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Rec. Nat. Prod. X:X (20XX) XX-XX Chemical composition, enantiomeric distribution and AChE-BChE activities of the essential oil of Myrteola phylicoides (Benth) Landrum, from Ecuador.

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Abstract: The volatile constituents of the essential oil (EO) of Myrteola phylicoides (Benth) Landrum, from Ecuador, extracted by steam distillation have been analyzed. A total of 37 compounds, representing 90.30% the total essential oil sample were identified. Monoterpenes hydrocarbons (53.06%) and sesquiterpene hydrocarbons (35.24%) were the principal groups of compounds. The major components were identified as α-pinene (30.94%), (E)-caryophyllene (21.93%), β-pinene (14.45%) and α-humulene (9.56%). The essential oil of M. phylicoides showed weak in vitro activity against AChE inhibition with IC50 value 60.8 µg/mL and a low BChE activity with IC50 value <250 µg/mL. This is the first report on the chemical composition of the essential oil of this specie.

Keywords: Myrteola phylicoides, Essential oil, AChE, BChE.

1. Introduction

The research of new products of natural origin has contributed significantly in the discovery of new substances with therapeutic properties [1, 2], due to the diverse range of secondary metabolites that they possess and the wide range of pharmacological activities that they show [3, 4]. In this sense, Ecuador stands out for having large plant biodiversity [5, 6, 7] and because most of its plants are unexplored relative to their pharmacological potential.

The Myrtaceae is a large family with approximately 140 genera and approximately 3500-5800 species [8] distributed mainly in the humid tropics, especially in South America, Australia and tropical Asia. These plants are characterized by having fibrous bark, mostly with a lower ovary, opposite leaves and possessing aromatic essential oils [9]. In Ecuador it is distributed in the

provinces of Azuay, Loja, Napo, Morona Santiago and Zamora Chinchipe [10]. For a long time, several species of this family have been used by indigenous communities of Ecuador in the preparation of traditional foods and beverages such as leaves of Myrcianthes fragrans (Sw.) McVaugh and Myrcianthes hallii (O. Berg) McVaugh that are aromatic additives of colada morada, which is typically drunk in the Day of the Dead or All Soul's Day [11, 12]. Likewise, the leaves from M. phylicoides are used by the "Saraguros ethnic group" in the Andean region of south Ecuador for the treatment fever, cold and "mal aire" (a supernatural disease caused by strong winds) [13, 14]. De la Torre et al. (2008) reported that the aqueous extract of the M. phylicoides mixed with water or milk is used to treat measles [15]. Previous studies have described antiradical, antioxidant and antiinflammatory properties of activity, the total phenolic content and the total content of anthocyanins, flavonols, antioxidants and anti-inflammatories and phenolic acids [16, 17]. Among some important data of the Myrteola genus has been described the inhibition of the proliferation of cancer cells of Myrteola nummularia [18].

Nevertheless, no phytochemical and pharmacological investigation exist which indicate the presence of bioactive compounds in this specie or in their essential oil. Therefore, we considered it interesting to investigate the chemical composition, enantiomeric distribution of the essential oil of M. phylicoides.

In addition, we have previously documented the effect of equatorian plants extract on acetyl- and butyryl-cholinesterases. These activities are of particular interest since theses enzymes play a key pivotal role in biological processes affected in neurological disorders as well as inflammatory context. In order to complete our data we present data on the effect of the essential oil of M. phylicoides on these enzymes [19].

This is the first report of the chemical composition, enantiomeric distribution and AChE-BChE activities of the essential oil of M. phylicoides (Benth) Landrum.

2. Materials and Methods

2.1 Plant material and preparation of the essential oil

The aerial parts of M. phylicoides were collected in “Patunadana” (9590410N, 17692602E) in San Lucas, Saraguro region in Loja Province, Ecuador on November 2017. The plant species was identified by Dr. Fani Tinitana of the UTPL Herbarium. The scientific name was based on the Catalogue of the Vascular Plants of Ecuador [10]. The plant was collected under permission n. 001-IC-FLO-DBAP-VS-DRLZCH-MA of the Ministry of Environment of Ecuador (MAE) and a voucher specimen of Myrteola phylicoides is conserved in the Herbarium HUTPL of the Universidad Técnica Particular de Loja under the code PPN-my-006.

The essential oil was hydro-distillated of fresh leaves in a Clevenger-type apparatus for four hours. Subsequently, the essential oil was tagged and stored in a brown vial at 4oC until analysis.

2.2 Physical properties of the essential oil

The physical properties of EO as: relative density (d20), refractive index (n20) and optical activity were determined as the means of three different experiments done at 20 °C, using a pycnometer (1 mL), a refractometer (model ABBE), and a polarimeter Hanon P 810, for relative density, refractive index and optical activity respectively.

2.3 Gas Chromatography Coupled Mass Spectrometry analysis

The GC-MS analysis of the essential oil composition was performed using an Agilent Chromatograph (6890N series), coupled to a mass spectrometer-detector (Agilent 5973 series). The spectrometer, controlled by the data system MSD-Chemstation D.01.00 SP1, operated in the electron impact (EI) mode (electron energy at 70 eV); electron multiplier 1600 V; scan rate: 2 scan/s; mass range: 40-350 m/z. The GC column was a non-polar capillary column, DB-5MS 5%-phenyl-methylpolysiloxane stationary phase (30 m × 0.25 mm, 0.25 µm film thickness, Agilent, USA); helium was the carrier gas at a flow rate of 1.0 mL/min in constant flow mode; the detector and injector temperatures were set at 250 °C.

The injector operated in split mode (40:1). The GC oven temperature was set at 60 °C for 5 min, then increased to 165 °C, with a gradient rate of 3 °C/min, followed by an increase to 250 °C with a gradient of 15 °C/min and held for 10 minutes. The ion source temperature was 250 °C. Samples were dissolved in dichloromethane (Fisher Scientific, relation 1:100 (v/v) and 1 µL of the solution was injected.

The constituents of the essential oil were identified, by comparing their Linear Retention Indices calculated (LRIcal) and Linear Retention Indices-Mass Spectra (LRI-MS) data present in literature [20]. The retention indices were determined according to Van Den Dool and Kratz [21]. The retention indices were calculated using a homologous series of hydrocarbons C10-C25 (from Fluka, purity 99%), which were analyzed by GC immediately after oil samples, under the same conditions. The identification was considered as acceptable in a range of ±14 units of LRI values, according to the injection of some standard compounds belonging to the different terpenic families.

2.4 Gas Chromatography Coupled Flame Ionization Detector analysis

Quantitative analysis of the essential oil was performed on an Agilent Technologies chromatograph (model 6890N series), using a flame ionization detector (FID). The percentage composition of the oil was determinate by correlating GC peak areas to the total chromatogram, with applying any correction factor, but normalizing with nonane as an internal standard. The analytical parameters were the same as the GC-MS analysis.

2.5 Enantioselective distribution

Enantioselective GC-MS analysis was performed using the following parameters: the MS operated in electron impact ionization mode at 70 eV, operated with a mass range of m/z 40-350 full scan mode. The ion source temperature was set at 200 °C. Helium was

the carrier gas at a flow rate of 1.0 mL/min. The

injector operated in split mode (40:1) at 200 °C. The oven thermal program was set at 50 °C for 2 min, and then increased to 220 °C, with a gradient rate of 2 °C/min and held for 2.0 min. A chiral capillary column based on cyclodextrin diethyl tertbutylsilyl--CDX (25m × 0.25mm dc × 0.25mm df) from Mega (Legnano, MI, Italy) was used. The essential oil samples were dissolved in dichloromethane (Fisher Scientific, relation 1:100 (v/v) and 1 µL of the solution was injected.

2.6 Cholinesterase Inhibition Test

Cholinesterase (ChE) activities were assayed following a colorimetric protocol adapted from Ellman et al. [22] ChEs efficiently catalyze the hydrolysis of acetylthiocholine (ATCh), the sulfur analog of the natural substrate of these enzymes. Upon hydrolysis, this substrate analog produces acetate ion and thiocholine. Thiocholine, in the presence of the highly reactive dithiobisnitrobenzoate (DTNB) ion, generates a yellow color, which can be quantitatively monitored by spectrophotometric absorption at 412 nm. All reagents were obtained from the Sigma-Aldrich trading house. A typical 200 µL inhibition assay volume contained phosphate buffered saline solution (pH 7.4), DTNB (1.5 mM), test sample in DMSO (1% v/v final). Both acetylcholinesterase from Electrophorus electricus (Type V-S, lyophilized powder, 744 U/mg solid, 1272 U/mg protein) and butyrylcholinesterase from equine serum (lyophilized powder, ≥900 unit's/mg protein) were dissolved in Phosphate Buffered Saline (PBS) pH 7.4 and used at 25 mU/mL for the assay. After 10 min of pre-incubation, the substrate acetylthiocholine iodide (1.5 mM) was added to start the reaction. During 1 h of incubation, 96-well microtiter plates were read on a PherastarFS (BMG Labtech) detection system. All measurements were made in triplicate. When possible, the IC50 values were calculated using the GNUPLOT package on line (www.ic50.tk, www.gnuplot.info). Donepezil was used as reference ChE inhibitor with an IC50 = 100 nM for AChE and 8500 nM for BChE. In this assay, we did not exclude the possibility of false-positive inhibition results previously described for high concentrations (<100 µg/mL) of amine or aldehyde compounds [23, 24].

3. Results and Discussion

3.1 Physical properties

The clear pale yellow essential oil was obtained in 0.15±0.02 % yield. Three physical properties were determined: refractive index (n = 1.49±0.002), relative density (d=0.91± 0.012 g/L), and optical rotation (-5.32±0.137 in CH2Cl2, c = 10.0).

3.2 Chemical Composition

This is the first report on the chemical composition of the essential oil of M phylicoides. The chemical composition of M. phylicoides essential oil is compiled in Table 1. The essential oil was analyzed by GC-MS and GC-FID. Thirty seven constituents were identified, which corresponding to 90.3% of all the oil, with the major constituents in the sample analyzed were identified as α-pinene (30.9%), (E)-caryophyllene (21.9%), β-pinene (14.5%), α-humulene (9.6%) and limonene (3.2%) (Table1). Monoterpenes hydrocarbons (53.1%) and sesquiterpene hydrocarbons (35.2%) were the principal groups of compounds, and oxygenated monoterpenes (1.3%) were the minor groups present in the essential oil.

Like in the EO of M. phylicoides we found high content of ?-pinene and ?-pinene compounds in other genera of Myrtheola such as: Blepharocalyx salicifolius from Bolivia, 34% of β-pinene and 17% α-pinene are reported, likewise in Eugenia rotundifolia the percentaje of α-pinene is 15.8% [25, 26]. Precedent

studies have shown that the α-pinene may have

anti-inflammatory effects in human chondrocytes, thus exhibiting potential antiosteoarthritic activity [27]

and antimicrobial potential [28]. The β-pinene shown phytochemicals properties against gram-positive bacteria [29]. In terms of applications, α- and β-pinene are most commonly used in solvents such as turpentine (a cleanin solvent), as well as in the fragrances industry as building blocks for artificial odorants [30].

(E)-Caryophyllene is used in spice blends, citrus flavors, soaps, detergents, creams and lotions, and in a variety of food products and beverages. (E)-Caryophyllene is also known for its anti-inflammatory and local anesthetic properties [31].

The α-humulene acts as an antibacterial agent and has anti-cancer and anti-inflammatory properties. In small quantities, it has been shown to kill the S.aureus bacteria. A study showed that humulene, especially when acting in concert with other terpenes and cannabinoids, killed cancer cells. The most recent studies concluded that α-humulene was as effective of an anti-inflammatory as the steroidal drug dexamethasone. Further still, α-humulene is frequently invoked as an appetite suppressant, which may lead to more widespread use in the future [32].

Table 1. Chemical composition of the essential oil from Myrteola phylicoides.

LRIcal LRIlit Compound % RSD 925 924

α-Thujene 0.09 0.02 934 931 α-Pinene 30.94 2.83 948 946 Camphene 0.63 0.09 979 974 β-Pinene 14.45 0.72 989 988 Myrcene 2.86 0.13 1006 1002 α-Phellandrene trace trace 1016 1014 α-Terpinene 0.11 0.01 1024 1020 p-Cymene 0.08 0.00 1029 1024 Limonene 3.16 0.10 1035 1026 1,8-

Cineole 0.78 1.26 1057 1054 ?- Terpinene 0.33 0.02 1084 1086 Terpinolene 0.40 0.02 1101 1095 Linalool 0.38 0.11 1105 1110 n-Nonanal 0.09 0.01 1119 1118 exo-Fenchol 0.07 0.01 1172 1164 3-Thujanol trace trace 1181 1174 Terpinen-4-ol 0.09 0.02 1218 1218 endo-Fenchyl acetate trace trace 1284 1282 (E)-Anethole trace trace 1293 1293 2-Undecanone 0.06 0.01 1323 1324 Myrtenyl acetate 0.03 0.00 1359 1373 Linalool isobutanoate trace trace 1374 1374 Isoledene 0.33 0.04 1405 1400 Sibirene 0.14 0.00 1422 1417 ???-Caryophyllene 21.93 0.70 1426 1407 Longifolene 0.09 0.02 1437 1439 Aromadendrene 1.22 0.05 1444 1445 Myltayl-4(12)-ene 0.13 0.00 1456 1452 ?-Humulene 9.56 0.43 1459 1449 Himachalene 0.09 0.00 1470 1475 ?-Gurjunene 0.16 0.02 1473 1478 ?-Muurolene trace trace 1488 1496 Viridiflorene 0.78 0.01 1501 1511 δ-Amorphene 0.66 0.03 1510 1513 y- Cadinene 0.08 0.01 1516 1522 δ-Cadinene 0.16 0.06 1538 1544 α-Caracolene 0.47 0.00 Monoterpenes hydrocarbons 53.1 Oxygenated

monoterpenes 1.3 Sesquiterpenes hydrocarbons 35.2 Others 0.7 Total 90.3 LRIcal Linear retention indices calculated in reference of a homologous series of n-alkanes on DB-5MS capillary column; LRIlit Linear Retention Indices from the literature [20]; % Relative percentage values are means of determinations with a Relative Standard Deviation (RSD%) below 5%; trace (>0.03%). RI = retention index; MS = mass spectroscopy.

3.3 Enantioselective GC-MS analysis

The enantiomeric distribution and enantiomeric excesses (e.e.) of essential oil were performed by enantioselective GC-MS on a cyclodextrine-based chiral stationary phase. Four couples of chiral monoterpenoids were detected (Table 2). These same compounds were previously reported in L. mutica essential oil [32]. The LRI of (+)-β-pinene, (-)-β-pinene were similar to reported for L. mutica [33] and N. dissecta [34], the (+)-β-pinene enantiomer were highly toxic to Candida albicans [35]

Table 2. Enantiomeric analysis of the components of Myrteola phylicoides essential oil.

Compound LRIcal Enantiomeric distribution % e.e. (%) (+)-β-pinene 961 99.79 99.57 (-)-β-pinene 969 0.21 (+)-linalool 1198 27.85 44.31 (-)-linalool 1209 72.15 (+)-terpinen-4-ol 1269 56.00 12.01 (-)-terpinen-4-ol 1272 44.00 (+)-α-terpineol 1312 72.65 45.31 (-)-α-terpineol 1325 27.35 LRIcal Linear retention indices calculated in reference of a homologous series of n-alkanes on MEGA-DEX DET Beta capillary column; e.e. enantiomeric excess.

Three couples and one enantiomerically pure chiral monoterpenoide were detected and baseline separated. (+)-β-pinene was detected as enantiomerically pure compound while (-)-linalool and (+)-α-terpineol were present in mixture with their enantiomers but with a very high ee value.

In contrast, the enantiomeric excess of (+)-terpinen-4-ol was almost racemic. These results further confirm that secondary metabolites canbe present in plants as enantiomeric mixtures.

3.4 Cholinesterase inhibition test

The essential oil showed a weak inhibitory activity against AChE and BChE with IC50 concentrations 60.8 µg/mL and <250 µg/mL, respectively. The study by Dohi et al., showed that the activity of α-pinene, the main component in some essential oils, does not contribute much to the activities against AChE [36]. For this reason, it is necessary to carry out new studies that allow to determine if any isolated compound of the essential oil of M. phylicoides present a marked activity on the enzymatic systems studied, this could also contribute to know the anti-AChE symbiotic effect that can exert each compound.

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