

Full Length Research Paper

# Preliminary phytochemical and biological screening of methanolic and acetone extracts from *Leonotis nepetifolia* (L.) R. Br.

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Accepted 28 May, 2012

This study is aimed at preliminary phytochemical analysis and determination of biological activity of methanolic extract (ME) and acetone extract (AE) from aerial parts of *Leonotis nepetifolia* (L.) R. Br. collected from Southern Brazil. Phytochemical analysis demonstrated the presence of terpene compounds, flavonoids, tannins, iridoids, sterols and fats. The extracts were inactive towards selected strains of Gram (+), Gram (-) bacteria and dermatophytes at concentrations up to 50 mg/ml, demonstrated antioxidative ability measured by ferric reducing ability of plasma (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) methods and exhibited cytotoxic *in vitro* activity towards Du145 human prostate cancer cell line.

**Key words:** *Leonotis nepetifolia*, antioxidant capacity, Du145 cells.

## INTRODUCTION

*Leonotis nepetifolia* (L.) R. Br. (*Lamiaceae*), known also under the name of Klip Dagga, Lion's Ear or Cordão-de-frade, is a widespread specie, especially on the African continent. Two varieties of that species are known: *L. nepetifolia* var. *nepetifolia* (with long orange hairs on the corolla) and *L. nepetifolia* var. *africana* (hairs are usually pale yellow coloured). The former is found growing wild also beyond the African continent: in Western India, South America and in Southern part of North America. *L. nepetifolia* var. *africana* almost entirely occurs in Africa (Iwarsson and Harvey, 2003; Venkat et al., 2011). The plant is used in traditional medicine in therapy of bronchial asthma, diarrhoea, fever, malaria and as an analgesic agent in menstrual pains; also to treat common cold and to alleviate cough (Clement et al., 2005, 2007; Maregesi et al., 2007; Lans, 2007). In India, the flowers are used in case of hardly healing wounds, scars and burns. To burn healing, the seeds are used as well (Kumar et al., 2007; Kala, 2005; Gupta et al., 2010).

Phytochemical investigations of this species have been focused so far on a diterpene fraction, where inter alia leonotinic acid, nepetefolin, methoxynepetefolin, leonotin, nepetefolinol and dehydronepetefolinol were identified; also the presence of a coumarin compound - 4,6,7-trimethoxy -5-methyl 2- chromene was noted (Govindasamy et al., 2002; Boalino and Tinto, 2004; Von Dreele et al., 1975; Purushothaman et al., 1976). In raw materials obtained from Japan, iridoids such as: geniposidic acid and its 10-O-trans-3,4-dimethoxycinnamyl- and 10-O-p-hydroxybenzoic - derivatives were identified (Takeda et al., 1999).

However, we report the results of preliminary phytochemical analysis of methanolic extract (ME) and acetone extract (AE) from *L. nepetifolia* var. *nepetifolia*, and the assessment of their antibacterial, antioxidative and cytotoxic activity.

## MATERIALS AND METHODS

### Plant material

Dried and crushed *L. nepetifolia* (L.) R. Br. herb was obtained from Alquimia da Terra Farmacia de Manipulacao Ltd in Gravatal, Santa

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Catarina region, Brazil, in 2009. ME and AE were prepared from the raw material using the method of hot extraction at solvent boiling temperature. The extracts obtained were analyzed using standard methods applied in order to identify various classes of compounds (Farmakopea, 2002; Wagner and Bladt, 2001; Harborne, 1998).

#### Antimicrobial activity examination

Dry ME and AE were dissolved in dimethyl sulfoxide (DMSO) (10 to 50 mg/ml). Their activity was analyzed towards the strains of Gram (-) bacteria: *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Escherichia coli*, Gram (+): *Staphylococcus aureus* and dermatophytes: *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Microsporum gypseum* and *Epidermophyton floccosum*. The disc diffusion method was used for the assessment of antimicrobial activity (Przondo-Morolaska, 2005).

#### Dermatophytes

The examined strains were cultured on Sabouraud medium, and next the suspension in 0.85% NaCl solution was prepared, corresponding to  $10^6$  cells/ml. Next, 100  $\mu$ l of prepared suspension was spread at the centre of agar plates (Sabouraud medium). The plates were left for 15 min, and then discs moistened with solutions of extracts in DMSO (10 to 50 mg/ml) were put on them. The plates were incubated for 5 to 7 days at 27°C. The growth inhibition zone around the discs was observed.

#### Bacteria

Twenty-four (24) h culture of examined strains on Sabouraud medium was prepared. The suspension in 0.85% NaCl solution was prepared from the culture (corresponding to density of  $0.5^\circ$  in McFarland scale). Further procedure was as previously described for dermatophytes. The plates were incubated for 24 h at a temperature of 37°C. The growth inhibition zone around the discs was observed.

#### Determination of total phenols

Total phenols were determined colorimetrically using the Folin–Ciocalteu reagent, as described previously (Paško et al., 2009). Total phenols assay was conducted by mixing 2.7 ml of de-ionized water, 0.3 ml of extracts, 0.3 ml 7 g/100 g  $\text{Na}_2\text{CO}_3$  and 0.15 ml Folin–Ciocalteu reagent. Absorbance of mixture was measured at 725 nm using the spectrophotometer Jasco UV-530. A standard curve was prepared with gallic acid. Final results were given as gallic acid equivalents (GAE).

#### Determination of ferric reducing ability of plasma (FRAP) activity

FRAP assay was carried out according to Benzie and Strain (1996), and modified to 48-well plates and automatic reader (Synergy-2, BioTek/USA) with syringe rapid dispensers (Benzie and Strain, 1996). Briefly, the oxidant in the FRAP assay (reagent mixture) consisted of ferric chloride solution (20 mmol/L), 2,4,6-tripyridyl-s-triazine (TPTZ) solution (10 mmol/L TPTZ in 40 mmol/L HCl) and acetate buffer (pH = 3.6) in a proportion of 5:5:10, respectively, and was freshly prepared. To each plate, 0.4 ml of acetate buffer (pH 3.6) was dispensed, followed by 50  $\mu$ l of sample (AE, ME), standard or blank. The plate was conditioned at the temperature of 37°C for 2 min, and then 0.2 ml of reagent mixture was added and shaken for 30 s; afterwards, absorbance at 593 nm was measured with

kinetic mode for 8 min. The final results were expressed as mmol  $\text{Fe}^{2+}$ /kg.

#### Determination of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical-scavenging activity was measured according to the method of Yen and Chen (1995) with modification by Paško et al. (2009). For the measurement of sample scavenging activity, 0.4 ml of methanolic acetate buffer was added to the cuvettes containing the increasing volumes of sample (for example, 0, 0.1, 0.2, 0.3, 0.45 and 0.6 ml) with adequate volumes of methanol to make total volume of 1 ml. Acetate buffer was made from 0.2 mol/L solutions of sodium acetate and acetic acid in methanol mixed at the volume ratio 7.9:2.1. The pH of the buffer was 5.2. 1 ml of DPPH stock solution (12 mg DPPH was dissolved in 100 ml of methanol; absorbance 1.3) was added to each cuvette, subsequently absorbance was measured after 24 h. The absorbance of the resultant solution was determined using Jasco UV-530 spectrometer (Japan) at 514 nm. The total antioxidant capacities were estimated as Trolox equivalents by interpolation to 50% inhibition.

#### Cytotoxic assay

The cytotoxic activity of ME and AE (in DMSO) was tested against Du145 human prostate cancer cells which were grown in Dulbecco's Modified Eagle Medium (DMEM) F12 medium supplemented with 10% heat inactivated calf serum and a mixture of antibiotics (37°, 5%  $\text{CO}_2$ ). Cells were transferred into 24-well microtiter plates (density  $1.5 \times 10^4$  cells/well) and incubated for 24 h. The extracts were added to the wells at concentrations from 10 to 200  $\mu$ g/ml. The positive controls were untreated cells cultured in media alone. Colchicine solution was served as a negative control. The cells were incubated for 24 or 48 h. The viability of the cells was tested using trypan blue dye. Cytotoxic activity was measured as a percentage of dead cells.

## RESULTS AND DISCUSSION

Preliminary phytochemical analysis of AE demonstrated the presence of terpene compounds, sterols and fats, whereas, in the ME terpenes, flavonoids, tannins, iridoids and sterols were identified.

The presence of tannins and iridoids - compounds of recognized antimicrobial activity, and also the use of *L. nepetifolia* in Brazilian traditional medicine for the treatment of infectious diseases, urged us to subject ME to an *in vitro* test against selected strains of bacteria and fungi: dermatophytes (*T. rubrum*, *T. mentagrophytes*, *M. gypseum* and *E. floccosum*), Gram (+) bacteria (*S. aureus*) and Gram (-) bacterias (*P. aeruginosa*, *Acinetobacter baumannii*, *E. coli*). No antimicrobial activity at concentrations up to 50 mg/ml of the extract was noted. Similar results were obtained for AE. Lack of antimicrobial effect was probably due to excessively low content of active compounds in tested extracts.

These results are consistent with results of the studies conducted in other countries. Lack of antibacterial activity of ME from *L. nepetifolia* collected in Puerto Rico towards *E. coli* and *S. aureus* was observed (Meléndez and

**Table 1.** *In vitro* cytotoxic activity of ME and AE towards Du145 human prostate cancer cell line.

Extract concentration (µg/ml)	Methanolic extract		Acetone extract	
	Dead cells 24 h (%)	Dead cells 48 h (%)	Dead cells 24 h (%)	Dead cells 48 h (%)
10	0	0	8.67	22.67
20	0.66	12.67	17.00	37.00
40	24.33	23.33	37.67	53.33
60	36.33	54.33	50.67	75.67
100	51.00	66.67	61.07	88.60
200	74.33	89.67	87.67	100

ED<sub>50</sub> value for colchicine = 1.24 +/- 0.03.

Capriles, 2006). Similarly, ethanol extract from Rwanda's *L. nepetifolia* did not exhibit antibacterial and antifungal activity (Cos et al., 2002).

As *L. nepetifolia* belongs to the subfamily Lamioideae which includes plants with high antioxidant capacity, the next stage of our studies on biological activity of raw material was determination of antioxidative potential (by determination of total polyphenols content) and analysis of total antioxidative ability of ME and AE using FRAP and DPPH methods. Moderate content of polyphenols in extracts was observed. Their average content in terms of milligram of gallic acid/g dw of the sample was 1.125 (mg GAE/g dw) for AE, and 6.4 (mg GAE/g dw) for ME.

Antioxidative activity of the extracts was correlated with total polyphenols content. ME and AE were characterized by low total antioxidative ability determined using FRAP method. After 8 min, it was  $43.25 \pm 1.16$  mM Fe<sup>2+</sup>/kg for ME, and  $5.53 \pm 0.64$  mM Fe<sup>2+</sup>/kg for AE. Similarly, the total antioxidant activity (DPPH) was higher in ME ( $60.6 \pm 4.7$  µM DPPH/g) than that in AE ( $14.4 \pm 2.2$  µM DPPH/g).

Studies on free radicals scavenging ability (DPPH method) conducted in Brazil on ME from overground parts of *L. nepetifolia*, originating from the northeastern part of that country also demonstrated its moderate antioxidative activity, half maximal inhibitory concentration (IC<sub>50</sub>) was 6.5 mg/ml (David et al., 2007).

However, it seems that the studies on antioxidant activity of Brazilian *L. nepetifolia* should be continued. In the studies conducted in India, it was found that the ethanolic extract from *L. nepetifolia* administered for 14 days to the Ehrlich Ascites carcinoma bearing mice brought back the altered levels of liver enzymatic and non-enzymatic antioxidants in dose-dependent manner (Gurunagarajan and Pemaiah, 2010). Also, glutathione level and activity of superoxide dismutase (SOD) and catalase were increased. It increased the life span of the experimental animals.

The last stage of the studies of the present paper was an analysis of *in vitro* AE and ME cytotoxicity towards Du145 human prostate cancer cell line (Table 1).

The value of median effective dose (ED<sub>50</sub>) for AE after 24 h of incubation was about 60 µg/ml, while after 48 h was about 40 µg/ml. 100% of cells mortality was

observed after 48 h at the concentration of 200 µg/ml of the extract. ED<sub>50</sub> value for colchicine after 24 h was 1.24 +/- 0.03 µg/ml. ME demonstrated moderate cytotoxic activity weaker than AE. The value of ED<sub>50</sub> for ME after 24 h of incubation was about 100 µg/ml, while after 48 h of incubation was about 60 µg/ml.

The results seem promising but need further studies on mechanism of action and isolation of active compounds responsible for cytotoxicity. Activation of apoptotic pathway has been proposed as mechanism of cytotoxic activity of the ethanolic extract of aerial parts of *L. nepetifolia* against Ehrlich Ascites carcinoma cells (Gurunagarajan and Pemaiah, 2011). The extract was found to produce considerable membrane damage and DNA fragmentation.

The aerial parts of *L. nepetifolia* are a source of labdane-type diterpenes. There are some reports on cytotoxic activity of such compounds isolated from different plants sources (Li et al., 2005; Mahaira et al., 2011). They are recognized as cell cycle inhibitors and apoptosis inducers. It is possible that this class of compounds is responsible for AE and ME cytotoxicity towards Du145 human prostate cancer cell line and they act via activation of apoptotic pathway.

## ACKNOWLEDGMENTS

The authors are grateful to Dr. Danuta Trojanowska from Department of Pharmaceutical Microbiology, Jagiellonian University, Medical College for conducting the studies on antimicrobial activity of the extracts.

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