UNIVERSIDAD DE SANTIAGO DE CHILE

BOLETÍN LATINOAMERICANO Y DEL CARIBE DE PLANTAS MEDICINALES Y AROMÁTICAS
18 (6): 586 - 594 (2019)
© / ISSN 0717 7917 / www.blacpma.usach.cl

Blacpma

Artículo Original | Original Article

Anti-leishmanial activity and identification of chemical constituents of *Scrophularia striata* essential oil by head space solid phase micro extraction gas chromatography mass spectrometry (HS-SPME GC-MS)

[Actividad anti-leishmanial e identificación de componentes químicos del aceite esencial de *Scrophularia striata* mediante microextracción en fase sólida en el espacio de cabeza seguido de cromatografía gaseosa acoplada a espectrometría de masas (HS-SPME GC-MS)]

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Abstract: The aim of the present study was to determine the main constituents of *Scrophularia striata* essential oil and to evaluate *in vitro* effect of essential oil on *Leishmania tropica* and *Leishmania major* promastigotes and axenic amastigotes. Chemical constituents of the extracted essential oil were separated by headspace solid-phase microextraction (HS-SPME) equipped with a PDMS/DVB fiber. The fiber was injected to gas chromatogram- mass spectroscopy (GC-MS) to determine their identity. Finally, after exposure of parasites to different concentrations of water soluble fraction of essential oil, viability of promastigotes and axenic amastigotes were investigated. Based on the HS-SPME results, 47 compounds representing 95.6% of the total oil, were identified in essential oil. Essential oil analysis showed that nonane (19.7%), α-terpineol (17.4%) and linalool (10.2%) were the most abundant compounds. This study indicates that water soluble fraction of *S. striata* essential oil has promising anti-leishmanial activity.

Keywords: Essential oil; HSPME GC/MS; *Leishmania tropica*; *Leishmania major*; *Scrophularia striata*.

Resumen: El objetivo del presente estudio fue determinar los principales componentes del aceite esencial de *Scrophularia striata* y evaluar el efecto *in vitro* del aceite esencial en promastigotes y amastigotes axénicos de *Leishmania tropica* y *Leishmania major*. Los componentes químicos del aceite esencial extraído se separaron mediante microextracción de fase sólida en el espacio superior (HS-SPME) equipado con una fibra PDMS/DVB. Para determinar su identidad la fibra se inyectó en un cromatógrafo de gases acoplado un espectrómetro de masas (GC-MS). Finalmente, después de la exposición de los parásitos a diferentes concentraciones de fracción soluble del aceite esencial en agua, se investigó la viabilidad de los promastigotes y los amastigotes axénicos. En base a los resultados de HS-SPME, se identificaron 47 compuestos que representan el 95.6% del aceite total en el aceite esencial. El análisis de aceites esenciales mostró que el nonano (19.7%), el α-terpineol (17.4%) y el linalol (10.2%) fueron los compuestos más abundantes. Este estudio indica que la fracción soluble en agua del aceite esencial de *S. striata* tiene una actividad antileishmanial prometedora.

Palabras clave: Aceite esencial; HSPME GC/MS; *Leishmania tropica*; *Leishmania major*; *Scrophularia striata*.

Recibido | Received: May 14, 2019
Aceptado | Accepted: July 27, 2019
Aceptado en versión corregida | Accepted in revised form: October 1, 2019
Publicado en línea | Published online: November 30, 2019
Este artículo puede ser citado como / This article must be cited as: S Bahrami, MHR Jalali, Z Ramezani, G Janeh. 2019 Anti-leishmanial activity and identification of chemical constituents of *Scrophularia striata* essential oil by head space solid phase micro extraction gas chromatography mass spectrometry (HS-SPME GC-MS). **Bol Latinoam Caribe Plant Med Aromat** 18 (6): 586 – 5594. **Https://doi.org/10.35588/blacpma.19.18.6.41**

INTRODUCTION

Leishmaniasis is one of the most important zoonotic diseases caused by parasites of the genus *Leishmania* in the family Trypanosomatidae. The clinical manifestations of leishmaniasis are classified: cutaneous, mucocutaneous, and visceral leishmaniasis, which is also known as kala-azar (Desjeux, 2004; Murray *et al*., 2005). Cutaneous leishmaniasis, the most common form, is a group of diseases with a varied spectrum of clinical manifestations, which range from small cutaneous nodules to gross mucosal tissue destruction. About 1.5 million new cases of cutaneous leishmaniasis occur each year. Cutaneous leishmaniasis is endemic in more than 70 countries worldwide and over more than 90% of cutaneous leishmaniasis cases occur in seven countries: Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria (Reithinger *et al*., 2007). *L. tropica* and *L. major* are the main etiologies of cutaneous leishmaniasis in Iran (Bahrami *et al*., 2011). Chemotherapy is the most effective treatment against leishmaniasis, due to the lack of an effective vaccine (Handman, 2001). The recommended first-line chemotherapy for different forms of leishmaniasis includes pentavalent antimonials like glucantime and pentostam. But, due to expenses, necessity for intravenous administration, toxicity, and the increase in parasite drug resistance treatment of leishmaniasis is often difficult. Also, these drugs have secondary effects on the renal, cardiac, and hepatic systems (Berman, 2003). Thus, development of new chemotherapeutic agents is important for the control of disease (Pandey *et al*., 2005). Indiscriminate application of synthetic chemicals has led to several ecological and medical problems due to hormonal imbalance, residual toxicity, teratogenicity, carcinogenicity, and spermatotoxicity (Kumar *et al*., 2007). Natural products (NPs) play an important role in drug discovery and about more than 50 percent of FDA-approved drugs were NPs or natural products derivatives. Plants are clearly a potential source of new antiprotozoal drugs. The biological activity of plant extracts has been attributed to compounds belonging to diverse chemical groups including alkaloids, flavonoids, phenylpropanoids, steroids, and terpenoids (Iwu *et al*., 1994; Rocha *et al*., 2005; Wang *et al*., 2009). According to Basso *et al*. (2005) natural products and their derivatives are the sources of 30% of the global pharmaceutical market.

The Scrophulariaceae family consists of 220 genera. *Scrophularia* genus is one of the large genera of the Scrophulariaceae. This genus is represented by 60 species in the flora of Iran and can be used as heart stimulant, circulatory stimulant and diuretic. Other traditional uses of this genus include antipyretic, febrifuge, antibacterial, anti-erythema, anticonstipation, antifurunculosis, ulcerous stomatitis and tonsillitis treatment (Pasdaran & Hamedi, 2017). From ancient times, in some regions of Iran, *S. striata* been used traditionally to treat eczema, wounds and ulcers. Among these traditional uses of the *Scrophularia*, anti-inflammatory and anti-infections' treatment in different types of diseases is common (Swiatek & Dombrowicz, 1975). Tasdemir *et al*. (2008) reported that crypthophilic acids from the aerial parts of *Scrophularia cryptophila* exerted significant anti-leishmanial activity against *L. donovani*. As far as the authors are aware, there are no published reports regarding the leishmanicidal effect of *S. striata* essential oils. The aim of the present study was to identify and quantify the *S. striata* essential oil components and to evaluate the leishmanicidal effect of the oil. To our knowledge, it is the first time that PDMS/DVB fiber is used to identify the chemical constituents of *S. striata* essential oil using head space solid phase micro extraction gas chromatography mass spectrometry (HS-SPME GC-MS)

MATERIALS AND METHODS

Preparation of plant material

Aerial parts of *S. striata* were collected from mountains of suburb of Ilam city, Ilam, Iran, 33°38′15″N 46°25′22″E. The plant species was identified and authenticated by Dr. R. Asadi, a plant taxonomist, at the Marvdasht University Herbarium, Fars, Iran. A voucher specimen (32586) has been deposited in the herbarium.

Extraction of the essential oil

Whole plants containing stem and seed were collected at full ripening stage, then dried under shade, cut into small pieces, ground mechanically using a commercial electric blender and subjected to hydro-distillation using a Clevenger-type apparatus (200 g, 2 h) according to the method recommended by the European Pharmacopoeia (Council of Europe, 1997). The present water soluble essential oils components were extracted by liquid-liquid extraction using heptane as the extracting solvent. The extracted samples were dried over anhydrous sodium sulfate, filtered, and stored in sealed vials at

4ºC for gas chromatography mass spectrometry (GC/Mass) analysis and anti-leishmanial uses.

Gas chromatography-Mass spectrometry analysis of the essential oil

Identification of essential oil was carried out on an Agilent 7890A GC (Palo Alto, CA, USA) with split/split less injection port equipped with an Agilent 5975 mass detector. The MS detector was operated in electron impact (EI) mode with ionization energy of 70 eV. Helium with purity of 99.999% was used as the carrier gas at a flow rate 1 mL min^{-1}. Mass spectrum range was 35-1050 amu. The compounds were separated on a 30 m × 250 µm HP-5 MS column with 0.25 µm film thickness. The GC column temperature was first 40ºC (t=0 min), then increased to 300ºC by 5ºC min^{-1} stay at the final temperature for 3 min. The injection port was set at 280ºC with split ratio of 50:1. The GC–MS interface, ion source and quadruple temperatures were set at 280, 150, and 230ºC, respectively. The solvent delay and total run time for analysis were 3 min and 56 min, respectively. Injection volume was 0.4 µL.

HS-SPME conditions

The plant materials were cut roughly with scissors (1–2 cm long) before subjection to HS-SPME. The SPME device (Supelco) coated with divinylbenzene/polydimethylsiloxane (PDMS/DVB, 65 µm) was used for head space analysis of the plant volatiles. Optimization of conditions was carried out using fresh plant (1 g in a 20 mL GC head space vial) and based on the number and the sum of total peak areas measured on GC-MS. HS-SPME and subsequent analyses were performed in triplicate. Before sampling, each fiber was reconditioned for 10 min in the GC injection port at 250ºC.

Identification and quantification of constituents

The quantitative data regarding the volatile constituents were obtained by peak-area normalization using an Agilent GC/MS operated under above mentioned conditions.

The retention index was calculated for all the volatile constituents by using chromatograms obtained for *n*-alkane (C7–C28) homologous series at the same GC-MS conditions. The peak assignment was performed by comparison of both mass spectrum and GC retention data available in library, as well as with the aid of commercial libraries containing retention indices and mass spectra of volatile compounds commonly found in essential oils (Skaltsa *et al*., 2003; Siegmund & Murkovic, 2004).

Culture of the parasites

The *Leishmania* strain used in this study was *L. tropica* (MHOM/AF/88/KK27) and *L. major* (MRHO/IR/75/ER). The promastigotes were grown at 26ºC in BHI medium plus 10% heat-inactivated fetal calf serum, pH 7.0 and 1% of Penicillin (50 u/ml) Streptomycin (50 µg/ml) solution. (Sigma, St. Louis, Mo., USA). The methods used for *in vitro* transformation of metacyclic promastigotes to axenic amastigotes were done according to our previous investigation (Bahrami *et al*., 2011). Therefore, amastigote-like forms were obtained in BHI medium supplemented with 20% FCS at pH 4.5 after 48 h of incubation at 37ºC in the presence of 5% CO_2. To evaluate the morphology of the transformed parasites, the axenic amastigotes were harvested from the culture, centrifuged at 400×g with 50 mM phosphate-buffered saline (PBS), applied to microscope slides, fixed and stained with Giemsa, and examined with an ordinary light microscope (Olympus Optical, NY, model BX60, Tokyo, Japan) at ×1,000.

Cell proliferation measurements by colorimetric MTT assay

Parasites susceptibility to essential oil was evaluated by following up the proliferation of parasites in the absence or presence of essential oil. Water soluble fraction of essential oil was added to the growth medium 24 h after starting the cultures with 10^6 parasites/ml. The essential oil concentration was ranged from 0.5 to 8% (0.5, 1, 2, 4 and 8%). Promastigotes and axenic amastigotes cultured at complete medium without treatment were used as negative control, treated with glucantime as positive control and complete medium without parasites and drugs were used as the blank. The plates were incubated at 25ºC for 48 h before MTT assay. All tests were performed in triplicates. The cytotoxic effects of *S. striata* against promastigotes and axenic amastigotes of *L. major* and *L. tropica* were investigated, using MTT [3-(4, 5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay as described by Lyu & Park, (2005). MTT colorimetric assay measures reduction of MTT dye (tetrazolium) into formazan by mitochondrial enzymes in viable cells. 48h after treatment, MTT was added to the cells in culture and the plate was incubated at 37ºC for 4 h. Finally, DMSO was added

to dissolve the formazan dye. The absorbance was measured at 570 nm. The viability percentage was calculated by: [(AT–AB)/(AC–AB)] ×100

Where, AB is OD of the blank well, AC is OD of the negative control and AT is OD of the treated cells. Results were expressed as the concentrations that inhibited parasite growth by 50% (IC_{50}) which were determined by linear regression analysis.

RESULTS

Analysis of essential oil

The composition of the essential oil obtained from the aerial parts of *S. striata* is compiled in Table No. 1, where the components are listed in order to their RI. Direct injection of the heptane extract and head space analysis of the herb showed the same components but different concentrations. However, HS-SPME is faster than direct injection of the heptane extract since extraction and analysis are simultaneous. Based on the HS-SPME results, 47 compounds representing 95.6% of the total oil, were identified in essential oil. The components of essential oil were separated into two classes, which were terpenoids (84.6%) and non-terpenoid (11%) compounds. Terpenoids were represented mainly by nonane (19.7%), α- terpineole (17.4%) and linalool (10.2%) as the most abundant compounds, respectively. Among non-terpenoid compounds, fatty acids constituted the main fraction of this part, with Isovaleric acid (9.3%) as the major components.

Anti-Leishmania activity assay

L. tropica promastigotes and axenic amastigotes were exposed to different concentrations of *S. striata* essential oil, and their proliferation was followed for 2 days. Results showed a concentration-dependent inhibition of the growth induced by the treatment. The 50% inhibitory concentration was 5.8% (58 mg/ml) and 2.1% (21 mg/ml) for promastigotes and axenic amastigotes of *L. tropica*, respectively after 48h of treatment. Treatment with *S. striata* essential oil resulted in a concentration-dependent inhibition of *L. major* promastigotes and axenic amastigotes viability with an IC_{50} of 6% (60 mg/ml) and 4.5% µM (45 mg/ml), respectively. Tables No. 2 and No. 3 showed the percentage of dead *L. tropica* and *L. major* promastigotes and axenic amastigotes evaluated by MTT assay following treatment with different concentrations of *S. striata* essential oil and in positive and negative control groups.

DISCUSSION

In most cases pentavalent antimonials remain an efficient anti-leishmanial treatment, but their parenteral route of administration, long duration of treatment and side effects limit their usages (Aronson *et al*., 1998). In patients with human immunodeficiency virus the antimonial drugs are inefficient and toxic (Domingo *et al*., 1996). Indiscriminate application of synthetic chemicals led to several ecological and medical problems due to residual toxicity, carcinogenicity, teratogenicity and hormonal imbalance. Moreover, the emergence of parasites resistant current chemotherapies highlights the importance of ne alternative therapeutic approaches for these parasites (Croft *et al*., 2006). Plants are an important source for drug discovery-particularly for parasites because of the long association between the coexistence of parasites, humans and herbal remedies (Srivastava *et al*., 2009a; Srivastava *et al*., 2009b). Essential oils which are accumulated in aromatic plants are chiefly used as flavors or fragrances, but currently a renewed interest in natural substances has focused attention on plants rich in bioactive compounds. Among these compounds, essential oils well known for their antimicrobial properties.

In this study chemical constituents of S. striata essential oil was identified using HS-SPME GC-MS. HS-SPME has several advantages including its avoidance of solvents, ease of automation, and small sample size requirements (Marsili, 2012). Based on the results, nonane (19.7%), α-terpineole (17.4%) and linalool (10.2%) were the most abundant compounds. These results were in contrast with Amiri *et al*. (2011) study that reported the linalool as the major constituent of *S. striata* essential oil. In Barati & Sani, (2017) study, result of GC-MS analysis of S. khorassanica showed that the main components of the essential oil were p-cymene (20.68%), palmitic acid (11.19 %), thymol (10.21%), linalool (7.56%), carvacrol (2.87%), γ-terpinene (3.15%) and β-elemene (1.7%). In Asgharian *et al*. (2015) study, Linalool (22.35%), geraniol (7.27%) and α-terpineol (5.25%) were the most abundant compounds in *S. subaphylla* essential oil. These findings showed that the genus *Schrophularia* had a remarkable variation in essential oil composition.

Table No. 1
Chemical composition of essential oil identified by headspace solid-phase microextraction (HS-SPME) gas chromatography- mass spectroscopy (GC-MS)

No	Component	Retention index	Percentage	
			GC-MS	HS-SPME
1	Cineol	778.3	1.8	3.3
2	Nonane	815.8	9.4	19.7
3	Isovaleric acid	845.4	4.2	9.3
4	1-Ethyl-2-methyl benzene	856.9	1	1.1
5	1,3 dimethyl Benzene	866.5	2.47	6.0
6	α- Terpineol	877	6.7	17.4
7	2methyl-2-Pentanol	920	0.1	0.1
8	α-Pinene	925	0.1	0.3
9	Decane	930.4	2.51	6.3
10	Camphene	945.2	0.2	0.3
11	α- Terpinene	956.9	0.1	0.2
12	1-Octen- 3- ol	962	0.1	0.1
13	ß-Myrcene	980	0.1	0.1
14	α-Phellandrene	1014	0.1	0.1
15	m-Cymene	1009.4	2.76	5.8
16	p-Cymene	1018.1	0.2	0.6
17	Limonene	1032.2	1.17	2.1
18	δ-Terpinene	1055	0.1	0.2
19	Linalool	1088	8	10.2
20	α-Thujene	1109	0.1	0.2
21	Isopulegol	1145	0.1	0.1
22	Butanoic acid	1165	0.3	0.4
23	Z-Geraniol	1210	0.2	0.3
24	Geraniol	1230	0.1	0.3
25	2-Decanol	1255	0.1	0.3
26	2-Undecanone	1273	0.1	0.1
27	Carvacrol	1298	0.1	0.1
28	γ-Damascone	1370	0.2	0.1
29	Z-ß-Damascenone	1377	0.1	0.2
30	ß-Elemene	1383	0.1	0.1
31	Caryophyllene	1426	1.9	3.1
32	Geranylacetone	1430	0.1	0.2
33	*trans*-Nerolidol	1537	0.1	0.1
34	Nerolidol B	1544	0.1	0.1
35	α-Bisabolol	1660	0.1	0.2
36	E,Z-Farnesol	1735	0.1	0.3
37	Myristic acid	1741	0.1	0.4
38	Benzyl benzoate	1760	0.2	0.5
39	Tetradecanedioic	1777	0.3	0.3
40	Palmitaldehyde	1791	0.1	0.3
41	Palmitic acid	1965	0.1	0.1
42	Phytol	2017	0.1	0.1
43	Heneicosane	2100	0.2	0.3
44	Linoleic acid	2105	0.2	0.3
45	Oleic acid	2117	0.1	0.1
46	Ethyl linoleate	2139	0.1	0.1
47	Indole	2669.4	2.3	3.7
	Total identified	-	**48.8**	**95.6**
	Terpenoid	-	**43.6**	**84.6**
	Non- terpenoid	-	**5.2**	**11**

Table No. 2
Percentage of dead *L. tropica* promastigotes and axenic amastigotes evaluated by MTT assay following treatment with different concentration of *S. striata* essential oil and in positive and negative control groups

Parasite stage	Essential Oil Different Concentration (%)					Negative control	Glucantime
	0.5	1	2	4	8		
Promastigotes	37.67±11.1[a]	40.28±8.9[a]	41.18±17.4[a]	48.52±12.1[a]	57.38±11.5[a]	3.8±0.8[b]	58.4±10.3[a]
Axenic amastigotes	54. 7±12.3[a]	56.3±20.1[a]	62.6±19.3[a]	65.7±18.3[a]	69.1±19.2[a]	5.8±0.8[b]	68.2±14.2[a]

Data are expressed as the mean ± SD and values in rows with different uppercase superscripts are significantly different ($p < 0.05$)

Table No. 3
Percentage of dead *L. major* promastigotes and axenic amastigotes evaluated by MTT assay following treatment with different concentration of *S. striata* essential oil and in positive and negative control groups

Parasite stage	Essential Oil Different Concentration (%)					Negative control	Glucantime
	0.5	1	2	4	8		
Promastigotes	36.23±5.4[a]	39.41±8.6[a]	42.34±6.1[a]	44.52±14.4[a]	51.65±9.6[a]	2.4±0.7[b]	51.6±8.6[a]
Axenic amastigotes	42.4±11.7[a]	44.7±17.2[a]	48.3±16.2[a]	51.5±12.1[a]	60.3±18.2[a]	4.8±0.9[b]	76.8±12.3[a]

Data are expressed as the mean ± SD and values in rows with different uppercase superscripts are significantly different ($p < 0.05$)

Nonane is a linear alkane hydrocarbon with the chemical formula C_9H_{20}. Different studies have shown that alkane hydrocarbon components are toxic for microorganisms. Changes in membrane lipid composition, modification of outer membrane lipopolysaccharide, inhibition of glucose transport system and cell wall constituent's alterations are the mechanisms of action attributed to these components (Sikkema *et al*., 1995). α- Terpineole, a monoterpene alcohol which is slightly water soluble with the chemical formula $C_{10}H_{18}O$ and linalool, an acyclic terpene alcohol which is appreciably water-soluble organic compound were the other obtained components in *S. striata* essential oil. Terpene alcohols have been known to have antibacterial (Inoue *et al*., 2004), molluscicidal (Lahlou *et al*., 2001), acaricidal (Born *et al*., 2012), insecticidal (Pandey *et al*., 2009), antifungal (Nagalakshmi *et al*., 2000) and antiparasitic (Moazeni *et al*., 2012) properties. Because of terpene alcohols lipophilic character, terpenes will preferentially partition from an aqueous phase into membrane structures. This results in membrane expansion, increased membrane fluidity and permeability, disturbance of membrane-embedded proteins such as ATPases or membrane receptors, inhibition of respiration, and alteration of ion transport processes (Sikkema *et al*., 1994; Sikkema *et al*., 1995). Therefore, they might exert their anti-leishmanial activity by interference with the energy metabolism of parasites potentiation of ATPase activity (Tamura & Iwamoto, 2004) and thus loss of energy reserves (Lateef *et al*., 2006).

Several investigations on *Scrophularia* have revealed various pharmacological activities such as wound healing, anti-inflammatory, antibacterial, cardiovascular, diuretic, protozoacidal, fungicidal, cytotoxic and anti-nociceptive (Akhmedov *et al*., 1969; Emam *et al*., 1997; Bermejo-Benito *et al*., 1998; Ghisalberti, 1998; Stevenson *et al*., 2002). According to Nokhodi *et al*. (2014) findings 25%

concentration of water soluble extract of *S. striata* could eliminate all promastigotes of *L. major*. Their study showed that *S. striata* was effective in healing of cutaneous leishmaniasis in BALB/c mice. Also, Zahiri *et al*. (2016) showed *S. striata* ethanolic extract 10% had anti leishmanial effects in both *in vivo* and *in vitro*. But there is not available data on leishmanicidal effects of *S. striata* essential oil. In the present study, leishmanicidal activity of *S. striata* essential oil was investigated. The 50% inhibitory concentration *S. striata* was 5.8% (58 mg/ml) and 2.1% (21 mg/ml) for promastigotes and axenic amastigotes of *L. tropica*, respectively. Treatment with *S. striata* essential oil resulted in a concentration-dependent inhibition of *L. major* promastigotes and axenic amastigotes viability with an IC_{50} of 6% (60 mg/ml) and 4.5% µM (45 mg/ml), respectively.

In conclusion, nonane, linalool and α-terpineol were the most abundant compounds in *S. striata* essential oil. Furthermore, this study indicates that water soluble fraction of *S. striata* essential oil has promising anti-leishmanial activity, but its mode of action remains incomplete at present and is an area for future research. To the best of our knowledge, it is the first time that HS-SPME is used to analyse the *S. striata* essential oils constituents.

ACKNOWLEDGMENTS

This study was supported by the research grant provided by Shahid Chamran University of Ahvaz.

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