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Chemical composition, acetylcholinesterase inhibitory and antifungal activities of *Pera glabrata* (Schott) Baill. (Euphorbiaceae)

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ABSTRACT − (Chemical composition, acetylcholinesterase inhibitory and antifungal activities of *Pera glabrata* (Schott) Baill. (Euphorbiaceae)). *Pera glabrata* (Schott) Baill. was selected for this study after showing a preliminary positive result in a screening of Atlantic Forest plant species in the search for acetylcholinesterase inhibitors and antifungal compounds. The bioassays were conducted with crude ethanol extract of the leaves using direct bioautography method for acetylcholinesterase and antifungal activities. This extract was partitioned with hexane, chloroform and ethyl acetate solvents. The active chloroform fraction was submitted to silica gel chromatography column affording 12 groups. Caffeine, an alkaloid, which showed detection limits of 0.1 and 1.0 μg for anticholinesterasic and antifungal activities, respectively, was isolated from group nine. After microplate analyses, only groups four, nine, 10, 11 and 12 showed acetylcholinesterase inhibitory activity of 40% or higher. The group 12 was purified by preparative layer chromatography affording four sub-fractions. Two sub-fractions from this group were analyzed by gas chromatography-mass spectrometry and gas chromatography-flame ionization detector. The first sub-fraction showed anticholinesterasic activity and contained two major compounds: 9-hydroxy-4-megastigmen-3-one (84%) and caffeine (6%). The second sub-fraction presented five major compounds identified as 9-hydroxy-4-megastigmen-3-one, isololiolide, (-) loliolide, palmitic acid and lupeol and did not show activity.

Key words - 9-hydroxy-4-megastigmen-3-one, biological activities, caffeine, Euphorbiaceae

Resumo – (Composição química, atividades inibidora da acetilcolinesterase e antifúngica de *Pera glabrata* (Schott) Baill. (Euphorbiaceae)). *Pera glabrata* (Schott) Baill. foi selecionada para este estudo a partir de uma triagem de espécies vegetais da Mata Atlântica na busca de substâncias com atividades anticolinesterásica e antifúngica. A técnica da bioautografia direta foi utilizada para a detecção das atividades anticolinesterásica e antifúngica. O extrato etanólico bruto obtido das folhas foi particionado com hexano, clorofórmio e acetato de etila. A fração clorofórmica ativa foi fracionada por cromatografia em coluna de sílica gel fornecendo 12 grupos. Do grupo nove foi isolado o alcalóide cafeína com limites de detecção de 0,1 e 1,0 μg para as atividades anticolinesterásica e antifúngica, respectivamente. Após bioensaio em microplaca, somente os grupos quatro, nove, 10, 11 e 12 apresentaram inibição da acetilcolinesterase maior ou igual a 40%. O grupo 12 foi purificado por cromatografia em camada delgada preparativa de sílica gel fornecendo quatro sub-frações. Duas sub-frações deste grupo foram analisadas por cromatografia a gás-espectrometria de massas e cromatografia a gás com detector de ionização de chama. A primeira sub-fração contém dois compostos majoritários: 9-hidroxi-4-megastigmen-3-ona (78%) e cafeína (6%), e apresentou atividade anticolinesterásica. A segunda sub-fração contém cinco compostos principais identificados como 9-hidroxi-4-megastigmen-3-ona, isololiolida, (-) loliolida, ácido palmítico e lupeol e não apresentou atividade.

Palavras-chave - 9-hidroxi-4-megastigmen-3-ona, atividades biológicas, cafeína, Euphorbiaceae

Introduction

Pera glabrata (Schott) Baill. is a species of the family Euphorbiaceae. This family presents cosmopolitan distribution, being more abundant in the tropical and subtropical regions, consisting of about 300 genus and 7,500 species (Cronquist 1981). In Brazil the family is represented by 72 genera and about 1,100 species present in all types of vegetation (Barroso *et al*. 1984). Euphorbiaceae are very well represented in the Brazilian flora, with species used as food (cassava – *Manihot esculenta* Crantz), as oil producer (castor-oil – *Ricinus communis* L.; tung-oil tree – *Aleurites fordii* Hemsl.), as rubber producer (rubber tree – *Hevea brasiliense* Müll. Arg.), as phytotherapeutic and as ornamental plants too (crown-of-thorns – *Euphorbia milii* Des Moul.) (Joly 1976, Mors & Rizzini 1966).

Pera benensis Rusby is considered a medicinal plant used in folk medicine by Chimane Indians from Bolivia for the specific treatment of cutaneous

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leishmaniasis popularly known as *espundia* (Fournet *et al*. 1992).

Pera glabrata is popularly known as *tamanqueira*, *sapateiro*, *pau-de-sapateiro* and *seca-ligeiro* (Lorenzi 2002). This evergreen and heliophyte species, which can reach 8.0 to 10.0 m height, is native to seasonal semi-deciduous forest and can be found in well-drained lands of hilltops as well as in riparian forest (Lorenzi 2002).

Plants are a valuable source of new natural products. Only a small proportion of the several hundred thousand plant species around the globe has been investigated both phytochemical and pharmacologically. The crucial factor for the ultimate success of an investigation into bioactive plant constituents is thus the selection of the plant material (Hostettmann *et al.* 1995). In view of the large number of plant species potentially available for study, it is essential to have efficient systems for the rapid chemical and biological screening of the plant extracts selected for investigation. Isolation of pure and pharmacologically active constituents from plants remains a long process and requires the collaboration of botanists, pharmacologists, chemists, pharmacognosists and toxicologists (Hamburger & Hostettmann 1991, Hostettmann 1998).

All the vegetal extracts or pure compounds isolated in phytochemical laboratories should be submitted to a large number of bioassays (Hostettmann 1998). So, the quick and efficient detection of biologically active substance is an aspect of great importance in the process of its discovery (Rios *et al*. 1988).

The search for antifungal drugs has received attention especially as a result of the crescent incidence of opportunist mycosis, mainly associated with AIDS and treatment with immunosuppressive drugs. There are few antifungal agents indicated for the systemic mycosis treatment but their efficacy is limited (Hamburger & Hostettmann 1991).

The advances obtained in the comprehension of evolution and molecular aspects of genesis of the Alzheimer's disease (AD) have shown that the use of acetylcholinesterase inhibitors must be the most efficient way of controlling its evolution and recovering of the presented symptoms (Francis *et al*. 1999, Quik & Jeyarasasingam 2000). Despite the fact that it is not used in folk medicine, *Pera glabrata* was chosen for this study based on the screening for the anticholinesterasic and antifungal activities assayed in our laboratory. The focus of this work is to study chemical and biological aspects of *Pera glabrata* extracts with potential anticholinesterasic and antifungal activities.

Material and methods

Study area – The *Parque Estadual das Fontes do Ipiranga* (PEFI) is located in the southeast region of the Municipal District of São Paulo in the frontier of the Municipal District of Diadema and several other districts of São Paulo and holds an area of approximately 526.38 ha. The flora is represented by native vegetation and characterized as an extension of the Atlantic hillside forest, without predominance of any plant family in particular. Some specimens of *Pera glabrata* (Schott) Baill. can be found in this park.

Plant material – *Pera glabrata* leaves were collected in PEFI in March 2006 and identified by Dr Inês Cordeiro, SP Herbarium, Instituto de Botânica. A voucher specimen (*Cordeiro 1775*) is deposited at the SP.

Preparation of extract – After drying and crushing, the leaves (3.5 kg) were extracted with 92% ethanol (0.5 L: 0.5 kg, 10 days) six times at room temperature. The extracts were filtered and concentrated under vacuum in a rotary evaporator and dried in a steam bath at 50 °C. The crude ethanol extract (104.90 g) was dissolved in methanol-water (6:4), and partitioned with hexane (150 mL, seven times), chloroform (150 mL, six times) and ethyl acetate (150 mL, six times) to yield hexane (37.43 g), chloroform (10.14 g), ethyl acetate (6.14 g) and water-methanol (24.03 g) fractions. The antifungal and anticholinesterasic activities assays of these fractions were performed by direct bioautography and microplate assays, respectively (Homans & Fuchs 1970, Rhee *et al.* 2001).

Bioassay guided fractionation – The chloroform fraction (10.14 g) was fractionated using column chromatography (CC) over silica gel (0.063 – 0.200 mm) using $CHCl_3$-MeOH gradient solvent system, affording 88 fractions (100 mL each) that were monitored by TLC silica gel eluted with $CHCl_3$: MeOH (9:1) and visualized under ultraviolet light (UV) at 254 nm and 366 nm. Fractions were pooled according to their inhibition of acetylcholinesterase and antifungal activities and the chromatographic profile on thin layer chromatography affording 12 groups. The groups were classified as G1 (fractions 1-16), G2 (17-21), G3 (22), G4 (23), G5 (24-27), G6 (28-32), G7 (33-40), G8 (41-45), G9 (46-48), G10 (49-51), G11 (52-62) and G12 (63-88). The groups G9, G10, G11 and G12 were the most active and presented crystals that were identified as described below. The active group G12 was further fractionated by preparative TLC (hexane-EtOAc, 4:6) giving four sub-fractions. The second and third sub-fractions were analyzed by gas chromatography and mass spectrometry (GC/MS).

Gas Chromatography / Mass Spectrometry (GC/MS) and CG/ FID – The identification of compounds was achieved by gas chromatography and mass spectrometry (GC/MS), using an Agilent GC (6890 Series) – quadrupole MS system (5973), with a fused silica capillary column (30 m × 0.25 mm

× 0.25 µm, coated with DB-5), EI operating at 70 eV. Injector and detector temperatures were set at 250 °C. The oven temperature program was 40 °C for 1 min, 40-240 °C at 10 °C min^{-1} and helium was employed as carrier gas (1 mL min^{-1}) and injector FID temperature 250 °C, injection volume 2 µl. The compound identification was performed by comparing mass spectra to a standard compound (caffeine), library Wiley 275 and literature data. The anticholinesterasic and antifungal activities of each group were tested and the most active groups presented crystals identified as caffeine by ^1H (300 MHz) and ^{13}C NMR ($CDCl_3$ – 75 MHz) spectra obtained in a Bruker DRX-300 spectrometer. The identification of the crystals was based on comparison with data of ^1H- and ^{13}C NMR spectra (Wehrli & Nishida 1979, Sousa *et al.* 1991). The ^1H NMR spectrum of crystal presented chemical shift (δ) signals at δ 7.57 (1H, *s*, CH), δ 4.00 (3H, *s*, NCH_3), δ 3.58 (3H, *s*, NCH_3), δ 3.40 (3H, *s*, NCH_3) and ^{13}C NMR spectrum presented signals at δ 141.3 (CH), δ 33.4 (NCH_3), δ 29.6 (NCH_3), δ 27.7 (NCH_3). The GC/MS analysis presented a total ions chromatogram (TIC) with retention time (RT) = 18.25 min of *m/z* 194 [M^+], 194 (100), 109 (55), 82 (25), 67 (38), 55 (33) confirming the caffeine structure.

Acetylcholinesterase activity by bioautography – The procedure recently reported by Marston *et al*. (2002) was used for this bioassay. Briefly, acetylcholinesterase type V (Sigma, product no. C 2888, 1000 U) was dissolved in Tris-hydrochloric acid buffer (pH 7.8) and stabilized by the addition of bovine serum albumin fraction V (0.1%, Sigma, product no. A-4503). TLC layers were spotted with 200 µg of plant extract, 50 µg of fractions groups (G1 to G12) and 1 µg of galanthamine (Sigma) was used as positive control. TLC layers were developed with $CHCl_3$: MeOH (9:1, v/v) and subsequently dried. The plates were then sprayed with the enzyme solution (6.66 U mL^{-1}), thoroughly dried and incubated at 37 °C for 20 min in moist atmosphere. Enzyme activity was detected by spraying the plate with a solution consisting of 0.25% of 1-naphtyl acetate in EtOH (5 mL) plus 0.25% aqueous solution of Fast Blue B salt (20 mL). Potential acetylcholinesterase inhibitors appeared as clear zones on a purple colored background.

Antifungal activity – *Cladosporium cladosporioides* (Fresen.) G.A. de Vries CCIBt 140 from the live collection of IBt was grown in potato dextrose agar for 12 days until sporulation. Ten microliters of a solution corresponding to 400 µg of crude extracts and 50 µg of fraction groups (G1 to G12) were applied on Al-backed silica gel F_{254} TLC layers (Merck) and run with $CHCl_3$: MeOH (9:1 v/v). The solvent was then completely removed and the plates were sprayed with a conidia suspension of *C. cladosporioides* (≥ 10^6 conidia mL^{-1}) in a glucose and salt solution (Homans & Fuchs 1970, Rahalison *et al*. 1994) and incubated for 48 h at 28 °C. After incubation, clear inhibition zones appeared against the dark green background of the chromatogram. Nystatin 1.0 µg (Sigma) was used as standard control. These bioautographic assays were performed in triplicate.

Acetylcholinesterase activity by microplate assay – Acetylcholinesterase activity was evaluated using a 96-well microplate reader (Rhee *et al.* 2001) based on Ellman's method (Ellman *et al*. 1961). In this method the enzyme hydrolyzes the substrate acetylthiocholine resulting in the production of thiocholine which reacts with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB, Sigma) to produce 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate which can be detected at 405 nm. In the 96-well plates, 25 µl of 15 mM acetylthiocholine iodide (ATCI, Sigma) in water, 125 µl of 3 mM DTNB in buffer C, 50 µl of buffer B, 25 µl of plant extract sample (20 mg ml^{-1} of crude extract in MeOH diluted 10 times with buffer A and 10 times in well to give a final concentration of 0.2 mg ml^{-1}) were added and the absorbance was measured at 405 nm every 30 s for three times. Then 25 µl of 0.22 U ml^{-1} of the enzyme acetylcholinesterase (AChE, Sigma) were added and the absorbance was read again every 10 min for two times. Any increase in absorbance due to the spontaneous hydrolysis of the substrate was corrected by subtracting the rate of the reaction before the addition of the enzyme from the rate of the enzyme reaction. Enzyme activity was calculated as a percentage compared to an assay using a buffer without any inhibitor (10% MeOH in Buffer A). The following buffers were used: buffer A (50 mM Tris-HCl, pH 8), buffer B (50 mM Tris-HCl, pH 8, containing 0.1% bovine serum albumin V fraction – BSA, Sigma), and buffer C (50 mM Tris-HCl, pH 8, containing 0.1 M NaCl and 0.02 M $MgCl_2.6H_2O$).

Results expression – Results from bioautographic assays were expressed as retention factor (Rt) of zones of growth inhibition of *C. cladosporioides* and inhibition zones of acetylcholinesterase. The quantitative results of acetylcholinesterase inhibition were represented as means ± standard error (SE) of the mean of one typical experiment performed in triplicate. The AChE inhibitory data were analyzed with the software package Prism version 3.0 (Graph Pad Inc., San Diego, USA). IC_{50} value is the mean ± SE of determinations performed in triplicate.

Results and discussion

As a result of the investigation of the crude ethanol extract of the leaves of *P. glabrata* evaluated for its antifungal and anticholinesterasic activities, it was found that this extract was active against *C. cladosporioides* and also showed anticholinesterasic activity.

The crude ethanol extract (104.90 g) partitioned between 80% aqueous MeOH and hexane, chloroform and ethyl acetate yielded fractions representing 35.4%, 9.5% and 5.8% of the total extract, respectively. Only the chloroform fraction showed significant antifungal and anticholinesterasic activities by TLC method at Rf 0.61. In microplate assay this fraction inhibited

the enzyme by 42% and it was selected for further purification (figure 1). It was fractionated by chromatographic procedure and the antifungal and anticholinesterasic activities of each fraction were tested by TLC bioassay. With $CHCl_3$ as eluent, 80 fractions (F1-F80) were obtained, and with $CHCl_3$/MeOH (99:1), eight fractions were obtained (F81-88). These fractions were pooled according to their chromatographic profile and anticholinesterasic activity in microplate assay, into 12 groups: G1 (1-16, 4.0 mg), G2 (17-21, 6.2 mg), G3 (22, 4.6 mg), G4 (23, 37.9 mg), G5 (24-27, 882.0 mg), G6 (28-32, 64.8 mg), G7 (33-40, 88.9 mg), G8 (41-45, 125.2 mg), G9 (46-48, 141.0 mg), G10 (49-51, 228.5 mg), G11 (52-62, 471.4 mg) and G12 (63-88, 231.4 mg). The groups (50 µg mL^{-1}) that inhibited the enzyme at 40% or at higher percentages were G4 (47 ± 2.7%), G9 (53 ± 0.2%), G10 (59 ± 0.4%), G11 (61 ± 2.4%), and G12 (64 ± 0.8%) (figure 2). Groups G3, G4, G6, G9, G10, G11 and G12 also displayed strong antifungal activity when 50 µg were tested by TLC bioassay (figure 3).

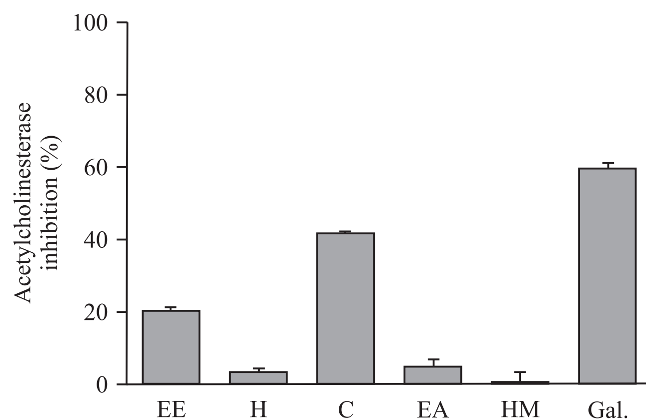

Figure 1. Acetylcholinesterase inhibitory activity of ethanol extract (200 µg mL^{-1}), different fractions (100 µg mL^{-1}) obtained from purification of ethanol extract of *Pera glabrata* and galanthamine (1 µM) (values represent the mean of triplicates with standard error). (EE = ethanol extract; H = hexane fraction; C = chloroform fraction; EA = ethyl acetate fraction; HM = hydromethanolic fraction; Gal. = Galanthamine).

The alkaloid caffeine was isolated from group nine to 12 as a white solid crystal with a yield of 0.02% leaf extract. The identification of this alkaloid was performed by ^{1}H and ^{13}C NMR, GC-MS and compared with a standard compound (caffeine).

To establish detection limits (DL) for the bioautographic assay, *i.e* the concentration that produced the spot with the least observable whiteness, varying concentrations of galanthamine and physostigmine (eserine), known acetylcholinesterase inhibitors, were applied onto a TLC plate and compared with caffeine. Galanthamine inhibited the enzyme up to 0.03 µg, while the least amount of physostigmine required for activity was 0.003 µg. The alkaloid caffeine inhibited the enzyme up to 0.1 µg and these results are in accordance with the bioautographic results obtained by Marston *et al*. (2002). The concentration of caffeine necessary to produce 50% of acetylcholinesterase inhibition (IC_{50}) was 17.7 µg mL^{-1}. These *in vitro* results are in accordance with recent researches that demonstrated that the consumption of caffeine-containing beverages appeared to possibly confer some protective effects against Alzheimer's disease (Maia & De Mendouca

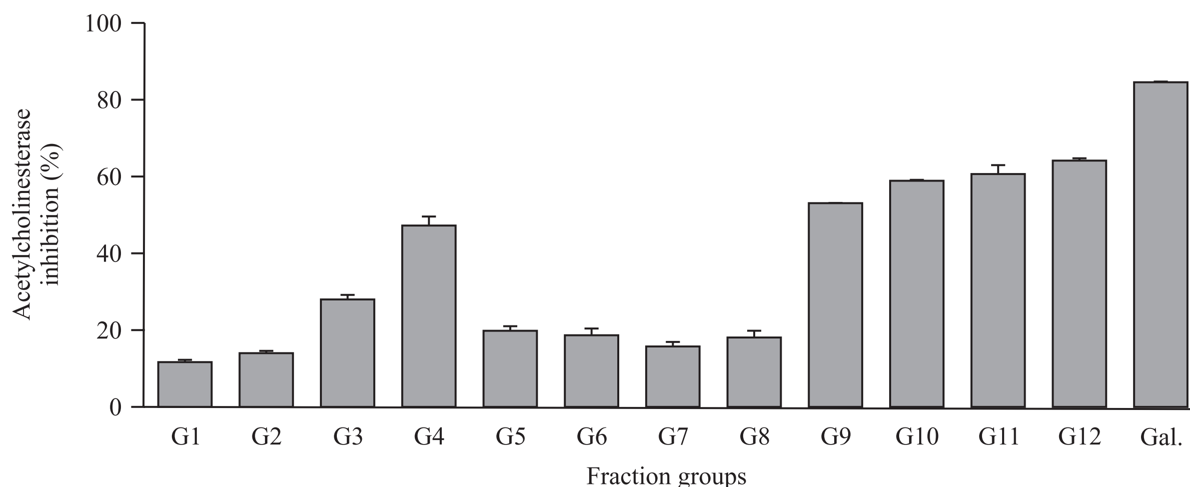

Figure 2. Acetylcholinesterase inhibitory activity of different fraction groups, G1 to G12 (50 µg mL^{-1}), obtained from chloroform fraction of *Pera glabrata* and galanthamine 1 µM (Gal.) (values represent the mean of triplicates with standard error).

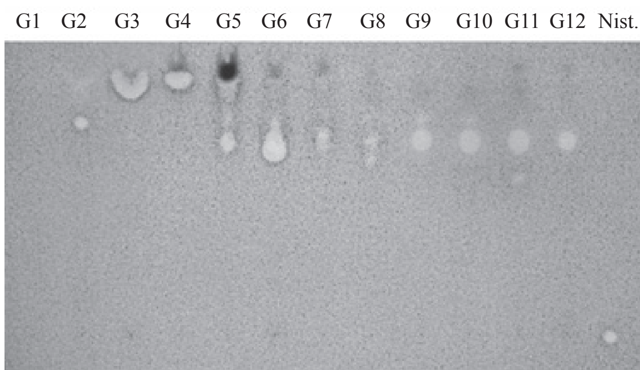

Figure 3. Antifungal activity of different fraction groups, G1 to G12 (50 µg mL^{-1}) obtained from chloroform fraction of *Pera glabrata* and nystatin 1 µg (Nist.).

2002). Caffeine has many molecular targets: release calcium through activating the ryanodine-sensitive calcium release channels of the endoplasmic and sarcoplasmic reticulum, inhibit phosphodiesterase and block adenosine A_1 and A_{2A} receptors (Daly 2007). In studies with model Alzheimer's mice, chronic intake of caffeine protected against cognitive impairment and resulted in reduced brain levels of β-amyloid protein (Arendash *et al*. 2006). Our data reinforce those obtained with the *in vivo* model, in which caffeine inhibits the enzyme acetylcholinesterase, improving the symptoms of the disease and being capable of retarding the neurodegenerative process in Alzheimer's disease. A study performed by Silva *et al*. (2008) showed that caffeine maternal treatment increased hippocampal AChE activity in 21-day-old pups, with no effect on mRNA expression.

For the antifungal activity against *Cladosporium cladosporioides* the detection limit of caffeine was 1 µg. Methylxanthines like caffeine may have ecological significance to the plant that produces them, influencing the interactions between organisms and benefiting the plant adaptation to adverse environment. The content of methylxanthines may be influenced by the stage of plant development, due to seasonal alterations, interventions of agronomic procedures and other factors (Rates 2001). The "chemical defense theory" proposes that the high concentrations of caffeine in young leaves, fruits and flower buds of a species act as a defense to protect young soft tissues from pathogens and herbivores (Ashihara *et al*. 2008). Nathanson (1984) described that caffeine is naturally an insecticide, larvicide and fungicide at concentrations found in plants. Another work realized by Yuvamoto & Said (2007) has shown that caffeine and caffeic acid exert a negative effect on germination, nuclear duplication cycle and on formation of the first septum of *Aspergillus nidulans* (Eidam) G. Winter. Ecological and pharmacological studies have demonstrated that caffeine has a broad range of effects, both as a research tool and as a key structure for the synthesis of molecules with therapeutic value (Daly 2007).

The group G12 was submitted to silica gel preparative chromatography affording four sub-fractions. The second sub-fraction (13.6 mg) with a blue fluorescence (UV 366 nm) showed two major compounds (figure 4) when analyzed by gas chromatography- FID and gas

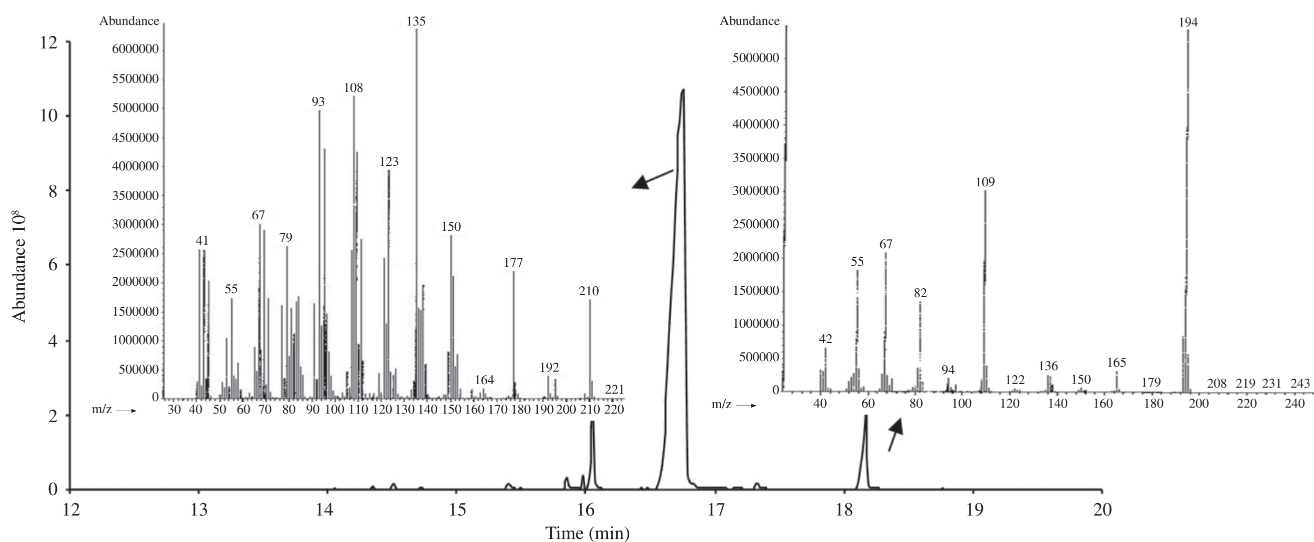

Figure 4. Total ion chromatogram (TIC) and mass spectra of 9-hydroxy-4-megastigmen-3-one (*m/z* 210) and caffeine (*m/z* 194). The fragmentation patterns of these substances are indicated by arrows inside the chromatogram.

chromatography- mass spectrometry. The ion at *m/z* 210 (RT = 16.8 min) had the fragmentation pattern *m/z* 210 [M^+], (percentage of relative intensity) 135 (100), 109 (66), 108 (80), 95 (66), 93 (76) and corresponded to literature data of 9-hydroxy-4-megastigmen-3-one (Miyase *et al*. 1988), and caffeine (RT = 18.2 min, 6%, *m/z* 194 [M^+] 194 (100), 109 (55),82 (25), 67 (38), 55 (33). Megastigman compounds were previously isolated from *Glochidion zeylanicum* (Gaertn.) A. Juss. (Euphorbiaceae) besides species of Apocynaceae (Otsuka *et al*. 2003). The third sub-fraction (4.2 mg) with a green fluorescence (UV 366 nm) had five major compounds: 9-hydroxy-4-megastigmen-3-one (RT = 11.6 min, *m/z* 210), isololiolide (RT = 12.0 min, *m/z* 196), (-) loliolide (RT = 12.3 min, *m/z* 196), palmitic acid (RT = 14.1 min, *m/z* 256) and lupeol (RT = 28.4 min, *m/z* 189). The three first compounds are norisoprenoids registered in species of seaweed and higher plants. Most importantly, this is the first report of the occurrence of (-)-loliolide in the genus *Pera* and the second occurrence in the family Euphorbiaceae. The first occurrence of this compound for Euphorbiaceae was described in *Alchornea glandulosa* Endl. & Poeppig (Conegero *et al*. 2003). Okunade & Wiemer (1985) demonstrated that (-)-loliolide isolated from *Xanthoxyllum setulosum* P. Wilson (Rutaceae) is a potent leafcutter repellent. The natural occurrence of loliolide is mainly reported for plant material (Ghosal *et al.* 1976). They have become especially known as flavor compounds in tea and tobacco (Bricout *et al*. 1967, Kodama *et al*. 1982, Roberts & Rohde 1972). Loliolide is also reported to occur in the marine mollusk *Dolabella ecaudata* (Pettit *et al*. 1980).

Lupeol was also found in another species of the genus *Pera*. Fournet *et al.* (1992) reported this compound in *P. benensis*, which shows antileishmaniasis activity.

In conclusion, this work demonstrated that leaves of *Pera glabrata* contain a great amount of caffeine that may act as a chemical defense against fungal pathogens and can enhance memory and learning of Alzheimer's disease patients.

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