Supporting Information

***Rec. Nat. Prod*. 9:4 (2015) 586-591**

Chemical Characterization and Acetylcholinesterase Inhibition Potential of Volatile Components of Aerial Parts of *Pluchea lanceolata* (DC.) Oliv. & Hiern

Pooja Srivastava[1], Joytshna[1], Debabrata Chanda[2] and Karuna Shanker[1]*

[1]Analytical Chemistry Department, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow-226015, India

[2]Molecular Bioprospection Department, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow-226015, India

Table of Contents **Page**

1. Essential oil extraction by hydrodistillation

The aerial parts of fresh plants were subjected to hydrodistillation in a Clevenger type apparatus (750 g each) for 5 hrs. The distillate was saturated with NaCl and the oil was extracted with *n*-hexane and dichloromethane. The solvent phase was then dried over anhydrous Na_2SO_4 and then the solvent distilled off at 35°C under vacuum using rotary vacuum evaporator (Buchi, Switzerland). The oil yield of plant material was 0.05% (v/w, fresh wt basis). The oil samples were stored at -20°C until analysis.

2. Gas Chromatography (GC-FID) and gas chromatography-mass spectrometry (GC-MS) analysis

The quantitative and qualitative analyses of the essential oils were performed by capillary GC and GC/MS, respectively. A PerkinElmer Autosystem XL gas chromatograph with flame ionization detector (GC-FID) was used, system fitted with an EQUITY–5 [bonded: poly (5% diphenyl/95% dimethylsiloxane), 60 m x 0.32 mm, film thickness 0.25µm, SUPELCO, USA). The column temperature ranged from 70-250°C, at 3°C/min and 250-320°C, at 6°C/min, with a final hold time of 5 min, using H_2 as carrier gas at 10 psi constant pressure, a split ratio of 1:50, an injection size of 0.03 µL and injector and detector (FID) temperatures of 280°C and 300°C, respectively. The percentage compositions were obtained from electronic integration measurements using flame ionization detection without taking into account relative response factors.

The sample was analyzed by GC-MS/ PerkinElmer turbomass quadrupole mass spectrometer (GC-MS) operating at 70 eV with a mass range of *m/z* 40-450 using bonded poly(5% diphenyl/95% dimethyl siloxane (EQUITY-5, 60 m x 0.32 mm, film thickness 0.25 µm, SUPELCO, USA) column.. The column temperature of 70-300°C was programmed at a rate of 3.0°C /min, with a hold time of 10 min. The oven temperature program was the same in as in GC while the injector temperature was 270°C, transfer line and ion source temperatures were 300°C, injection size 0.03µL neat, split ratio 1:50 using He as carrier gas at 10 psi constant pressure. The identification of the compounds was achieved on the basis of retention time, Kovats Indexes, literature reported retention index using a homologous series of *n*-alkanes (C_8-C_{25} hydrocarbons, Polyscience Corp. Niles IL), co-injection with standards (Sigma Aldrich), mass spectra library search (NIST, Wiley and Nbs), and by comparing with the mass spectral literature data.

3. NMR spectroscopy

^{1}H NMR and ^{13}CNMR spectral analysis was performed with 300 MHz NMR (Avance, Bruker, Switzerland) equipped with a 5 mm probe, in $CDCl_3$ with reference to tetramethyl silane (TMS) as internal standard.^{1}H NMR spectra of the *Pluchea lanceolata* essential oil (Figure 1S) was recorded with the following parameters: pulse width, 6.7 µs (flip angle 30°); acquisition time, 5.30s for 65K data table; relaxation delay D1: 3.5 s; spectral width (SW) of 6172 Hz (20 ppm); digital resolution of 0.09 Hz/pt. The number of accumulated scans was 20 (around 100 mg of the oil sample in 500µL of $CDCl_3$).^{13}CNMR spectra of the *P. lanceolata* essential oil (Figure 1S) was recorded with the following parameters: pulse width, 11.5µs (flip angle 30°); acquisition time, 1.82s for 65K data table with a spectral width of 17985 Hz (238ppm); digital resolution, 0.27 Hz/pt. The number of accumulated scans was 7168 (around 100 mg of the oil sample in 500µL of $CDCl_3$).

The whole essential oil was used for NMR analyses without any prior fractionation, following the previous methodology [1]. The identification was based on comparison of the signals in the oil spectrum with those of reference spectra compiled (Figure 2S), in the laboratory spectral library, with

the help of laboratory-made database (SDBS and ChemDraw Ultra 8.0). Compounds were identified considering three parameters viz. number of identified carbons, number of overlaying of signals, and difference of chemical shift of each resonance in the mixture and in the reference spectra.

4. *Ex-vivo* cholinesterase inhibitory activity of the essential oil in the mouse brain homogenate

Male Swiss albino mice (4 in no) of 20g body weight were taken for the study. The protocol of the present study was approved by the Institutional animal ethics committee. The animals were then sacrificed by cerebral dislocation and the frontal cortices were dissected out and kept at −80 ^{0}C until use. Estimation of cholinesterase activity was performed on the basis of the colorimetric method as described earlier [2-4]. Briefly, the frozen cortex was weighed and homogenized in 10-times volume of 0.1M phosphate buffer (pH 7.4) containing 1% Triton-X-100. After centrifugation at 15,000g at 4°C for 20 min, the clear supernatant was collected and served as the enzyme source. Protein content of the homogenate was estimated by Lowry's method [5]. Cholinesterase inhibitory activity of the essential oils were carried out following a pre-incubation for 30 min of the tissue homogenate with the essential oils serially two fold diluted in 20% cremophore®EL in distilled water. Vehicle control samples were treated with 20% cremophore®EL in distilled water and physostigmine was used as positive control. Briefly, 3µL of the drug solution/vehicle/serially diluted oil sample was added to 50µL of tissue homogenate in a 96-well flat bottom microtitre plate and incubated for 30 mins at 37^{o}C. A parallel set of experiment was carried out in triplicate to estimate the basal cholinesterase activity wherein brain tissue homogenate received no treatment before enzyme assay. The reaction was started by adding 20µL of 10mM 5,5'-dithiobis-(2-nitrobenzoic acid), 20µL of 30mM acetylthiocholine, and 160µL of phosphate buffer. The spectrophotometric absorption at 405nm during a 3-min incubation period at 25°C was quantitatively measured using a microplate reader (Spectramax; Molecular Devices, USA) and expressed as nmol ACh hydrolyzed/min/mg tissue.

5. Statistical analysis

Unless otherwise indicated, all determinations were conducted in triplicate, and results were presented as the mean ± standard deviation (SD) .The statistical analysis was performed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA).

Table S1 : Major volatile composition of Essential oil of *Pluchea* species.

Species	Origin/	Parts	Major component	References
P. carolinensis	Western Cuba	Flower	Selin-11-en-4α-ol,	[6]
P. quitoc	South of Brazil	Leaves	Sesquilavandulyl acetate, sesquilavandulol and α-gurjunene	[7]
	Brazil	Leaves	δ-Cadinene, 1,8-cineole	[8]
P. sagittalis	Argentina	Leaves and stem	Camphor, salicylic acid, m-cresol, eugenol and methyleugenol	[9]
	Brazil	Aerial	α-Pinene, β-pinene , limonene, 1,8-cineole, isomenthon	[10]
	Argentina	Aerial	α-Pinene, camphene, β-pinene, d-limonene, 1,8-cineole, p-cymene, linalool, camphor, bornyl acetate, caryophyllene, α-terpineol, borneol, citronellol, geranyl acetate and geraniol	[11]
	Argentina	Aerial	Caryophyllene, α-humulene	[12]
	Argentina	Aerial	α-Pinene ,camphene , β-pinene, d-limonene, 1,8-cineole, p-cimene, linalool, l-camphor, bornyl acetate, caryophyllene, α-terpineol, borneol, and geranyl acetate	[13]
	Argentina,	Aerial	α-Pinine, camphene, cineol, p-cymene, linalol, l-camphor, α-terpineol, borneol, caryophyllene, and humulene	[14]
	Argentina	Aerial	d-Camphene, p-cymene, d-camphor, and sesquiterpenes	[15]
P. lanceolata	India	Aerial	Linalool, α –terpineol, linalylacetate , α-copaene, *trans*-caryophyllene, epi-cubebol, *trans*-α-bergamotene, spathulenol, 1,6-dimethyl-4-(1-methylethyl)	present study

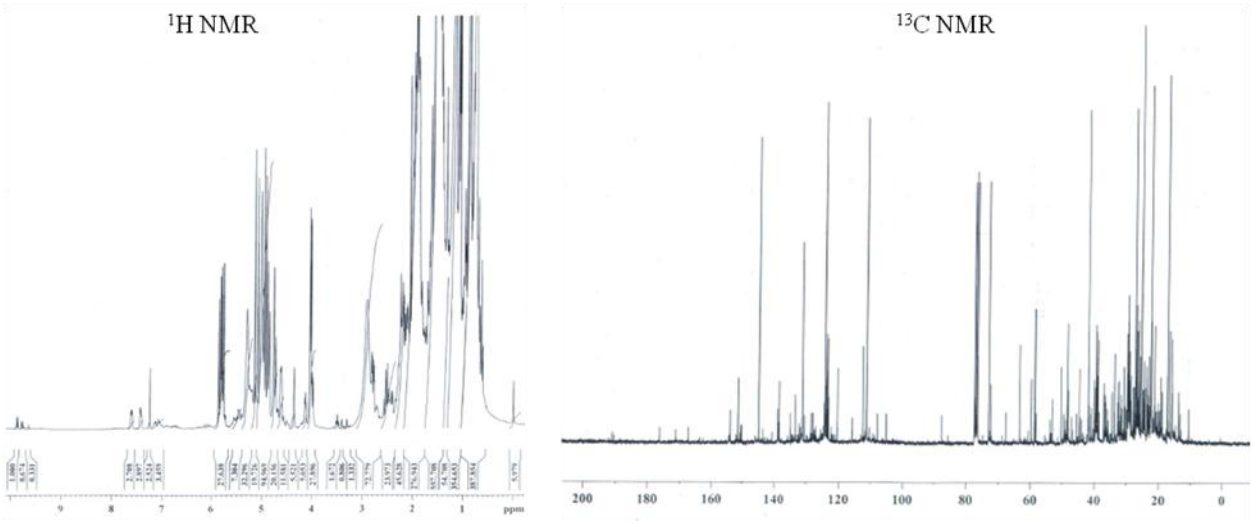
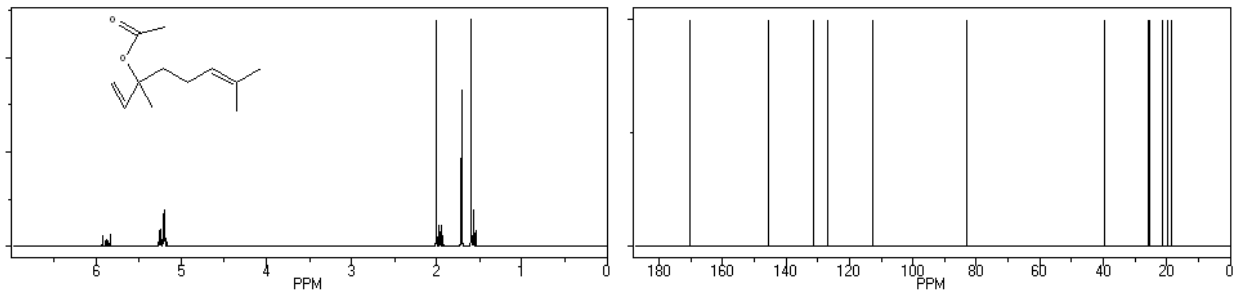
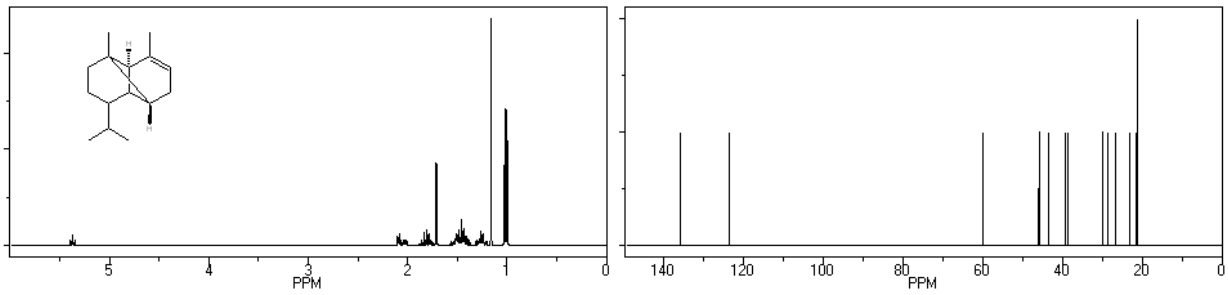


Figure 1S : A representative ^{1}H and ^{13}C NMR spectra of essential oil of *P. lanceolata*.

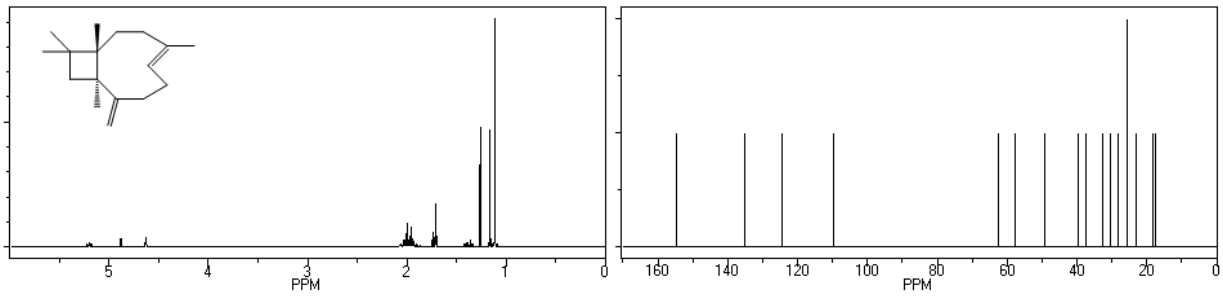
Linalylacetate



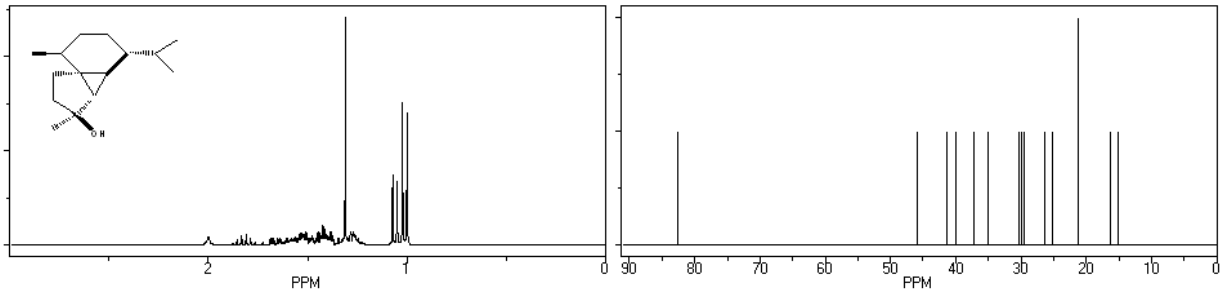
Alpha-copaene



Trans caryophyllene



Epi-cubebol



Contd……

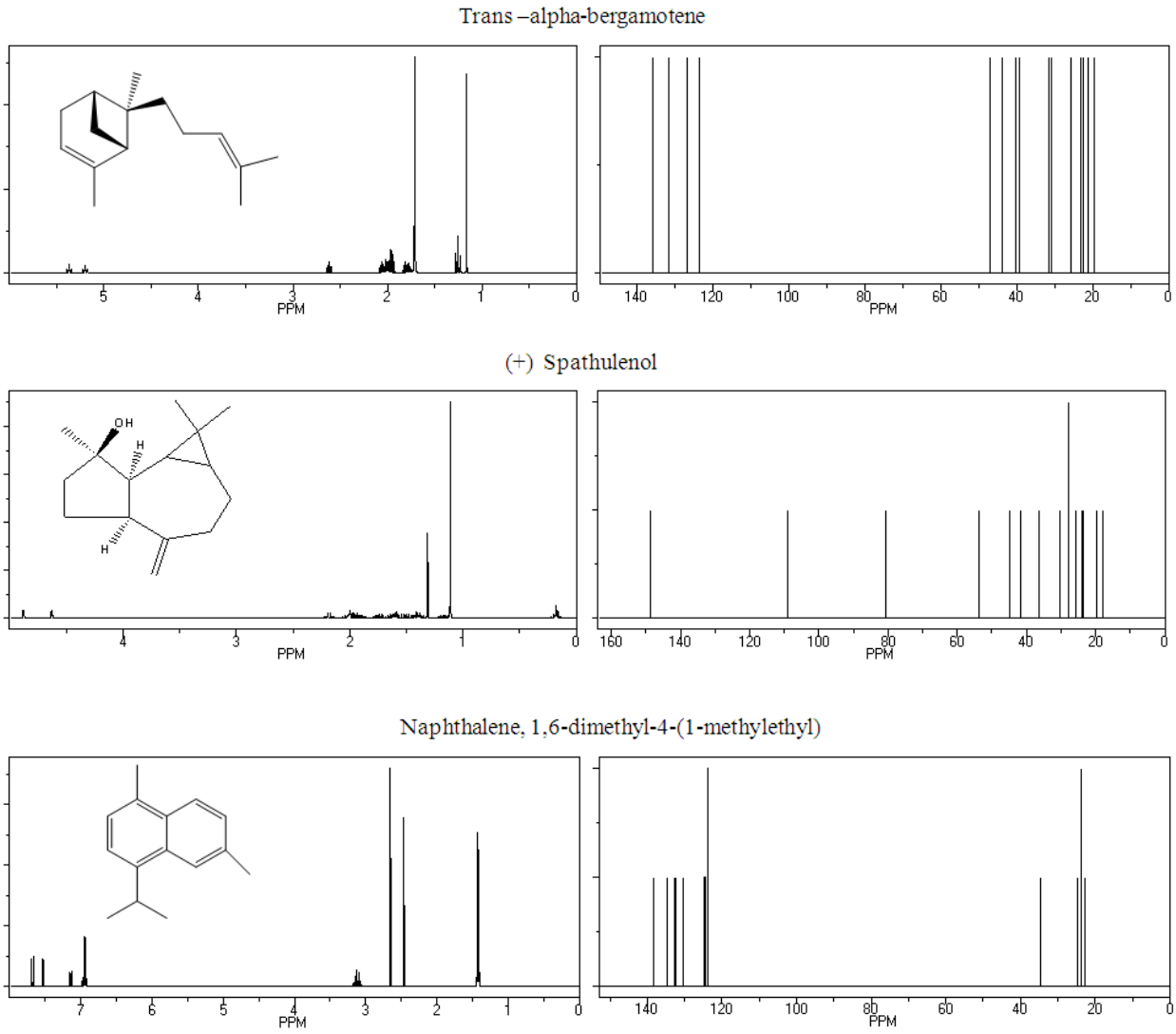


Figure 2S : Computer generated (Chemdraw Ultra 8.0) ^{1}H and ^{13}C NMR spectra of major components of essential oil of *P. lanceolata*.

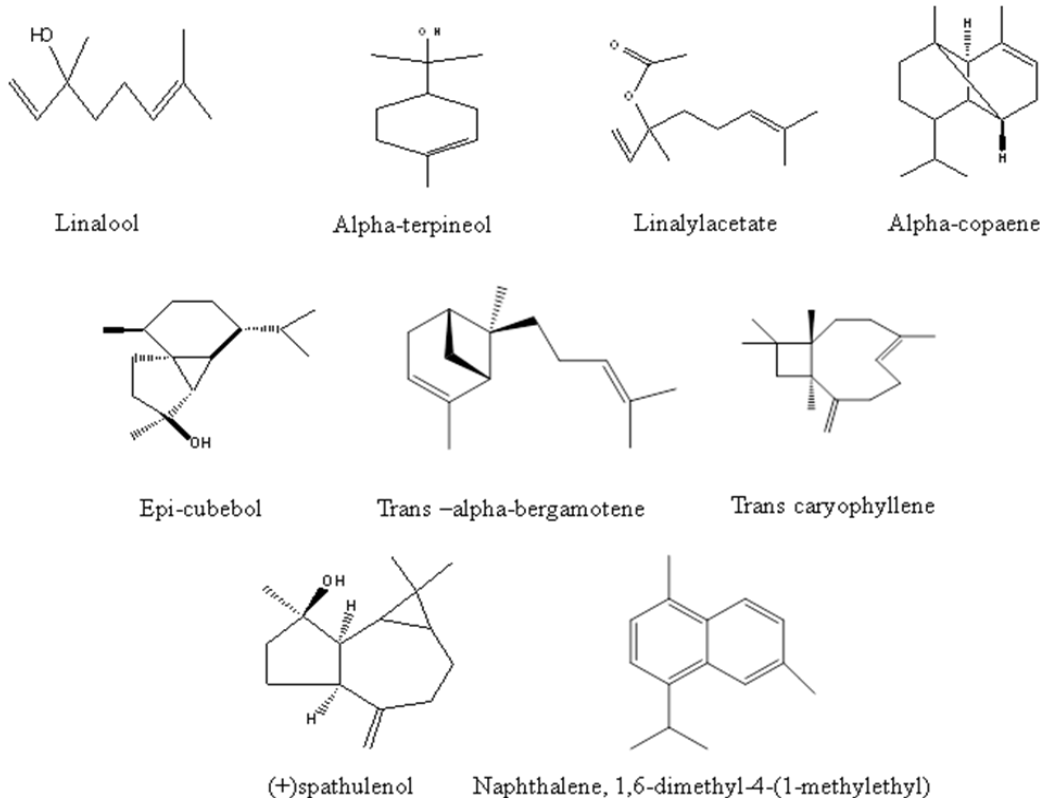


Figure 3S : Major compound of essential oil of *P. lanceolata* aerial part identified using RI, GC-MS (NIST, Wiley & Nbs libraries) and NMR spectral database (laboratory made).

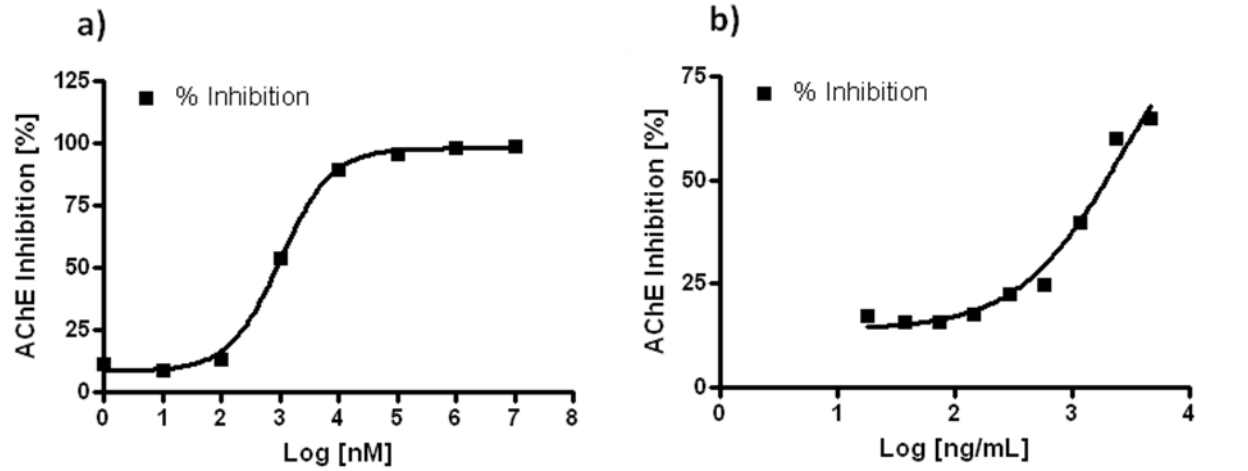


Figure 4S : Acetylcholinesterase inhibition by (a) physostigmine and (b) non-polar fraction of *P. lanceolata*

References

[1] A. Khadri, M.L.M. Serralheiro, J.M.F. Nogueira, M. Neffati, S. Smiti and M.E.M. Araujo (2008). Antioxidant and antiacetylcholinesterase activities of essential oils from *Cymbopogon schoenanthus* L. Spreng. Determination of chemical composition by GC-mass spectrometry and C-13 NMR, *Food Chem.***109,** 630-637.

[2] G.L. Ellman, K.D. Courtney, J.J.Andres and R.M. Feather-Stone, (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**, 88–95.

[3] Q. Zhao, Y. Murakami, M. Tohda, R. Obi, Y. Shimada and K. Matsumoto (2007). Chotosan, a Kampo formula, ameliorates chronic cerebral hypoperfusion-induced deficits in object recognition behaviors and central cholinergic systems in mice. *J. Pharmacol. Sci.* **103**, 360–373.

[4] M. Yamada, M. Hayashida, Q. Zhao, N. Shibahara, K. Tanaka, T. Miyata and K. Matsumoto (2011). Ameliorative effects of Yokukansan on learning and memory deficits in olfactory bulbectomized mice. *J. Ethnopharmacol.* **135**, 737–746.

[5] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall (1951). Protein measurement with Folin phenol reagent. *J. Biol. Chem*. **193**, 265–275.

[6] J.A. Pino, W.H. Perera**,** R. Sarduy**,** R.Oviedo and C.E. Quijano' (2009). Essential oil from flowers of *Pluchea carolinensis* (Jacq.), G. Don. *J. Essen. Oil Res.* **21,** 45-47.

[7] E. Simionatto, C.Z. Stüker, C. Porto, I.I. Dalcol, U.F. da Silva, A.F. Morel, E. L. Simionatto and A.W. Júnior (2007). Essential oil of *Pluchea quitoc* dc. (Asteraceae), *J. Essen. Oil Res.* **19**, 494-497.

[8] A.M.C. Arriaga, F.C.V. Cordeiro, J.Q. Lima, J.N. Vasconcelosa, E.M.A. Feitosaa and M.A. Neto (2006). Constituents of the essential oil of *Pluchea quitoc* DC, *J. Essen. Oil Res.* **18**, 693-694.

[9] E.C.J. Talenti (1982). Study of the essential oil of *Pluchea sagittalis* (Lamarck) Cabrera. *Essenze Derivati Agrumari.* **52**, 21-51.

[10] L. Bauer, N.C.S. de Siqueira, G.A.A. Brasil-e-Silva, C.T.M. Bacha, B.M.S. Sant'ana and N.O. Sieckowski (1979). Essential oil of *Pluchea sagittalis* Lam. Compositae of Rio Grande do Sul [Brazil], *Tribuna Farmaceutica*. **47,** 85-88.

[11] E.C.J. Talenti (1974). Essential oil of *Pluchea sagittalis* of Santa Rosa, Province of Santa Fe1, Argentina. In: Sixth International Congress of Essential Oils, San Francisco, California. USA

[12] E.C.J. Talenti and L.R. De Vottero (1974). Presence of caryophyllene and humulene in the essential oil of *Pluchea sagittalis.* Anales de la Sociedad Cientifica Argentina. **198**, 11-22.

[13] E.C.J. Talenti, J.A. De Orellana and L.J.J. Priano (1975). Essential oil of *Pluchea sagittalis*: Its composition. *Anales de la Sociedad Cientifica Argentina*. **199**, 31-45.

[14] E.C.J. T alenti, R. Manzi, F.A. Tedone, E. Aringoli and R.A. Yunes (1969). Methodological study of *Pluchea sagittalis.* Ecological and anatomo-histological features. Determination and identification of the main components of the essential oil. Revista de la Facultad de Ingenieria Quimica, Universidad Nacional del Litoral. **38,** 251-267.

[15] G.A. Fester, E.A. Martinuzzi, J.A. Retamar and A.I.A. Ricciardi, (1958). Essential oils from Argentine plants*. Boletin de la Academia Nacional de Ciencias* (Argentina). **40,** 189-208.