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Chemical composition, *in vitro* cytotoxic and antioxidant activities of the essential oil of Peruvian *Minthostachys mollis* Griseb

[Composición química, actividades citotóxicas y antioxidantes *in vitro* del aceite esencial de *Minthostachys mollis* Griseb peruano]

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Abstract: The composition of the essential oil obtained by hydrodistillation from *Minthostachys mollis* Griseb (Lamiaceae) aerial parts was determined by GC and GC/MS. Menthone (13.2%), pulegone (12.4%), cis-dihydrocarvone (9.8%) and carvacrol acetate (8.8%) were the main essential oil components. The cytotoxic activity of the essential oil was in vitro measured using the MTT colorimetric assay. IC_{50} values were calculated on healthy non-tumor cells (HEK-293) and three human cancer cell lines (T24, DU-145 and MCF-7). In such latter cells, the estimated values were around 0.2 mg/mL. In addition, the antioxidant activity was determined by interaction with the stable free radical 2,2”-diphenyl-1-picrylhydrazyl. The essential oil was almost devoid of antioxidant activity indicating that its anti-proliferative action relies on other unknown mechanism.

Keywords: *Minthostachys mollis*; Essential oil; Chemical composition; Cytotoxicity; Antioxidant activity; Lamiaceae.

Resumen: La composición del aceite esencial obtenido por hidrodestilación a partir de partes aéreas de *Minthostachys mollis* Griseb (Lamiaceae) se determinó mediante GC y GC/MS. Mentona (13.2%), pulegona (12.4%), junto con cis-dihidrocarvona (9.8%) y acetato de carvacrol (8.8%) fueron los principales componentes del aceite esencial. La actividad citotóxica del aceite esencial se midió in vitro utilizando el ensayo colorimétrico MTT tanto en células sanas no tumorales (HEK-293) como en tres líneas celulares de cáncer humano (T24, DU-145 y MCF-7). Los valores de IC_{50} calculados fueron de alrededor de 0.2 mg/mL. Además, se determinó la actividad antioxidante por su interacción con el radical libre 2,2"-difenil-1-picrilhidrazilo. El aceite esencial tiene baja actividad antioxidante, lo que indica que su acción antiproliferativa depende de otro mecanismo desconocido.

Palabras clave: *Minthostachys mollis*; Aceite esencial; Composición química; Citotoxicidad; Actividad antioxidante; Lamiaceae..

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INTRODUCTION

Essential oils (EOs) are volatile compounds extracted from the leaves, flowers, fruits, stalks, roots, and resins of plants and are widely used since antiquity in medicine, pharmaceutical, perfumery, cosmetic, and in many food applications (Li *et al*., 2015; Al-Tamimi *et al*., 2016). EOs are now attracting increasing interest in the scientific community and a particular interest has been focused on their anticancer and antioxidant activities of essential oils from extraction of plants (Yan *et al*., 2017; Dar *et al*., 2011).

According to some authors, the genus *Minthostachys* include 17 species (Epling and Játiva 1963; Schmidth-Lebuhn, 2008). *Mintostachys mollis* (Lamiaceae) is a sub aromatic shrub that grows over a wide range of altitudes in Venezuela, Colombia, Ecuador, Peru and Bolivia (Alkire *et al*., 1994). It is known with the popular name of "muña" and "chancua" in the central and in northern of Peru respectively (Schmidt-Lebuhn, 2008). Numerous uses of *Minthostachys* from southern Peru have been described, they include seasoning, tea, medicine against colds and stomachache, preservation of stored potatoes (Ugarte *et al*., 1984); against flea infestations and rheumatism (Joyal, 1987); and against bronchitis, asthma, headache, to induce menstruation, and as a condiment (White, 1982).

Table No. 1 shows that a great variation in chemical composition have been observed in EOs derived from seeds, leaves and flowers of *Minthostachys mollis* isolated by hydrodistillation. Likely the collection in different locations and seasons may explain such great variability but pulegone was the monoterpene identified in all essential oil from different origins (33 - 85%).

Table No.1
Main constituents of the essential oils of *Minthostachys mollis* from different geographical origins previously reported

Retention Indices Apolar[a]	Retention Indices Polar[a]	Main component	Percentage (%)	Origin	Part of plant	Type of isolation	Reference
No determined		Neomenthol	29.3	Quito (Ecuador)	Flower	Hydrodistillation	Alkire *et al*., 1994
Not determined		Pulegone	79.32	San Rafael (Venezuela)	Leaves	Hydrodistillation	Rojas & Usubillaga, 1995
Not determined		Pulegone	39.6	Los Condores (Argentina)	Seeds	Not referred	Chebel *et al*., 1998
Not determined		Pulegone	42.8	Córdoba (Argentina)	Leaves and stems	Hydrodistillation	Valladares *et al*., 2002
Not determined		Pulegone	85	Córdoba (Argentina)	Leaves	Hydrodistillation	Banchio *et al*., 2005
Not determined		Pulegone	57.1 – 76.3	Padre Monti El Cajón	Aerial Parts	Hydrodistillation	Elchosa *et al*., 2007 Van Baren *et al*., 2014
		Carvacrol	19.9 – 23.5	Rio Nio			
		Limonene	35.7 – 43.3	La Florida			
		Carvacryl acetate	39.9	Chorrillos			
		Dihydrocarvone	38.4 – 57.2	Tafi del Valle			
		Linalool	68.5 – 71.7	Gonzalo region			
		Carvacryl acetate	31.4	Siambon (Argentina)			
Not determined		Pulegone Menthone	33.48 26.68	Lima (Perú)	Leaves	Hydrodistillation	Armas *et al*., 2016
Not determined		Carvacrol	21.24	Pamplona Norte, (Colombia)	Leaves	Hydrodistillation	Torrenegra-Alarcon *et al*., 2016

[a]GC Column

Although EO chemical composition of *Minthostachys mollis* have been previously investigated, to date there are no data in the literature about their cytotoxic and antioxidant activities. Three main objectives have been defined as aim of the study. First, to determine a chemical characterization of *Minthostachys mollis* essential oil. Second, to investigate its *in vitro* cytotoxic activity against three human cancer cell lines (T24, DU-145 and MCF-7) and in healthy non-tumor cells (HEK-293). Finally, to determine the antioxidant activity by its interaction with the stable free radical 2,2”-diphenyl-1-picrylhydrazyl.

MATERIAL AND METHODS

Plant material

Minthostachys mollis Griseb plants were collected in January 2017 in the Community of Chugur at 2648 m above sea level, in the province of San Marcos, Department of Cajamarca, Perú (Figure No. 1). Once collected, the specimen was identified from the Herbarium Truxillense de la Facultad de Ciencias Biológicas de la Universidad Nacional de Trujillo. A voucher sample under accession HUT 59477 was deposited in this herbarium.

Essential oil isolation

Fresh aerial parts (50 g) were subjected to hydrodistillation for 3 h using a Clevenger-type apparatus. The obtained oil was dried over anhydrous sodium sulfate and after filtration, stored protected stored at +4ºC until tested and analyzed.

Gas chromatography analysis

The essential oil was analyzed on a Perkin Elmer Clarus 400 gas chromatograph equipped with two flame ionization detectors (FIDs), a data handling system and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (polydimethylsiloxane, 30 m x 0.25 mm i.d., film thickness 0.25 μm; J & W Scientific Inc., Rancho Cordova, CA, USA) and a DB-17HT fused-silica column [(50% phenyl)-methylpolysiloxane, 30 m x 0.25 mm i.d., film thickness 0.15 μm; J & W Scientific Inc.]. Oven temperature was programmed, 45-175° C, at 3° C/min, subsequently at 15° C/min up to 300° C, and then held isothermal for 10 min; injector and detector temperatures, 280° C and 300° C, respectively; carrier gas, hydrogen, adjusted to a linear velocity of 30 cm/s. The samples were injected using split sampling technique, ratio 1:50. The volume of injection was 0.1 μL of a pentane-oil solution (1:1). The percentage composition of the oil was computed by the normalization method from the GC peak areas, calculated as mean values of two injections from oil, without using correction factors.

Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS analysis of the essential oil was conducted on a Perkin Elmer Clarus 600 gas chromatograph, equipped with DB-1 fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 μm; J & W Scientific, Inc.), and interfaced with a Perkin-Elmer Clarus 600T mass spectrometer (software version 4.1, Perkin Elmer, Shelton, CT, USA). Injector and oven temperatures were as above; transfer line temperature, 280°C; ion source temperature, 220° C; carrier gas, helium, adjusted to a linear velocity of 30 cm/s; split ratio, 1:40; ionization energy, 70 eV; scan range, 40-300 m/z; scan time, 1 s. The identity of the components was assigned by comparison of their retention indices, relative to C9-C21 *n*-alkane indices and GC-MS spectra from a home made library, constructed based on the analyses of reference oils, laboratory-synthesized components and commercial available standards.

Cytotoxic assays

Cell lines and cell cultures

Human cancer cell lines bladder (T24), prostate (DU-145), breast (MCF-7) and non-tumor HEK-293 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cultures were maintained at a density of 1-2 x 10^5 cells/ml and the medium was changed at 48- to 72-h intervals. They were cultured in high-glucose Dulbecco's modified Eagle medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). All cultures were kept at 37°C in 95% air/5% CO_2 at 100% humidity. Phosphate-buffered saline (PBS) was purchased from Gibco. Cells were incubated at the indicated times at 37°C with or without essential oil at various concentrations.

Cell survival assays

The cytotoxicity of the essential oil was assessed by following the reduction of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan blue (Mosmann, 1983). Cells were

seeded into 96-well plates at a density of 10 000 cells/well for 24 h and then incubated for 48 h with or without the essential oil. Doxorubicin was used as standard chemotherapeutic agent (positive control). Cells were then washed twice with warm PBS and incubated with MTT (0.5 mg/mL) for 2 hours at 37°C. Blue formazan crystals were solubilized by adding 100 µl DMSO/well, and the optical density of coloured solutions was subsequently read at 550 nm. Results are expressed as % of MTT reduction compared to untreated control conditions. The IC_{50} values were calculated using the GraphPad Prism software (San Diego, CA, USA).

Free radical scavenging activity

Free radical scavenging activity of EO were determined by using a stable free radical, namely DPPH (2,2"-diphenyl-1-picrylhydrazyl), according to a slightly modified method of Blois, 1958. Indeed, since DPPH has an unpaired electron its delocalization, by reaction with an antioxidant substance gives a violet color to the DPPH solution. By donating a hydrogen radical, DPPH is stabilized producing a decrease in absorbance (Castañeda *et al.*, 2008; Molyneux, 2004). DPPH solution was prepared at the concentration of 0.024 mg/mL of DPPH in ethanol. During assays, 1 mL of the EO (0.5 and 1 mg/mL) was mixed with 1 mL DPPH solution. Simultaneously, a control (Trolox®) was prepared without EO. The mixture was incubated at room temperature for 30 min and further reading on a TECAN micro plate reader plate at 515 nm. The percentage of free radical scavenging activity was expressed as percent inhibition from the given formula:

$$\% \text{ inhibition of DPPH radical} = \frac{\text{Abs. of control} - \text{Abs. of sample} \times 100}{\text{Abs. of control}}$$

The assay was performed in triplicate.

RESULTS AND DISCUSSION

As part of our research program focused on the evaluation of the popular use of medicinal plants of the Chilean and Peruvian Andean highlands, the biological activities of several plants has been investigated (Rojo *et al*., 2006; Rojo *et al*., 2009; Benites *et al*., 2009; Benites *et al*., 2011).

In this context *Minthostachys mollis* Griseb plants were collected in the Community of Chugur, province of San Marcos, Department of Cajamarca, Perú (Figure No. 1). The EO yield of *Minthostachys mollis* obtained from hydrodistillation of plant was calculated based on a moisture-free basis as 0.98% (w/w).

The results of the GC and GC/MS analyses are listed in Table No. 2. Identification of the components was based on the comparison of the GC retention indices (RI) on polar and non-polar columns, determined relative to the retention time of a series of *n*-alkanes with linear interpolation with those standards and with our essential oils database. The GC analysis of the EO showed detection of seventy-three compounds, accounting for 98.2% of the total composition. Oxygen-containing monoterpenes were the major constituents (76.2%), while the monoterpene hydrocarbons were present in concentrations of 17.6%. The sesquiterpene hydrocarbons were 3.3%, while oxygen-containing sesquiterpenes were 0.8%. In addition, other components were present in low concentrations in oil (0.3%).

Figure No. 1
Picture of *Minthostachys mollis,* from Cajamarca, Perú

Comparing the present data (Table No. 2) with those previously reported in literature (Table No. 1), the EO of *Minthostachys mollis* collected in northern of Peru shows menthone (13.2%), pulegone (12.4%), *cis*-dihydrocarvone (9.8%) and carvacrol acetate (8.8%) as the main constituents (Figure No. 2). However, in same species from different regions, pulegone was the major oxygenated identified terpene. To note that the yield and the compounds of EO bearing plants has been shown to be strongly influenced by environmental factors such as temperature, photoperiod, light intensity, nutrition, genotype, stage of development, moisture, and salinity (Sangwan *et al*., 2001)

Table No. 2
Percentage composition of the essential oil isolated from *Minthostachys mollis* collected in Cajamarca, Perú

Compound	RI[a]	Relative content (%)	Identification method
α-Thujene	924	1.3	RI, MS
α-Pinene	930	2.0	RI, MS
Camphene	938	0.1	RI, MS
Sabinene	958	0.4	RI, MS
1-Octen-3-ol	961	t	RI, MS
β-Pinene	963	0.7	RI, MS
Myrcene	975	0.4	RI, MS
α-Terpinene	1002	0.2	RI, MS
p-Cymene	1003	5.6	RI, MS
1,8-Cineole	1005	4.6	RI, MS
β-Phellandrene	1005	t	RI, MS
Limonene	1009	5.5	RI, MS
cis-β-Ocimene	1017	0.1	RI, MS
trans-β-Ocimene	1027	0.3	RI, MS
γ-Terpinene	1035	1.0	RI, MS
trans-Sabinene hydrate	1037	0.2	RI, MS
Linalool	1074	1.8	RI, MS
Menthone	1120	13.2	RI, MS
Citronellal	1121	0.4	RI, MS
Isomenthone	1126	1.0	RI, MS
cis-Isopulegone	1134	0.1	RI, MS
Terpinen-4-ol	1148	0.2	RI, MS
cis-Dihydrocarvone	1159	9.8	RI, MS
α-Terpineol	1159	0.2	RI, MS
trans-Dihydrocarvone	1164	0.6	RI, MS
Dihydrocarveol	1167	1.0	RI, MS
Pulegone	1210	12.4	RI, MS
Carvone	1210	0.6	RI, MS
Piperitone	1211	6.7	RI, MS
cis-Piperitone epoxide	1211	7.8	RI, MS
Bornyl acetate	1265	0.2	RI, MS
Thymol	1275	1.2	RI, MS
Carvacrol	1286	3.3	RI, MS
Dihydrocarveol acetate	1288	0.3	RI, MS
Piperitenone	1289	t	RI, MS
Thymol acetate	1330	0.9	RI, MS
Piperitenone oxide	1330	0.7	RI, MS
α-Terpenyl acetate	1334	t	RI, MS
Carvacrol acetate	1348	8.8	RI, MS
Geranyl acetate	1370	0.2	RI, MS
β-Bourbonene	1379	0.2	RI, MS
β-Caryophyllene	1414	2.1	RI, MS
Aromadendrene	1428	0.1	RI, MS
α-Humulene	1447	0.4	RI, MS
Germacrene-D	1474	0.2	RI, MS
Bicyclogermacrene	1487	0.3	RI, MS
Spathulenol	1551	0.8	RI, MS
β-Caryophyllene oxide	1561	t	RI, MS

a RI - Retention index as determined on the DB-1 column using the homologous series of n-alkanes (C_9-C_{21}); t - trace (<0.05)

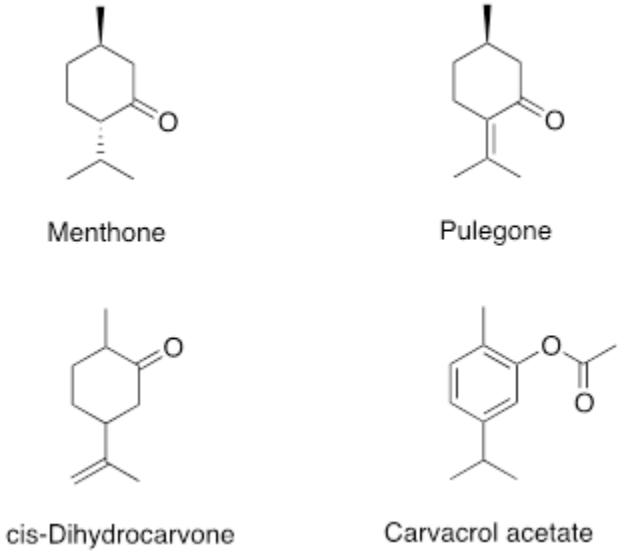

Figure No. 2
Structures of abundant compounds identified in the essential oil of *Minthostachys mollis*

Data presented here is the first report about a growth inhibitory activity by the essential oil of Minthostachys mollis on three human tumor cell lines (T24, DU-145 and MCF-7), and non-tumor cells (HEK-293). Indeed, by using the MTT assay, the IC50 was calculated and values around 200 µg/mL were obtained (Table No. 3). Although such values are rather lower to those obtained by using Doxorubicin, a currently used antitumor drug, they are comparable to IC50 values reported for other essential oils (Afoulous *et al*., 2011; Xu *et al*., 2015).

Table No. 3
The cytotoxic and antioxidant activities of the essential oil of *Minthostachys mollis*

Treatments	Cytotoxic activity IC_{50} (µg/mL)				Antioxidant activity
	T24	DU-145	MCF-7	HEK-293	DPPH
Essential oil	614.10 ± 11.84	722.40 ± 20.39	481.60 ± 19.39	687.60 ± 33.50	4834 ± 9.91
DOXO	0.46 ± 0.08	0.70 ± 0.02	0.05 ± 0.003	4.27 ± 0.34	-
TROLOX	-	-	-	-	4.33 ± 2.86

Cells were seeded into 96-well plates at a density of 10 000 cells/well for 24 h and then incubated for 48 h with or without the essential oil. At the end of the incubation, aliquots of cells suspension were taken and the MTT test was performed as described in the Experimental section. Results are expressed as means values ± SEM (n=6). DOXO=doxorubicin

Currently, the antioxidant activity has been widely used as parameter to characterize biological effects of a given molecules. Such activity is related to compounds capable to protect a biological system against deleterious effects caused by excessive formation of reactive oxygen species (Alam *et al*., 2013; Lopez-Alarcon & Denicola, 2013). In this context, Table No. 3 shows that the essential oil of *Minthostachys mollis* has poor antioxidant activity against DPPH (IC_{50} value>4,000 mg/L). These results suggest that compounds identified in the essential oil of *Minthostachys mollis* have a low antioxidant activity.

CONCLUSIONS

Based on previous results, it can be concluded that menthone and pulegone, which were identified as main constituents of the essential oils from *Minthostachys mollis* Griseb, are likely involved in the cytotoxicity against cancer cells. Such activity do not rely on their lower antioxidant ability.

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