Journal of Medicinal Plant Research

Volume 9 Number 17, 3 May, 2015
ISSN 2009-9723

Academic Journals

ABOUT JMPR

The Journal of Medicinal Plant Research is published weekly (one volume per year) by Academic Journals.

The Journal of Medicinal Plants Research (JMPR) is an open access journal that provides rapid publication (weekly) of articles in all areas of Medicinal Plants research, Ethnopharmacology, Fitoterapia, Phytomedicine etc. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles published in JMPR are peerreviewed. Electronic submission of manuscripts is strongly encouraged, provided that the text, tables, and figures are included in a single Microsoft Word file (preferably in Arial font).

Submission of Manuscript

Submit manuscripts as e-mail attachment to the Editorial Office at: jmpr@academicjournals.org. A manuscript number will be mailed to the corresponding author shortly after submission.

The Journal of Medicinal Plant Research will only accept manuscripts submitted as e-mail attachments.

Please read the **Instructions for Authors** before submitting your manuscript. The manuscript files should be given the last name of the first author.

Editorial Board

Journal of Medicinal Plants Research

Table of Contents: Volume 9 Number 17, 3 May, 2015

ARTICLES

Research Articles

academicJournals

Vol. 9(17), pp. 561- 568, 3 May, 2015
DOI: 10.5897/JMPR2014.5392
Article Number: 25C1E7C53062
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Anti-carcinogenic effects of ethanolic extracts from root and shoot of *Lupinus angustifolius* on breast carcinoma cell lines MCF-7 and BT20

Stapel*, J.[1], Oppermann, C.[2], Richter, D. U.[1], Ruth, W.[2] and Briese, V.[1]

[1]Medical Centre Rostock, University Gynaecological Clinic, Germany.
[2]Faculty of Mathematics and Science, University of Rostock, Germany.

Received 11 February, 2014; Accepted 30 April, 2015

The identification of medicinal plants and derived natural products for development as anti-cancer agents is of long standing interest. We have investigated the anti-proliferative properties of *Lupinus angustifolius* on breast cancer and, in particular, whether the extracts of roots and shoots of *L. angustifolius* can be considered as candidates for primary and secondary cancer prevention. Ethanolic extracts of roots and shoots of *L. angustifolius* were analysed for their substance classes by pyrolysis-field ionization mass spectrometry. Various concentrations of these extracts (0.1, 1, 10, 25, 50, 100 and 200 µg/ml, incubation period: 24 h) were studied with respect to their effects on cell proliferation and cytotoxicity against the breast cancer cell lines MCF-7 (ER-α+, ER-β+) and BT20 (ER-α-, ER-β-). *L. angustifolius* ethanolic root and shoot extracts inhibited cell proliferation in the MCF-7 and BT20 cells; root extract: strongest inhibition MCF-7 (200 µg/ml 71.03 ± 12.62%); BT20 (200 µg/ml 99.72 ± 6.48%), IC_{50}-values: MCF-7 (52.3 ± 9.39 µg/ml); BT20 (66.86 ± 7.02 µg/ml); shoot extract: strongest inhibition MCF-7 (200 µg/ml 64.7 ± 10.07 %); BT20 (200 µg/ml 86.32 ± 9.19%), IC_{50}-values: MCF-7 (18.06 ±4.49 µg/ml); BT20 (70.27 ± 0.76 µg/ml). Thus, extracts of *L. angustifolius* roots and shoots have anti-tumour activity against receptor-positive and receptor-negative breast cancer cell lines.

Key words: Anti-tumour activity, Lupinus angustifolius, breast cancer, MCF-7, BT20

INTRODUCTION

Breast cancer is the one of the most frequently diagnosed cancer in women and ranks second as a cause of cancer deaths in the female population (after lung cancer) (American cancer Siciety, 2012; Globocan, 2013; Centers for Disease Control and Prevention, 2013). It was estimated to cause approximately 421,000 new cases and 129,000 deaths in Europe alone in 2008 (Ferlay et al., 2010). In the United States, breast cancer is one of the leading causes of cancer death of women (40,676 women, 2009) (Centers for Disease Control and Prevention, 2013). Natural product research has revealed a large variety of phytochemicals that have been shown to be successful against breast cancer in several epidemiological and *in vitro* studies. More than 60% of

*Corresponding author. E-mail: janastapel@rocketmail.com.

currently used anti-cancer drugs were originally derived from natural sources such as plants, marine organisms and microorganisms (Aruoma et al., 2006). Various studies have suggested the potential of medicinal plants as anti-cancer drug candidates (Abarzua et al., 2012; Cragg et al., 2005; Pandey et al., 2009; Richter et al., 2013).

Lupinus angustifolius (family Fabaceae) is used in the food industry in Germany with regard to won protein and fibre (PlantProFood, 2013). Some studies have shown that lupin seeds have an immunotropic activity (Błaszczyk et al., 1994) and anti-bacterial properties (Oomah et al., 2006), whereas others indicate that lupin protein has hypocholesterolemic effects (Weisse et al., 2010), binds insulin *in vitro* and reduces plasma glucose levels (Magni et al., 2001). To date, no investigations into the possible anti-cancer effect of extracts from the root and shoot of the blue lupin *Lupinus angustifolius* have been described. Of the plants that we have examined, the blue lupin contains primarily isoprenoids, sterols, terpenes, flavonoids and polyphenols in its shoots and roots. We have used ethanolic extracts of the shoots and roots of blue lupin to determine whether they influence the cell proliferation of the breast cancer cell lines MCF-7, an estrogen receptor (ER)-positive control cell line, and BT20, an ER-negative cell line. MCF-7 and BT20 cells are useful for *in vitro* breast cancer studies, because the cell lines have retained several ideal characteristics of the mammary epithelium. Our data suggest that extracts of shoots and roots of *L. angustifolius* exhibit a breast-cancer-restraining effect in these receptor-positive and receptor-negative cell lines. Thus, roots and shoots of blue lupin might contain potential agents for the primary and secondary therapy of mammacarcinomas.

MATERIALS AND METHODS

Plants

Roots and shoots were harvested from the blue lupin, *Lupinus angustifolius (*type: Boregine), at the Saatzucht Steinbach GmbH & Co KG in Germany, during the month of July, 12 weeks after being sown. The plant was authenticated at the Herbarium unit of the Institute of Biosciences, University of Rostock, Rostock, Germany. Blue lupin roots and shoots were stored at -80°C at the University of Rostock in the University Gynaecological Clinic.

Microwave extraction

Microwave energy is a non-ionizing radiation that causes molecular motion by the migration of ions and the rotation of dipoles (Chen et al., 2008; Gu et al., 2008). Many reports have been published on the application of microwave-assisted extraction of secondary metabolites from plants (Chattip et al., 2008; Chen et al., 2007; Guo et al., 2008). The main advantages of microwave-assisted extraction are the considerable reduction in time and solvent as compared with conventional techniques. Samples of 3 g plant material (roots and shoots) from *L. angustifolius* were extracted with 15 ml ethanol (absolute) in a microwave oven (CEM Discover). Extraction took place under the following conditions: time, 10 min; temperature, 60°C; energy level, 800 W.

Pyrolysis-field ionization mass spectrometry (screening)

An analysis of the substance classes contained within blue lupin root and shoot extracts obtained by microwave extraction was carried out by pyrolysis-field ionization mass spectrometry at the University of Rostock in the Department of Soil Science. For field ionization, an emitter was used that was activated by a pyrolytic process within a benzonitrile atmosphere, with a partial pressure of approx. 80 Pa and under high voltage (4.8kV FI Emitter), by means of a Wolfram filament heated to white heat, leading to the formation of bristle-like carbon needles. The assay material was weighed in a quartz crucible, which was introduced, via a rod, into the ionic source, and the sample was ionized. The ions were detected by a MassSpectrometry MAT 900 S, which had a double-focus sector-field analyser.

Cell culture

MCF-7 and BT20 cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The breast cancer cell lines MCF-7 (ER-α +, ER-β +) and BT20 (ER-α-, ER-β-) were cultivated in the University Gynaecological Clinic at Rostock. MCF-7 cells were grown in phenol-red-free DME-medium (PAA), with 10% fetal calf serum (FCS), Amphotericin B (250 µg/ml; PAA), penicillin/streptomycin (100×; PAA). BT20 cells were grown in RPMI medium (PAA), with 10% fetal calf serum (FCS), Amphotericin B (250 µg/ml; PAA), penicillin/streptomycin (100x; PAA). Cells were cultivated at 37°C under 5% CO_2 in an incubator. Cells were passaged every 2 to 3 days by using trypsin and ethylenediaminetetraacetic acid (EDTA).

Assessment of cell proliferation and cytotoxicity

The cell proliferation and cytotoxicity of the human breast carcinoma cell lines MCF-7 and BT20 treated with various concentrations of ethanolic extracts from *L. angustifolius*, 17-β estradiol and tamoxifen were analysed by using the 5' bromodeoxyuridine (BrdU) cell proliferation enzyme-linked immunosorbent assay (ELISA) kit (colorimetric) and lactate dehydrogenase (LDH) cytotoxicity detection kit as recommended by the manufacturer (Roche, Germany). The test conditions were optimized in preliminary experiments, with the optimal cell number being 5×10^5 cells/well. The experiments were divided into four groups: (1) negative control with 1 µl absolute ethanol, (2) 17-β estradiol and (3) tamoxifen as positive controls and various concentrations (4) of the ethanolic extracts (see above). Each group consisted of 4 replicate wells in 96-well plates. The negative control with 1 µl absolute ethanol is hereafter referred to as the control. Table 1 shows the final concentration of the ethanolic extracts and controls in the assays.

Cell proliferation (cell proliferation BrdU assay, Roche)

Proliferating cells were revealed by the BrdU cell proliferation test (Roche). The cells were grown in 96-well plates: 100 µl cell suspension (5×10^5 cells/ml) was pipetted into each well of a 96-well plate and incubated for 24 h at 37°C under 5% CO_2. Subsequently, 1 µl of the test substance was added followed by 24 h of incubation at 37°C under 5% CO_2. Every well, except for the background controls, received 10 µl marker solution (1:100 dilution with sterile medium) and the cells were further incubated for 3 h at

Table 1. Final concentrations of ethanolic extracts and controls in the assays.

Lupin root extracts (µg/ml)	Lupin shoot extracts (µg/ml)	17-β estradiol	Tamoxifen	Ethanol absolute
200	200	10^{-9} mol/l	10^{-4} mol/l	1%
100	100	0.00027 µg/ml	37.15 µg/ml	-
50	50	-	-	-
25	25	-	-	-
10	10	-	-	-
1	1	-	-	-
0.1	0.1	-	-	-

37°C under 5% CO_2. Removal of the medium from the plates by sharply knocking the inverted plates was followed by incubation of the cells in 200 µl FixDenat for 30 min. The FixDenat was removed by knocking the inverted plates and the cells were further incubated for 60 min with the antibody solution. The cells were then washed three times with 200 µl washing buffer (1:10 dilution) and then incubated in 100 µl substrate solution (tetramethylbenzidine) for 10 to 15 min. The reaction was stopped with 1 M H_2SO_4. Absorption at a wavelength of 450 nm was measured in an ELISA reader (BioRad, Hercules, CA, USA).

$$\text{Cell proliferation (\%)} = \frac{\text{Absorbance sample}}{\text{Absorbance control}} \times 100$$

Cytotoxicity assay (LDH Cytotoxicity detection kit, Roche)

Cytotoxic effects on the cells were identified with a LDH cytotoxicity detection kit (Roche). The assay for the quantification of cell death and cell lysis was based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant. LDH activity is determined in a coupled enzymatic reaction; during this reaction, the tetrazolium salt INT is reduced to formazan. This formazan dye is easy to assay, since it is water-soluble and has a broad absorption maximum at approximately 500 nm. The increase in supernatant LDH activity is directly correlated to the amount of formazan formed over time. Samples taken from the cell suspension were pipetted into 96-well plates at 100 µl/well (5×10^5 cells/ml) for later treatment with each test substance and as controls. Cells were then incubated for 24 h at 37°C under 5% CO_2 in an incubator. The original medium (with 10% FCS) was exchanged for 198 µl/well fresh medium (1% FCS). Aliquots of 2 µl test substance were added to the relevant wells or 2 µl vehicle (absolute ethanol) was added to the wells as a negative control (medium plus cells plus absolute ethanol). As a positive (100% cell lysis) control, a well reaction mixture composed of 200 µl Triton X-100 and medium containing 1% FCS (at 1:46) were pipetted into four untreated wells. The cells were incubated for 24 h at 37°C under 5% CO_2 in the incubator. Subsequently, 100 µl cell suspension was removed and replaced by 100 µl well reaction mixture (at 1:46) and the reaction was stopped by the addition of 50 µl 1 M N HCl per well. The absorption of formazan was measured at a wavelength of 490 nm (reference 620 nm) in an ELISA reader and was evaluated with the program microplate manager.

$$\text{Cytotoxicity (\%)} = \frac{\text{(Absorbance of the sample - Absorbance of the negative control)}}{\text{(Absorbance of the high control - Absorbance of the negative control)}}$$

Statistical analysis

Statistical calculations were carried out with the Microsoft Office Excel 2007, Sigma Plot 11.0 and SPSS 10.0 for the Windows software package. Results are expressed as the means ± S.E.M. of three independent experiments. Student´s t-test and one way analysis of variance were used for statistical analysis; $P < 0.01$ was considered to be significant. IC_{50}-values (half-maximal inhibitory concentration) were calculated with Sigma Plot 11.0.

RESULTS

Pyrolysis-field ionization mass spectrometry (screening)

An analysis of the substance classes of the blue lupin root and of the blue lupin shoot components obtained by microwave extraction was carried out by pyrolysis-field ionization mass spectrometry (Figure 1). The substance class of LIPID (alkanes, alkenes, aldehydes, alcohols, fatty-acids, n-alkyl esters, waxes, fats) was the most strongly represented in the crude extract of lupin root (29.4%) and in that of lupin shoot (32.1%). ISOPR (isoprenoids, sterols, terpenes, carotenoids) gave complete ion strength (%) of 25.4% in the crude extract of lupin root and 25.1% in the crude extract of lupin shoot. Polyphenole (polyphenols) exhibited complete ion strength (%) of 10.4% in the crude extract of lupin root and 8.6% in the crude extract of lupin shoot.

Anti-proliferous activity of *Lupinus angustifolius* extracts (BrdU assay)

Blue lupin root and shoot extracts induced a significant

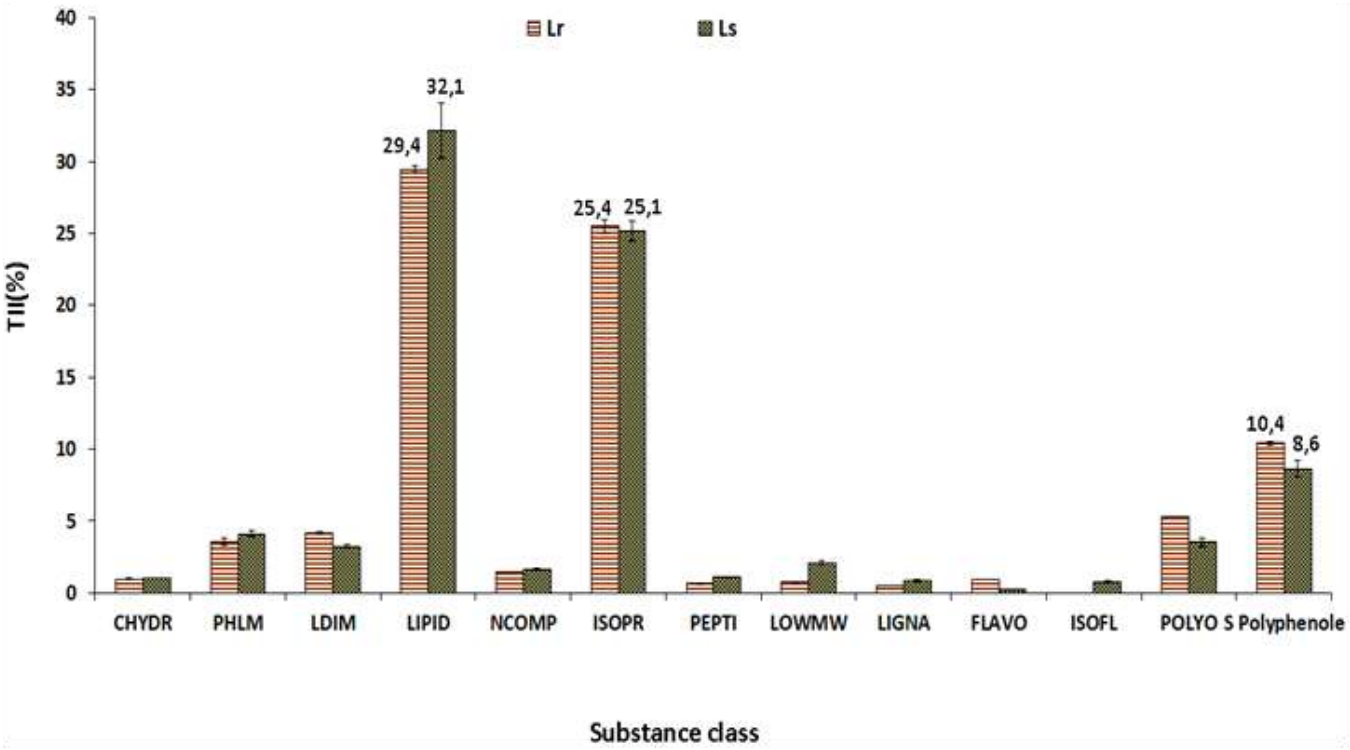

Figure 1. Pyrolysis-field ionization mass spectrometry (screening)**.** ITMS-cESI Full ms (90.00-2000.00) of ethanolic lupin extracts. Results are expressed as the mean ± SEM of 3 independent experiments. CHYDR = carbohydrates, PHLM = monolignols, LDIM = lignin dimers, LIPID = alkanes, alkenes, aldehydes, alcohols, fattyacids, n-alkyl esters, waxes, fats, NCOMP = compounds containing nitrogen, ISOPR = isoprenoids, sterols, terpenes, carotenoids, PEPTI =peptides and free amino acids, LOWMW = low-molecular compounds m/z 15-56, LIGNA = lignans, FLAVO = flavones, ISOFL = isoflavones, POLYO S = other polyphenols, Polyphenole = polyphenols. Lr = lupin root extract, Ls = lupin shoot extract.

reduction in the cell proliferation of MCF-7 and BT20 cells in a dose-dependent manner (Figure 2A and B). Strong inhibition of cell proliferation was seen at a concentration of 200 µg/ml in MCF-7 (64.7 ± 10.07%) and BT20 (86.32 ± 9.19%) cells (Figure 2A and B). The strongest inhibition was shown by the blue lupin root extract with a concentration of 200 µg/ml in MCF-7 (71.03 ± 12.62%) and BT20 (99.72 ± 6.48%) cells (Figure 2A and B). The addition of 17-β estradiol as a control at a concentration of 10^{-9} mol/l (0.00027 µg/ml) did not affect the proliferation of MCF-7 and BT20 cells. However, application of a tamoxifen control at a concentration of 10^{-4} mol/l (37.15 µg/ml) reduced growth by about 90% in MCF7 and in BT20 cells.

Cytotoxicity activity of *L. angustifolius* extracts (LDH assay)

Blue lupin root extract showed significant cytotoxicity in the MCF-7 breast cancer cell line at concentrations of 200 µg/ml (47.26 ± 6.44%), 100 µg/ml (44.98 ±10.50%) and 50 µg/ml (40.33 ± 23.44%). This extract had a lower cytotoxicity in the BT20 cells: 200 µg/ml (44.19 ± 9.78%) and 100 µg/ml (22.44 ± 5.08%). Blue lupin shoot extract had a low significant effect with regard to cytotoxicity on MCF-7 and BT20 cells. A significant cytotoxicity on MCF-7 cells was observed at 200 µg/ml (20.39 ± 10.10%), 100 µg/ml (21.14 ± 4.84%) and 50 µg/ml (26.89 ± 14.88%). Cytotoxicity in BT20 cells was seen at 200 µg/ml (17.63 ± 9.38%) (Figure 3A and B). The control treatment with 17-β estradiol at a concentration of 10^{-9} mol/l (0.00027 µg/ml) had no cytotoxicity effect on MCF-7 and BT20 cells. On the contrary, the tamoxifen control at a concentration of 10^{-4} mol/l (37.15 µg/ml) induced a strong cytotoxicity of 90-100% in MCF-7 and BT20 cells (Figure 3A and B).

IC_{50}-values of *L. angustifolius* extracts

Figure 4 shows the IC_{50}-values of *L. angustifolius* extracts. The IC_{50}-values have the following order: IC_{50}-values for MCF-7 with shoot extract (18.06 ± 4.49 µg/ml) < IC_{50}-values for MCF-7 with root extract (52.30 ± 9.39 µg/ml) < IC_{50}-values for BT20 with root extract (66.86 ± 7.02 µg/ml) < IC_{50}-values for BT20 with shoot extract < (70.27 ± 0.76 µg/ml).

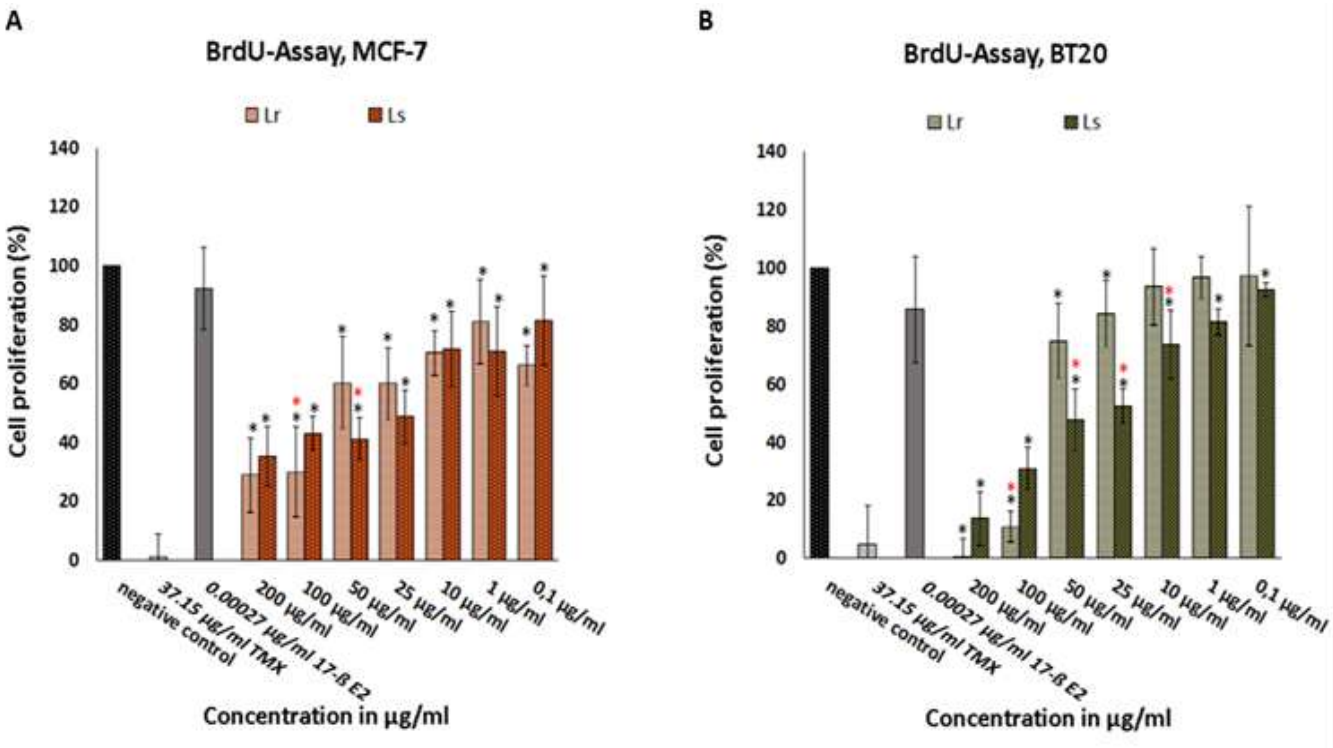

Figure 2. The effect of *Lupinus angustifolius* (Boregine) root and shoot extracts on cell proliferation (%). MCF-7 (A) and BT20 (B) cells were incubated for 24 h with root and shoot extracts (200-0.1 µg/ml). Results are expressed as the mean ± SEM of 3 independent experiments, four-fold for each experimental point. Analysis by t-test (*) and one-way analysis of variance (*) was used to compare cell proliferation in MCF-7 and BT20 cells cultured with various concentrations of lupin extracts with controls. $P < 0.01$ was regarded as statistically significant. Lr = lupin root extract, Ls = lupin shoot extract, TMX = Tamoxifen, 17-β E2 = 17-β estradiol.

DISCUSSION

The search is on for naturally based anti-proliferative and chemopreventive agents that can act as alternatives to chemically synthesized drugs and those are potentially less toxic and produce fewer side effects (Abrahim et al., 2012). In this study, we have tested the effects of ethanolic extracts from *L. angustifolius* on cell proliferation and cytotoxicity in the breast cancer cell lines MCF-7 and BT20. Our results demonstrate that blue lupin root and shoot extracts contain substance classes with potential anti-tumour effects (Figure 1). The substance class of LIPID (alkanes, alkenes, aldehydes, alcohols, fatty-acids, n-alkyl esters, waxes, fats) is the most strongly represented in the crude extract of the lupin root (29.4%) and in the crude extract of the lupin shoot (32.1%). Various studies have shown that mono- and poly-unsaturated fatty-acids (omega-3 fatty-acids and oleic acid (18:1) possesses anti-carcinogenic characteristics with respect to breast cancer (Carrillo et al., 2012; Mac Lennan and Ma, 2010). In addition, Menendez et al. (2006) have demonstrated that alpha-linolenic acid inhibits HER-2, which is overexpressed in breast cancer cells (Menendez et al. 2006). Our analysis of the substance classes have shown ISOPR (isoprenoids, sterols, terpenes, carotenoids) compounds make up 25.4% of the crude extract of the lupin root and 25.1% of the crude extract of the lupin shoot. The triterpenoid saponins from soybean (*Glycine max*) and lupin (*Lupinus angustifolius*) inhibit, for example, the sialyltransferases in tumour cells (Hsu et al., 2005; Zhang and Popovich, 2010). Our results show polyphenols as being 10.4% of the crude extract of the lupin root and 8.6% of the crude extract of the lupin shoot. According to Markaverich et al. (2011) and Rahman et al. (2011), the polyphenols luteolin, quercetin, chrysin and kaempferol are able to inhibit the growth of breast cancer cells.

Approximately 70% of all breast cancer types exhibit the expression of ER. In these cases, estrogen is mainly responsible for tumour growth (Rice et al., 2006). ER-negative breast cancer accounts for approximately 20% of all newly diagnosed breast malignancies; these are ER-β-, PR- and HER-2-negative (Rakha et al., 2009; Reis-Filho et al., 2008). MCF-7 is known to express both ER subtypes (ER-α, ER-β), PR and HER-2 (Brooks et al., 1973; Brandes and Hermonat, 1983). In contrast, BT20 has been established as being receptor-negative with respect to ER-α, ER-ß, PR and HER-2 (Castles et

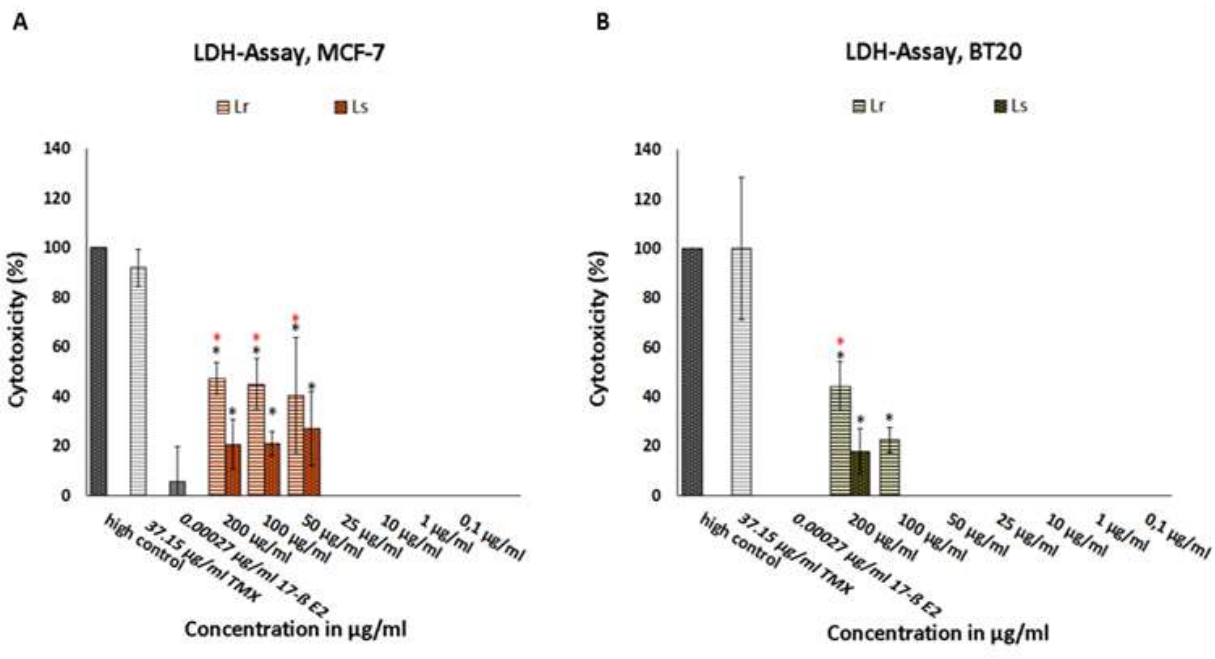

Figure 3. The effect of *Lupinus angustifolius* (Boregine) root and shoot extracts on cytotoxicity (%). MCF-7 (A) and BT20 (B) cells were incubated for 24 h with root and shoot extracts (200-0.1 µg/ml). Results are expressed as themean ± SEM of 3 independent experiments, four-fold for each experimental point. Analysis by t-test (*) and one-way analysis of variance (*) was used to compare cell proliferation in MCF-7 and BT20 cells cultured with various concentrations of lupin extracts with controls. $P < 0.01$ was regarded as statistically significant. Lr = lupin root extract, Ls = lupin shoot extract, TMX = Tamoxifen, 17-β E2 = 17-β estradiol

al., 1993; Grigoriadis et al., 2012). The results of the present study have revealed that natural *L. angustifolius* extract might constitute a potential anti-tumour compound against receptor-positive and receptor-negative breast cancer. Blue lupin root and shoot extracts inhibit cell proliferation and exhibit cytotoxicity in MCF-7 and BT20 breast cancer cell lines in a concentration-dependent manner (Figures 2, 3A and B). The strongest inhibition of cell proliferation is shown by the blue lupin root and the blue lupin shoot extracts in receptor-negative BT20 cells.

The blue lupin root extract shows strong cytotoxicity in both tumour cell lines at high concentrations. Whether the measured cytotoxicity is induced by apoptosis or necrosis cannot be distinguished by the LDH assay, because this assay is based on the measurement of cytoplasmic enzyme activity released by damaged cells (Roche, 2013). LDH is a soluble cytosolic enzyme that is released into the culture medium following the loss of membrane integrity resulting from either apoptosis or necrosis (Caymanchem, 2013). Furthermore, whether lupin root extract reduces the cell proliferation of MCF-7 and BT20 cells by necrosis or apoptosis also cannot be determined by the LDH assay. MCF-7 and BT20 cells were incubated for 24 h with blue lupin root and shoot extracts, and the effects on both cell proliferation and cytotoxicity were studied. Compared with the inhibition of cell proliferation, blue lupin root and shoot extracts had a low significant effect with regard to cytotoxicity on MCF-7 and BT20 cells. Further study needs to be conducted with longer incubation time to examine cytotoxicity in these cells.

Tamoxifen, used as a positive control in the BrdU and LDH assays, is one of the most extensively used drugs for the treatment of breast cancer (Yang et al., 2013). It is a nonsteroidal agent (selective ER modulator) with potent anti-estrogenic properties and it competes with estrogen for binding sites in breast and other tissues (Abarzua et al., 2012). Tamoxifen is used as a hormonal therapy for patients who exhibit ER-positive breast cancer (Yang et al., 2013). However, such tamoxifen use might involve risks related to the endometrium and ovaries (Mofrad et al., 2010). In the work presented here, tamoxifen induces a strong inhibition of cell proliferation and strong cytotoxicity in MCF-7 and BT20 cells (Figures 2, 3A and B). The inhibitory effects of tamoxifen in the ER-positive and ER-negative cells supports numerous previous results showing that the greatest portion of the growth-inhibitory effect of tamoxifen *in vitro* is not ER-dependent (Zheng et al., 2007; Todorova et al., 2011). However, the addition of 17-β estradiol as a control at a concentration of 10^{-9} mol/l (0.00027 µg/ml) does not affect cell proliferation or show cytotoxicity in MCF-7 and B20 cells. However, further phytochemical studies are needed to determine the main anti-cancer molecule or molecules in these blue lupin roots and shoot extracts. Moreover, additional studies are necessary to elucidate the mechanisms that are responsible for the observed effects

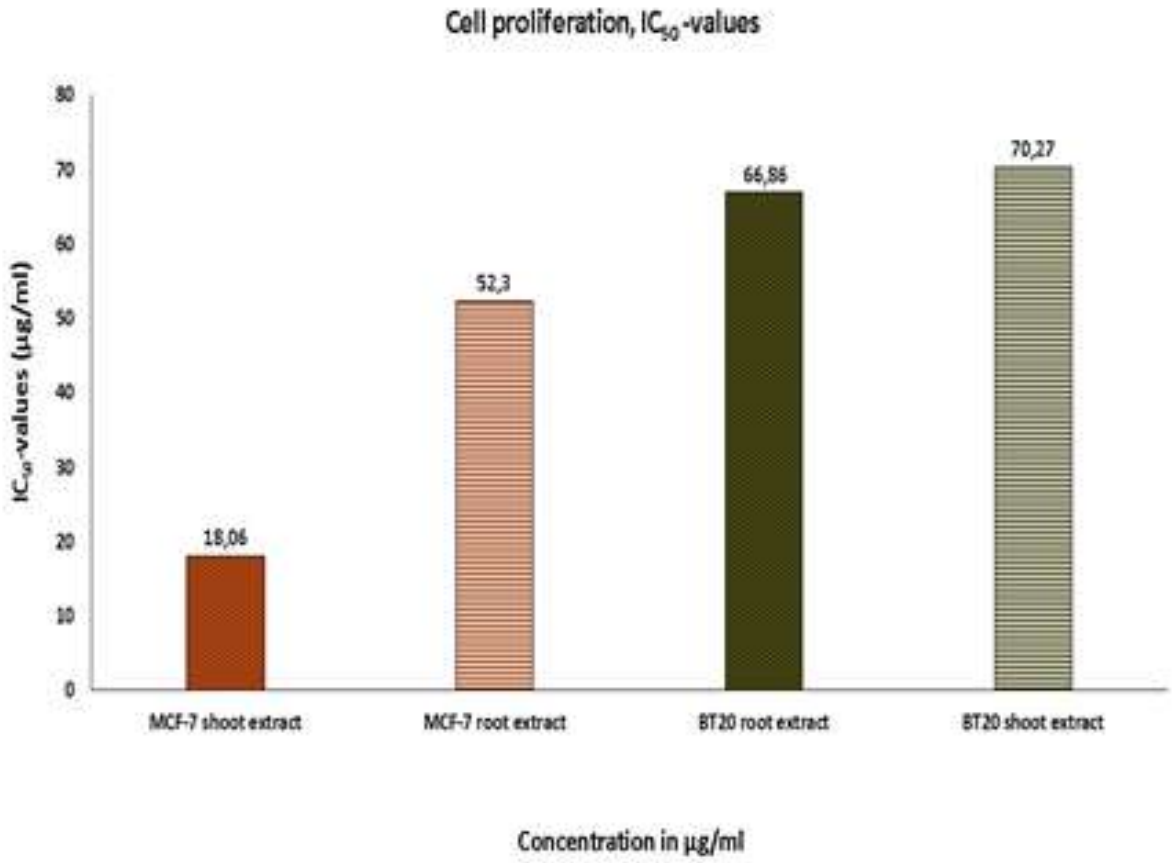

Figure 4. The IC_{50}-values of *Lupinus angustifolius* extracts**.** The IC_{50}-values show the half-maximal inhibitory concentration as calculated with Sigma Plot 11.0.

of the blue lupin extracts in breast cancer cells and benign cells and to determine the optimal dose for therapeutic use, possibly by using the xenograft mouse model.

ACKNOWLEDGEMENTS

This research project was supported by the following research grants: BMBF-project PlantsProFood; Germany.

Conflicts of Interest

All authors report no conflicts of interest.

REFERENCES

Abarzua S, Serikawa T, Szewczyk M, Richter DU, Piechulla B, Briese V (2012). Antiproliferative activity of lignans against the breast carcinoma cell lines MCF 7 and BT 20. Arch. Gynecol. Obstet. 285(4):1145-51.

Abrahim NN, Kanthimathi MS, Abdul-Aziz A (2012). Piper betle shows antioxidant activities, inhibits MCF-7 cell proliferation and increases activities of catalase. BMC Complement. Altern. Med. 12:220.

American Cancer Society (2012). Cancer Facts & Figures 2012. Atlanta: American Cancer Society. Available at: http://www.cancer.org/research/cancerfactsfigures/cancerfactsfigures/cancer-facts-figures-2012

Aruoma OI, Sun B, Fujii H, Neergheen VS, Bahorun T (2006). Low molecular proanthocyanidin dietary biofactor Oligonol: Its modulation of oxidative stress, bioefficacy, neuroprotection, food application and chemoprevention potentials. Biofactors 27(1-4):245-65.

Błaszczyk B, Stobiecki M, Kowalczyk-Bronisz SH, Gulewicz K, Szymaniec S (1994). Immunotropic activity of lupin seeds extracts and fractions from Lupinus angustifolius and Lupinus albus. Arch. Immunol. Ther. Exp. (Warsz). 42(2):147-53.

Brandes LJ, Hermonat MW (1983). Receptor status and subsequent sensitivity of subclones of MCF-7 human breast cancer cells surviving exposure to diethylstilbestrol. Cancer Res. 43(6):2831-2835.

Brooks SC, Locke ER, Soule HD (1973). Estrogen receptor in a human cell line (MCF-7) from breast carcinoma. J. Biol. Chem. 248(17):6251-3.

Carrillo C, M del Cavia M, Alonso-Torre SR (2012). Antitumor effect of oleic acid, mechanisms of action. A review. Nutr. Hosp. 27(5):1860-1865.

Castles CG, Fuqua SA, Klotz DM, Hill SM (1993). Expression of a constitutively active estrogen receptor variant in the estrogen receptor-negative BT-20 human breast cancer cell line. Cancer Res. 53(24):5934-9.

Caymanchem (2013). LDH Cytotoxicity Assay Kit Item Number 10008882 [pdf]. Retrieved from https://www.caymanchem.com/pdfs/10008882.pdf.2013

Centers for Disease Control and Prevention (2013). Breast Cancer.

Retrieved from: http://www.cdc.gov/.2013
Chattip P, Wanchai DE, Artiwan S (2008). Extraction of flavonoids and carotenoids from Thai silk waste and antioxidant activity of extracts. Sep. Purif. Technol. 62(2):444-448.
Chen LG, Jin HY, Ding L, Zhang HR (2008). Dynamic microwave-assisted extraction of flavonoids from Herba Epimedii. Separation Purif. Technol. 59(1):50-57.
Chen Y, Xie MY, Gong XF (2007). Microwave-assistedextraction usedfor the isolation of total triterpenoid saponins from Ganoderma atrum. J. Food Eng. 81(1):162-170.
Cragg GM, Newman DJ (2005). Plants as a source of anti-cancer agents. J. Ethnopharmacol. 100(1-2):72-9.
Ferlay J, Parkin DM, Steliarova-Foucher E (2010). Estimates of cancer incidence and mortality in Europein. Eur. J. Cancer 46(4):765-81.
Globocan (2013). Data Source and Methods. Retrieved from: http://globocan.iarc.fr/Pages/DataSource_and_methods.aspx
Grigoriadis A, Mackay A, Noel E, Jun Wu P, Natrajan R, Frankim J, Reis-Filo JS, Tutt A (2012). Molecular characterisation of cell line models for triplenegative breast cancers. BMC Genomics 13:619.
Gu ZY, Chen DM, Han Y (2008). Optimization of carotenoids extraction from Rhodobacter sphaeroides. LWT Food Sci. Technol. 41(6):1082-1088.
Guo Z, Jin Q, Fan G (2008). Microwave-assisted extraction of effective constituents from a Chinese herbal medicine Radix puerariae. Anal. Chim. Acta 436(1):41-44.
Hsu CC, Lin TW, Chang WW, Wu CY, Lo WH, Wang PH, Tsai YC (2005). Soyasaponin-I-modified invasive behavior of cancer by changing cell surface sialic acids. Gynecol. Oncol. 96(2):415-22.
Mac Lennan M, Ma DWL (2010). Role of dietary fatty acids in mammary gland development and breast cancer. Breast Cancer Res. 12:211.
Magni C, Sessa F, Accardo E, Vanoni M, Morazzoni P, Scarafoni A, Duranti M (2001). Conglutin gamma, a lupin seed protein, binds insulin in vitro and reduces plasma Glucose levels of hyperglycemic rats. J. Nutr. Biochem. 15(11):646-50.
Markaverich BM, Shoulars K, Rodriguez MA (2011). Luteolin Regulation of Estrogen Signaling and Cell Cycle Pathway Genes in MCF-7 Human Breast Cancer Cells. Int. J. Biomed Sci. 7(2):101-111.
Menéndez JA, Vázquez-Martín A, Ropero S, Colomer R, Lupu R (2006). HER2 (erB-2)-targeted effects of the omega-3 polyunsaturated fatty acid, alpha-linolenic acid (ALA, 18:3n-3), in breast cancer cells: the fat features of the Mediterranean diet as an anti-HER2 cocktail. Clin. Transl. Oncol. 8(11):812-20.
Mofrad MH, Shandiz FH, Roodsare FV, Moghiman T (2010). Evaluation of Ovarian Cysts in Breast Cancer Cases on Tamoxifen. Asian Pac. J. Cancer Prev. 11(1):161-4.
Oomah BD, Tiger N, Olson M, Balasubramanian P (2006). Phenolics and Antioxidative Activities in Narrow-Leafed Lupins (*Lupinus angustifolius* L.). Plant Foods Hum. Nutr. 61(2):91-7.
Pandey G, Madhuri S (2009). Some medicinal plants as natural anticancer agents. Pharmacogn. Rev. 3(6):259-263.
Rahman S, Salehin F, Igbal A (2011). *In vitro* antioxidant and anticancer activity of young *Zingiber officinale* against human breast carcinoma cell lines. Complement. Altern. Med. 11:76.
Rakha EA, Ellis IO (2009).Triple-negative/basal-like breast cancer: Review. Pathology 41(1):40-7.
Reis-Filho JS, Tutt AN (2008).Triple negative tumours: A critical review. Histopathology 52(1):108-18.
Rice S, Whitehead SA (2006). Phytoestrogens and breast cancer–promoters or protectors? Endocr. Relat. Cancer 13(4):995-1015.
Richter DU, Abarzua S, Chrobak M, Vrekoussis T, Weissenbacher T, Kuhn C ,Schulze S, Kupka MS, Friese K, Briese V, Piechulla B, Makrigiannakis A, Jeschke U, Dian D (2013). Effects of Phytoestrogen Extracts Isolated from Pumpkin Seeds on Estradiol Production and ER/PR Expression in Breast Cancer and Trophoblast Tumor Cells. Nutr. Cancer 65(5):739-45.
Roche (2013). Cytotoxicity Detection Kit^{PLUS} (LDH) [pdf]. Retrieved from: http://www.clontech.com/US/Products/Cell_Biology_and_Epigenetics/Cell_Biology_Kits/Cytotoxicity_LDH_detection_kit
Todorova VK, Kaufmann Y, Luo S, Klimberg VS (2011). Tamoxifen and raloxifene suppress the proliferation of estrogen receptor-negative cells through inhibition of glutamine uptake. Cancer Chemother. Pharmacol. 67(2):285-91.
Weisse K, Brandsch C, Zernsdorf B, Nkengfack Nembongwe GS, Hofmann K, Eder K, Stangl Gl (2010). Lupin protein compared to casein lowers the LDL cholesterol:HDL cholesterol-ratio of hypercholesterolemic adults. Eur. J. Nutr. 49(2):65-71.
Yang G, Nowsheen S, Aziz K, Georgakilas AG (2013). Toxicity and adverse effects of Tamoxifen and other anti-estrogen drugs. Pharmacol. Ther. 139(3):392-404.
Zhang W, Popovich DG (2010). Group B oleanane triterpenoid extract containing soyasaponins I and III from soy flour induces apoptosis in Hep-G2 cells. J. Agric. Food Chem. 58(9):5315-9.
Zheng A, Kallio A, Harkonen P (2007). Tamoxifen-induced rapid death of MCF-7 breast cancer cells is mediated via extracellularly signal-regulated kinase signaling and can be abrogated by estrogen. Endocrinology 148(6):2764-77.

academicJournals

Vol. 9(17), pp. 569-575, May, 2015
DOI: 10.5897/JMPR2014.5751
Article Number: E682DE453063
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Antioxidant activity, total phenolic and flavonoids contents in Stachytarpheta cayennensis, (Rich.) Vahl. (Verbenaceae)

Sideney Becker Onofre[1], Zípora M. Q. Santos[2], Francini Y. Kagimura[3] and Shaiana P. Mattiello[4]

[1]Center of Exact and Environmental Sciences - Postgraduate Program in Technology and Innovation Management (PPGTI), Universidade Comunitária da Região de Chapecó (UNOCHAPECÓ) Av. Senador Attílio Fontana, 591-E EFAPI - 89809-000 - Chapecó - Santa Catarina - Brazil.
[2]Laboratory of Chemistry and Biochemistry of the Universidade Paranaense (UNIPAR) - Francisco Beltrão – Paraná - Brazil.
[3]Department of Biochemistry and Biotechnology - Postgraduate Program in Chemical and Biochemical Processes of the Universidade Tecnológica Federal do Paraná (UTFPR) - Pato Branco - Paraná - Brazil.
[4]Molecular Biology Laboratory, Postgraduate Program in Cellular and Molecular Biology of the Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre - Rio Grande do Sul – Brazil.

Received 29 January, 2015; Accepted 29 April, 2015

In recent years, a substantial amount of evidence has pointed to the key role of free radicals and other oxidants as the main culprits for aging and degenerative diseases associated with aging, such as cancer, cardiovascular diseases, cataract, decline of the immune system and brain dysfunctions. The objective of this work was therefore to detect variations in total phenol and flavonoid content, and in the antioxidant activity of samples of *Stachytarpheta cayennensis*. Dried and crushed samples were submitted to static maceration extraction to obtain the hexane, ethyl acetate and ethanol fractions. Phytochemical prospecting and an assessment of the content of phenolic constituents and of the antioxidant activity were carried out. The data was analyzed according to the mean ± standard deviation and submitted to analysis of variance followed by Tukey's test to establish the degree of significance ($p < 0.05$). Flavonoids, tannins, coumarins, terpenoids and steroids, alkaloids and anthraquinones were detected in the samples. The content of total flavonoids varied between 2.69 ± 0.49 and 6.21 ± 0.67 g/100 g, while the total phenols ranged from 1.83 ± 0.06 to 15.33 ± 0 .44 g/100 g. The extracts produced EC_{50} between 38.60 ± 5.42 and 288.44 ± 22.12 µg/ml. The results indicate that the use of solvents of different polarities in the extraction process is an important strategy to detect variations in the levels of total phenols and flavonoids, and of antioxidant activity, in samples of *S. cayennensis*.

Key words: Phenols, flavonoids, free radicals, secondary metabolites.

INTRODUCTION

Aerobic organisms survive thanks to oxidative reactions using the oxygen (O_2) in the atmosphere. And although such reactions allow for the continuity of life, they also threaten it, since these reactions enable the formation of

reactive oxygen species (ROS) (Soares, 2002). ROS are extremely reactive molecules, many of which manifest themselves as free radicals. These species have a free electron in their last molecular orbital. Although they are part of the body's defense mechanism, they can also cause changes in the cells, acting on the cellular components, such as the fatty acids of membranes, cellular proteins and nucleic acids (Alves, 2007). These ROS can oxidize biomolecules, compromising many biochemical processes through the rupture of cell integrity, mutations, loss of molecular recognition and/or enzymatic activity, for example (Andrade Junior, 2005). Such cell damage may be associated with the origin of several diseases, such as arthritis, arteriosclerosis and neuro-degenerative disorders (Galvez et al., 2005).

It is known that ROS are strongly involved in the photo deterioration processes of the skin induced by UV light. Ultra-violet (UV) solar radiation contributes to the photo deterioration of the skin, causing skin cancer, photo-aging, photo-sensitization and other associated pathologies (Degáspari and Waszczynsky, 2004; Ishitsuka et al., 2005). Organisms have therefore developed biological adaptations that provide antioxidant protection against ROS. These defenses are fundamental for the existence of an oxidative equilibrium in organisms, because recent evidence has pointed to the essential involvement of ROS in biochemical processes. The main antioxidants present in human plasma are the proteins with thiol groups (SH), uric acid, ascorbic acid, tocopherols and carotenoids (Barreiros, 2006). Organisms are not fully protected by their endogenous antioxidant defenses. As such, the absorption of exogenous antioxidants, through the diet, for example, is required to maintain the oxidative balance and health of the human body. Various studies have confirmed the presence of antioxidants in various food items (Roesler, 2007). Flavonoids are natural substances with variable phenolic structures. More than 4,000 flavonoids have already been identified, with the flavonols, flavones, anthocyanidins and isoflavones being the most numerous. One of their most striking characteristics is their ability to act as antioxidant, hijacking free radicals and ROS (Machado, 2008). The antioxidant effect of flavonoids has been attributed to the reducing power of the phenolic group, which reduces free radicals and produces the phenoxyl radical, which, in turn, is stabilized by resonance. This capacity is influenced by the number of hydroxyls present, by their positions, and by the positions of glycosylation of these molecules (Wilmsen et al., 2005).

Phenolic compounds are the most abundant antioxidants in the human diet, and may reach up to 1 g of daily consumption (Cerqueira et al., 2007). Among these phenolic compounds, one of the most important classes is composed of the hydroxycinnamic acids, with caffeic acid as main representative, which occurs esterified to quinic acid and is known as chlorogenic acid in this form (Santos et al., 2007). There are 133 species of *Stachytarpheta cayennensis* (L.C. Rich) Vahl, which belongs to the genus *Stachytarpheta* (Verbenaceae). It is distributed throughout Brazil. The species of this genus are generally shrubs, branching subshrubs or, in rare cases, herbs that range from 0.5 to 1.5 m in height, although certain species may reach up to 4 m (Salimena-Pires and Giulietti, 1998). Its flowers are arranged in a spiral along the axis of the inflorescence in a very compact way, reaching up to 60 cm in length. Its corrolas are quite striking and easily located at a distance in the field. Usually they are blue, but they can have several colors depending on the species, such as red, violet, orange, white or black (Costa, 1960).

S. cayennensis (L.C. Rich) Vahl, is an erect, perennial, branching, somewhat angular, fibrous subshrub that is very resistant to traction. It usually has opposite, ovate leaves with a distinct petiole and serrated and indented edges, an acute or subacute apex, a slightly wrinkled appearance, green color, terminal inflorescence with linear stalks, sessile flower with a gamosepalous calyx, pilose on the dorsum, a corolla with five petals welded at the base, of a lilac coloring, with an androecium with two fertile stamens and two staminodes. *S. cayennensis* (Rich.) Vahl, popularly known as verbena, belongs to the family Verbenaceae (Pio Correa, 1984). This species is found in the tropical and subtropical Americas, from Mexico to Brazil (Lopes, 1977; Troncoso, 1979), and it has been used in traditional medicine as an anti-inflammatory, analgesic, antipyretic, hepatoprotective and laxative agent, and in the treatment of gastric disorders (Mathias and Emily, 1993; Mesia-Vela et al., 2004). Crushed leaves and roots have also been applied in the treatment of skin lesions (Caribe and Campos, 1991), including in ulcerated lesions caused by *Leishmania* sp. (Moreira et al., 1998; Moreira et al., 2002). Some of the effects suggested by the population have already been demonstrated experimentally, such as the anti-inflammatory, antioxidant, analgesic, gastro-protective, antibacterial and antifungal activity (Schapoval et al., 1998; Mesia-Vela et al., 2004; Duarte et al., 2004; Falcão et al., 2005). Its chemical composition includes alkaloids, glycosides (verbenalin and verbenin) tannins, saponins, flavonoids, steroids, quinones, phenolic compounds and gluconic acid (Mathias and Emily, 1993).

The objective of this work was therefore to detect variations in total phenol and flavonoid content, and in antioxidant activity, of samples of *S. cayennensis* (Rich.) Vahl. (Verbenaceae) collected in the southwest region of

*Corresponding author. E-mail: beckerside@unochapeco.edu.br. Tel: +55 (46) 3055-6465, +55 (46) 99739131.

Paraná- Brazil, submitted to extraction with solvents of different polarities.

MATERIAL AND METHODS

Collection and preparation of the raw material

The samples (A, B and C) of the aerial parts of the plant "verbena" (gervão-roxo) Stachytarpheta cayennensis, (Rich.) Vahl, belonging to the family Verbenaceae, were collected in the municipality of Francisco Beltrão Paraná, Brazil, during its flowering period (Spring). All harvesting was performed on different points of the same place in the month of November, 2013. The samples were exsiccated in the Laboratory of Chemistry and Biochemistry of the Universidade Paranaense (UNIPAR), Francisco Beltrão Campus Paraná, and Brazil. One voucher specimen was deposited in the Herbarium of UNIPAR under the number 12.643. The plants were then stored in a dehumidification chamber at a temperature of 24°C during 45 days for drying. After this period, the leaves were separated and crushed, obtaining the plant biomass (powder) for the preparation of the aqueous extract. The aqueous extract of S. cayennensis was prepared in the Laboratory of Chemistry and Phytochemistry of the União de Ensino do Sudoeste do Paraná (UNISEP), Dois Vizinhos Paraná, Brazil. The isolation and growth of the yeasts, in addition to the microbiological tests, were performed at the Laboratory of Microbiology of the Universidade Paranaense (UNIPAR) - Francisco Beltrão Campus Paraná, Brazil.

Extraction process

Samples of dried and crushed *S. cayennensis* were extracted by static maceration using solvents of increasing polarity: hexane, ethyl acetate and ethanol. The hexane (EH), ethyl acetate (EA) and ethanolic (EE) extracts were then filtered through filter paper and the solvents removed through evaporation, obtaining dry extracts.

Preparation of stock solutions

The stock solutions with a concentration of 5 mg/ml were prepared based on 0.250 g of each dry extract solubilized in Dimethyl sulphoxide (DMSO). The dilution of each solution produced concentrations of 1mg/ml for the realization of the tests.

Phytochemical prospecting

Chemical classes of secondary metabolites have been investigated in the extracts of *S. cayennensis* through identification reactions according to Matos (1997): flavonoids (reactions with $AlCl_3$, H_3BO_3, NaOH 1N, and Shinoda reactions), tannins (reactions with lead acetate, copper salts, iron salts, alkaloids and gelatin), coumarins (reaction with KOH 5%), steroidal heterosides (Kedde, Libermann-Burchard and Baljet reactions), saponins (foam index), alkaloids (Bertrand, Bouchardat, Dragendorff and Mayer reactions) and anthraquinones (Borntraeger reaction).

Determination of total phenol content

Total phenol contents were quantified by the spectrophotometric method using the Folin-Ciocalteu reagent (Sousa et al., 2007) and garlic acid as standard. A sequence of five test tubes, in triplicate, 0.01 (tube 1), 0.02 (tube 2), 0.03 (tube 3), 0.04 (tube 4) and 0.05 ml (tube 5), was prepared from a stock solution of garlic acid 1 mg/ml. 5 ml of the diluted Folin-Ciocalteau reagent, 4 ml of sodium carbonate and water were then added to make up the volume of 10 ml. The tubes were placed in the dark for 1 h at room temperature. The readings were performed with a spectrophotometer at 773 nm and the absorbances were used to obtain the calibration curve and coefficient of determination (R2) by the least squares method. Solutions of the extracts were prepared for the acquisition of the absorbances that were substituted in the curve equation, determining the levels of total phenols.

Determination of total flavonoid content

The quantification of total flavonoids was done with the spectrophotometric method according to Sobrinho et al., (2008). A 0.5 mg/ml solution of rutin was prepared. Aliquots of 0.02, 0.05, 0.1, 0.2 and 0.3 ml of this solution were transferred to test tubes, in triplicate, and added to 0.12 ml of glacial acetic acid, 2 ml of pyridine: ethanol (2:8), 0.4 ml of ethanol, 0.5 ml of aluminum chloride 8%, and water to obtain a final volume of 5 ml. The readings were performed with a spectrophotometer at 418 nm and the absorbances were used to obtain the calibration curve and coefficient of determination (R2) by the least squares method. Solutions of the extracts were prepared for the acquisition of the absorbances that were substituted in the curve equation, determining the levels of total flavonoids.

Antioxidant activity

The antioxidant activity of the extracts was determined through the spectrophotometric method using the free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) as described by Mensor et al., (2001). Stock solutions of dry extracts and rutin (positive control) at 1 mg/ml in ethanol 98% were prepared and diluted to various µg/ml concentrations for the spectrophotometric readings. A solution of 0.3 mM DPPH was also prepared to perform the test. After 60 min of reaction, the ability of the extracts and of the rutin to reduce 2,2-diphenyl-1-picrylhydrazyl to 2,2-diphenyl-1-picryl hydrazine was verified. The color change from purple to yellow was detected through the decrease in absorbance in a spectrophotometer at a wavelength of 520 nm. Based on the absorbance readings, the percentage of antioxidant activity (%AA) corresponding with the amount of DPPH reduced by the extracts was determined. After obtaining the antioxidant activity, the half maximal effective concentration (EC_{50}) of extracts was obtained by linear regression analysis using the least squares method, obtaining the equation of the curve and the coefficient of determination (R^2). The tests were performed in triplicate.

Statistical analysis

The results were demonstrated through the mean ± standard deviation. Analysis of variance (ANOVA) followed by Tukey's test was used to measure the degree of significance for $p < 0.05$.

RESULTS AND DISCUSSION

The identification reactions of chemical classes of secondary metabolites revealed the presence of flavonoids, tannins, coumarins, terpenoids and steroids, alcaloides and anthraquinones in samples of *S. cayennensis* (Table 1). In the three samples (A, B and C), however, it was observed that the classes of constituents

Table 1. Phytochemical prospecting of the samples A, B and C of *Stachytarpheta cayennensis*, (Rich.) Vahl.

Class	Reactions	(A)			(B)			(C)		
		EH	EA	EE	EH	EA	EE	EH	EA	EE
Flavonoids	$AlCl_3$	-	-		-	-	-	-	-	+
	H_3BO_3	-	+	+	-	-	+	-	-	+
	NaOH	-	+	+	-	+	+	-	+	+
	Shinoda	-	-	-	-	-	-	-	-	-
Tannins	Lead acetate	-	+	-	-	-	+	-	+	-
	Copper salt	-	+	-	-	-	+	-	+	-
	Iron Salts	-	-	-	-	-	-	-	-	-
	Alkaloids	-	-	-	-	-	-	-	-	-
	Gelatin	-	-	-	-	-	-	-	-	-
Coumarins	KOH	-	-	-	-	-	-	-	-	-
Terpenoids/Steroids	Baljet	+	+	+	+	+	+	+	+	+
	Kedde	-	-	+	-	-	+	-	+	+
	Liebermann-Burchard	+	-	+	+	-	+	+	+	+
Saponins	Foam index	-	-		-	-	-	-	-	-
Alkaloids	Dragendorff	+	-	+	+	-	+	+	+	+
	Mayer	-	-	-	-	-	-	-	-	-
	Bertrand	-	-	-	-	-	-	-	-	-
	Bouchardat	-	-	-	-	-	-	-	-	-
Anthraquinones	Borntraeger	-	-	-	-	-	-	-	+	+

Hexane extract (EH); Ethyl acetate extract (EA); Ethanolic extract (EE); (+) positive reaction; (-) negative reaction.

were identified according to the polarity of the solvent used in the extraction. Since flavonoids and tannins are more polar. For example, their reactions were positive in the ethyl acetate and ethanolic extracts (Tiwari et al., 2011). Negative reactions are indicative of the absence or low levels of constituents in the analyzed extracts. The absence of a constituent, such as of saponins, may be a result of it not being present, or of the decreased gene expression of enzymes involved in the biosynthesis of the secondary metabolite (Pichersky and Gang, 2000). Unlike the results shown in Table 1, saponins were found in a study of seasonal variation (Borella et al., 2006). Chemical prospecting also demonstrates, through the absence or presence of reactions, the difference between the analyzed extracts (Table 1). This means that the use of solvents of different polarities in the extraction process is an important strategy to detect variations in the chemical composition of the samples of S. cayennensis. In addition, it is possible that temporal and spatial variations arising from seasonality, circadian rhythm and development, temperature, the availability of water, ultraviolet radiation, nutrients, air pollution, induction by mechanical stimuli or attack by pathogens, among others, are related to the alteration in the synthesis of special metabolites (Gobbo-Neto and Lopes, 2007). The results showed the presence of different chemical classes, which may be associated with the biological activities of S. cayennensis, especially those related to free radicals. After defining the wavelength with a maximum absorption at 773 nm, the calibration curve of garlic acid ($y = 0.115x + 0.005$) was obtained to quantify total phenol contents. The absorbance values of extracts were replaced in this equation, determining the total phenol contents, which ranged from 1.83 ± 0.06 to 15.33 ± 0.44 g/100g equivalent to garlic acid (Table 2). In sample A, the highest total phenol content was obtained in the ethanolic extract, while samples B and C had more significant levels in the ethyl acetate extracts.

The ethyl acetate extract revealed a higher total phenol content for samples B and C ($p < 0.05$), probably due to the affinity of these substances with the employed solvent. Phenolic substances have a higher affinity with polar solvents, such as ethanol and ethyl acetate (Spagolla et al., 2009; Tiwari et al., 2011). In this sense,

Table 2. Mean total phenol content of the extracts of the samples of *Stachytarpheta cayennensis*, (Rich.) Vahl.

Extracts	Total Phenols g/100g		
	Sample A	Sample B	Sample C
Hexane	1.83 ± 0.06	1.87 ± 0.18	1.59 ± 0.05
Ethyl acetate	4.71 ± 0.03	5.95 ± 0.05	15.33 ± 0.44
Ethanolic	7.55 ± 0.10	6.99 ± 0.02	8.23 ± 0.08

The means differ among themselves after ANOVA followed by Tukey's Test for $p < 0.05$.

Table 3. Mean total flavonoid content of the extracts of the samples of *Stachytarpheta cayennensis*, (Rich.) Vahl.

Extracts	Total Flavonoids g/100g		
	Sample A	Sample B	Sample C
Hexane	---	---	---
Ethyl acetate	2.69 ± 0.49	3.58 ± 0.43	6.21 ± 0.67
Ethanolic	4.78 ± 0.59	---	3.92 ± 0.47

The means differ among themselves after ANOVA followed by Tukey's Test for $p < 0.05$.

the extraction process was fundamental to identify the difference in total phenol levels in the products analyzed. This difference may be related to intrinsic or extrinsic environmental factors that influence the biosynthesis of special metabolites in plants (Gobbo-Neto and Lopes, 2007). When the total phenol contents in the ethyl acetate extract are considered, it is possible that the main phenolic constituents found in this extract are tannins and flavonoids, as shown in Table 1. The result for total phenols in the ethyl acetate extract therefore corroborates the findings of the phytochemical prospecting. The absorbance values of the extracts of samples of *S. cayennensis* were replaced in the equation of the calibration curve of rutin ($y = 0.0104x + 0.0593$) and the levels of total flavonoids equivalent to rutin were determined, producing a variation of 2.69 ± 0.49 to 6.21 ± 0.67 g/100 g (Table 3). The ethyl acetate and ethanolic extracts of sample C had higher quantities of flavonoids. As expected, the hexane extracts did not reveal the presence of flavonoids.

Although the samples were taken from the same plant species, it is clear that they differ in the levels of flavonoids, which is an active substance group found in S. cayennensis (Borella et al., 2006; Saldanha et al., 2013). This difference is related to the use of solvents of different polarities in the extraction process, which influenced the clarity with which the variation of flavonoids in the samples could be detected. In addition, factors associated with temporal and spatial variations can change the synthesis of special metabolites, including flavonoids (Gobbo-Neto and Lopes, 2007). The results of this study corroborate those described by Borella et al., (2006) who showed variation in the flavonoid contents in different samples of *Baccharis trimera*. It is important to note that the changes in the levels of total flavonoids may cause a variation in biological activities. The levels of total constituents in plant derivatives depend on the extraction process and its variables, such as the solvent used. The polarity of the solvent interferes with the extraction, potentially extracting flavonoid glycosides in the ethanolic extract or free flavonoids in the ethyl acetate extract (Cechinel Filho and Yunes, 1998). The solvent can therefore direct the isolation of certain groups of flavonoids. Some free flavonoids, such as quercetin, luteolin and nepetin, were isolated in the ethyl acetate extract of *S. cayennensis* (Verdi et al., 2005). Table 4 presents the half maximal effective concentrations (EC50) of the rutin standard and the hexane, ethyl acetate and ethanolic extracts of the three samples of *S. cayennensis*. The values shown represent the effective concentrations able of reducing 50% of the DPPH present in the solution. The EC50 of the extracts varied between 38.60 ± 5.42 and 288.44 ± 22.12 µg/ml. Rutin produced an EC_{50} of 15.44 ± 0.39 µg/ml. All samples showed less antioxidant activity than the rutin standard.

The hexane extracts of samples A and C produced EC_{50} values above 320 µg/ml, the highest concentration used in this assessment, showing less antioxidant activity due to the low extraction of phenolic substances. The EC_{50} of the hexane extract of sample B was equal to 288.44 ± 22.12 µg/ml. This can be explained by the higher content of total phenols. Polar solvents, such as ethanol and ethyl acetate, extract greater quantities of phenolic substances with antioxidant activity. These substances, such as flavonoids, can react with the free

Table 4. Antioxidant activity of the extracts of aerial parts of *Stachytarpheta cayennensis*, (Rich.) Vahl. with the DPPH test.

Extract	Total flavonoids g/100 g			
	Sample A	Sample B	Sample C	Standard
Hexane	> 320	288.44 ± 22.12	> 320	---
Ethyl acetate	153.12 ± 19.32	128.13 ± 8.90	47.10 ± 2.64	---
Ethanolic	38.60 ± 5.42	67.57 ± 1.16	75.44 ± 8.84	---
Rutin	---	---	---	15.44 ± 0.39

The means differ among themselves after ANOVA followed by Tukey's Test for $p < 0.05$.

radicals, neutralizing their oxidant effect.

The results of this study corroborate the data obtained by other authors, who have correlated the content of phenolic substances and the antioxidant potential of plant extracts. By observing the total phenolic compound content of the crude methanolic extract and fractions of the leaves of the species *Palicourea rigida*, Rosa et al. (2010) found that despite the low activity presented by the crude extract (500 ppm), the ethyl acetate fraction showed moderate activity (192 ppm) and the highest total phenolic content among the fractions tested. Similarly, by analyzing the content of total phenols and antioxidant activity of five medicinal plants, Souza et al. (2007) concluded that three species (*Terminalia brasiliensis, Cenostigma macrophyllum* and *Copernicia cerifera*) showed a positive relationship between phenol contents and antioxidant capacity, as analyzed by the DPPH method.

Conclusion

The use of solvents with different polarities in the extraction process was crucial to detect variations in the chemical composition and antioxidant activity in samples of *S. cayennensis*. In addition, the antioxidant potential of *S. cayennensis* involves mechanisms of sequestration of free radicals, particularly in the more polar extracts, which produced higher amounts of phenolic constituents.

Conflicts of interest

The authors declare there are no ethical, publishing of financial conflicts of interest regarding the data of this study.

REFERENCES

Alves CQ (2007). Avaliação da atividade antioxidante de flavonoides. Diálog. Ciênc. 5(12):1-8.

Andrade Junior DR (2005). Os radicais livres do oxigênio e as doenças pulmonares. J. Braz. Pneumol. 31(1):60-68.

Barreiros ALBS, David JM, David JP (2006). Estresse oxidativo: relação entre geração de espécies reativas e defesa do organismo. Quim Nova. 29(1):113-123.

Borella JC, Duarte DP, Novaretti AAG, Menezes Junior A, França SC, Rufato CB, Santos PAS, Veneziani RCS, Lopes NP (2006). Variabilidade sazonal do teor de saponinas de Baccharis trimera (Less.) DC (carqueja) e isolamento de flavona. Rev. Braz. Farmacogn. 16(4):557-561.

Caribe J, Campos JM (1991). Plantas que ajudam o homem. Guia Prático para Época. Atual. Assoeste. Cascavel. Brasil. P 120.

Cechinel Filho V, Yunes RA (1998). Estratégias para a obtenção de compostos farmacologicamente ativos a partir de plantas medicinais. Conceitos sobre modificação estrutural para otimização da atividade. Quím. Nova. 21(1):99-105.

Cerqueira FM, Medeiros MHG, Augusto O (2007). Antioxidantes dietéticos: controvérsias e perspectivas. Quim. Nova 30(2):441-449.

Costa OA (1960). Estudo farmacognóstico do gervão. Rev. Braz. Farm. 41(11/12):615-650.

Degáspari CH, Waszczynskyj N (2004). Propriedades antioxidantes de compostos fenólicos. Visão Acad. 5(1):33-40.

Duarte MCT, Figueira GM, Pereira B, Magalhães PM, Delarmelina C (2004). Atividade antimicrobina de extratos hidroalcoólicos de espécies da coleção de plantas medicinais CPQBA/UNICAMP. Rev. Braz. Farmacognosia 14(1):6-8.

Falcão HS, Lima IO, Santos VL, Dantas HF, Diniz MFFM, Barbosa-Filho JM, Batista, LM (2005). Review of the plants with anti-inflammatory activity studied in Brazil. Rev. Braz. Farmacognosia 15:381-39.

Gobbo-Neto L, Lopes NP (2007). Plantas medicinais: fatores de influência no conteúdo de metabólitos secundários. Quím. Nova 30(2):374-371.

Ishitsuka K, Hideshima T, Hamasaki M, Raje N, Kumar S, Hideshima H (2005). Honokiol overcomes conventional drug resistance in human multiple myeloma by induction of caspase-dependent and -independent apoptosis. Blood 106:1794-1800.

Lopes SP (1977). Flora de Venezuela -Verbenaceae. Ed. Universidad de Los Andes, Venezuela.

Machado H (2008). Flavonóides e seu potencial terapêutico. Bol. Cent. Biol. Reprod. 27(1/2):33-39.

Mathias LA, Emily A (1993). Tapping and Amazonian plethora: four medicinal plants of Marajó Island, Pará-Brazil. J. Ethnopharmacol. 40:53-75.

Matos FJ (1997). Introdução à fitoquímica experimental. Fortaleza: Editora UFC. Florianópolis. Brazil. P 428.

Mensor LL, Menezes FS, Leitão GG, Reis AS, Santos TC, Coube CS, Leitão SG (2001). Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytother. Res. 15(2):127-130.

Mesia-Vela S, Souccar C, Lima-Landman MT, Lapa AJ (2004). Pharmacological study of Stachytarpheta cayennensis Vahl in rodents. Phytomedicine 11:616-624.

Moreira RCR, Costa JML, Saldanha AC, Silva AR (1998). Projeto Buriticupu Maranhão II. Plantas usadas como terapêutica da leishmaniose tegumentar americana na região de Buriticupu-Maranhão. Rev. Soc. Bras. Med. Trop. 31(1):126-134.

Moreira RCR, Rebêlo JMM, Gama MEA, Costa JML (2002). Nível de conhecimento sobre Leishmaniose Tegumentar Americana (LTA) e uso de terapias alternativas por populações de uma área endêmica da Amazônia do Maranhão, Brasil. Cad. Saude Publica 18:187-195.

Pichersky E, Gang DR (2000). Genetics and biochemistry of secondary metabolites in plants: an evolutionary perspective. Trends Plant Sci.

5(10):439-445.
Pio Correa M (1984). Dicionário das plantas úteis do Brasil. v. III. Rio de Janeiro: Impressa Nacional. P 646.
Roesler R (2007). Atividade antioxidante de frutas do cerrado. Rev. Ciênc. Tecnologia Alimentos 27(1):53-60.
Rosa EA, Silva BC, Silva FM, Tanaka CMA, Peralta RM, Oliveira CMA, Kato L, Ferreira HD, Silva CC (2010). Flavonóides e atividade antioxidante em Palicourea rigida, Rubiaceae. Rev. Braz. Farmacognosia 20(3):484-488.
Saldanha LL, Vilegas W, Dokkedal AL (2013). Characterization of Flavonoids and Phenolic Acids in Myrcia bella Cambess. Using FIA-ESI-IT-MSn and HPLC-PAD-ESI-IT-MS Combined with NMR. Molecules 18(3):8402-8416.
Salimena-Pires FR, Giulietti AM (1998). Flora da Serra do Cipó, Minas Gerais: Verbenaceae. Bol. Bot. Univ. São Paulo. 17:155-186.
Santos MH, Batista B, Duarte SMS, Abreu CMP, Gouvêa CMCP (2007). Influência no processamento e da torrefação sobre a atividade antioxidante do café (Coffea arabica). Quim. Nova. 30(3):604-610.
Schapoval EES, Winter DE, Vargas MR, Chaves CG, Raquel BJA, Zuanazzi ATH (1998). Anti-inflammatory and antinoceptive activities of extracts and isolated compounds from *Stachytarpheta cayennensis*. J. Ethnopharmacol. 60:53-59.
Soares SE (2002). Ácidos fenólicos como antioxidantes. Rev. Nutr. 15(1):71-81.
Sobrinho TJSP, Silva CHTP, Nascimento JE, Monteiro JM, Albuquerque UP, Amorim ELC (2008). Validação de metodologia espectrofotométrica para quantificação dos flavonoides de Bauhinia cheilantha (Bongard) Steudel. Rev. Farm. Bioquim Univ. Sao Paulo 44(4):683-689.
Sousa CMM, Silva HR, Vieira-Jr GM, Ayres MCC, Costa CLS, Araújo DS, Cavalcante LCD, Barros EDS, Araújo PBM, Brandão MS, Chaves MH (2007). Fenóis totais e atividade antioxidante de cinco plantas medicinais. Quím. Nova. 30(2):351-355.
Spagolla LC, Santos MM, Passos LML, Aguiar CL (2009). Extração alcoólica de fenólicos e flavonóides totais de mirtilo “Rabbiteye” (Vaccinium ashei) e sua atividade antioxidante. Rev. Ciênc. Farm. Básica Aplicada 30(2):187-191.
Tiwari P, Kumar B, Kaur M, Kaur G, Kaur H (2011). Phytochemical screening and extraction: A review. Int. Pharm. Sci. 1(1):98-106.
Troncoso NS (1979). Verbenaceae. In: Burkart A (Org.) Flora Ilustrada de Entre Rios, pt. 5. INTA, Buenos Aires. pp. 229-294.
Verdi LG, Brighente IMC, Pizolatti MG (2005). Gênero Baccharis (Asteraceae): Aspectos químicos, econômicos e biológicos. Quím. Nova 28(1):85-94.
Wilmsen PK, Spada DS, Salvador M (2005). Antioxidant activity of the flavonoid Hesperidin in chemical and biological systems. J. Agric. Food Chem. 53(12):4754-4761.


Journal of
Medicinal Plant Research
Related Journals Published by Academic Journals
African Journal of Pharmacy and Pharmacology
Journal of Dentistry and Oral Hygiene
International Journal of Nursing and Midwifery
Journal of Parasitology and Vector Biology
Journal of Pharmacognosy and Phytotherapy
Journal of Toxicology and Environmental Health Sciences
academicJournals