphor, α- pinene and 1, 8- cineol in the areal part oils were responsible for the antibacterial activity.

Table 2. Minimum inhibitory concentration (MICs) of essential oils of *A.annua* L. using agar dilution method

Bacteria	MIC (mg/ml)
Bacillus subtilis	*0.25*
Escherichia coli	*0.50*

Conclusions

In this study, an MW-SPME/GC-MS technique was successfully performed for the determination of volatile compounds in *A.annua* L. Twenty-five compounds were identified in *A.annua* L.using proposed method. Compared with conventional Hydrodistillation method, MW-SPME/GC-MS is a simple, rapid, solvent-free and efficient method for analysis of volatile compounds in *A.annua* L. and other dried plant tissues. R.S.D values obtained less than 9% show that MW-SPME couple with GC-MS has good precision. Furthermore, the oil tested represent an inexpensive source of natural antibacterial substances for use in pathogenic systems to prevent growth of bacteria.

Acknowledgment

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