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Full Length Research Paper

Antioxidant activity and total phenolic contents of some date varieties from Saravan Region, Baluchistan, Iran

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In this study, fruits of six soft cultivars of date palm *(Phoenix dactylifera* L.) commonly grown in Saravan (Sistan and Baluchistan province) were analysed for their antioxidant activities using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) methods. As well, total phenolic contents were evaluated by Folin-Ciocalteu reagent. The antioxidant power of fruits was in range of 236.82 to 579.54 µg/ml for various cultivars in DPPH assay. The antioxidant activity of samples was between 9.34 and 24.64 mg/ml in FRAP method. The total phenolic contents ranged from 2.980 to 8.454 mg gallic acid equivalents (GAE) per g dry weight (dw). The highest total phenolic contents was found at Zardan, with mean value of 8.454 ± 0.52 mg (GAE)/g dw and antioxidant activity was 24.64 ± 4.31 (mg/g dw) and 236.82 ± 11.14 (µg/ml) in FRAP and DPPH respectively. Radical scavenging correlation analyses indicated that there was a significant relationship (r=-0.974 and r=0.975, P<0.01) between both antioxidant methods and the total phenolic contents of various cultivars. The results of the experiments showed that fruits of date palm have high phenolic contents and suitable antioxidant potential. The antioxidant activity may be linked to phenolic contents of these cultivars but complementary investigations are suggested for determining active elements.

Key words: Antioxidants, date palm, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), total phenolic content.

INTRODUCTION

One of the initial plants that have a long historical use with sustaining mankind life is date palm *(Phoenix dactylifera* L., Arecaceae) mainly distributed in the desert and semi-desert areas of the world (Bhat and Al-Daihan, 2012; Ghahraman, 1998; Hajian, 2005). In Iran, date palm has been cultivated in 13 provinces. Sistan and Baluchistan province, with 45988 hectares of dry and irrigated groves, has first position of date cultivation in the country (Jahad Agricultural Organizations Reports). In this province, Saravan has many date palm gardens. More than 70 cultivars of date palm exist in this region.

Date palm is used as a multi-purpose tree by native

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people of Saravan. It is used as food, animal feed, medicine, buildings material, fencing, different household articles, decoration and handcrafts, and therefore it is respected as a “life” tree among the Baluch tribes (Sadeghi and Kuhestani, 2014).

The fruit of this species is used as medicine for cough, blood purgation, anemia, and constipation. There are some reports of the enhancing effect of date fruit on haemagglutinating antibody titers, plaque-forming cell counts in spleen, and macrophage migration index as an index of cell-mediated immunity (Vayalil, 2002). An important carcinogenic agent are free radicals, inhibited by antioxidants. The significance of antioxidants has been increasing because of their high capability in scavenging free radicals associated to variety of many damaging diseases (Haider et al., 2013). Antioxidants are molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Antioxidants play an important role in providing protection to humans against infection and degenerative diseases (Sadeghi et al., 2014). Wide investigation has been done for identification of natural antioxidant that may be used for prevention of cancer. Some researchers have reported the antioxidant activity of date palm. Date fruits possessed a variety of enzymatic and non-enzymatic antioxidant compounds and have anti-mutagenic and anticancer activities with varying amount in different cultivars (Abbas et al., 2008; Biglari et al., 2008; Awad et al., 2011). *In vitro* studies have shown that the aqueous extract of date fruit is a powerful scavenger of hydroxyl radicals and superoxide and to restrain protein oxidation and iron-induced lipid per-oxidation in the rat brain homogenate in a concentration dependent manner (Mohamed and Al-Okbi, 2004; Allaith and Abdul, 2005; Al-Qarawi et al., 2008; Vayalil, 2002). In spite of high medicinal value of date palm, biological studies on the health benefits are inadequate in Iran. Antioxidant capacity of some date varieties from Bushehr and Abadan was investigated by Khanavi et al. (2009) and Siahpoosh et al. (2012), respectively. Moreover, Shams Ardekani determined the antioxidant activity and total phenolic compounds of 14 different varieties of date palm (*P. dactylifera* L., Arecaceae) seed extracts. It is essential that compositional and biological studies in plant foods be carried out to take into account various factors such as cultivars, seasons and pre- and post-harvest conditions that may affect the chemical composition of plant foods. In this study, the ethanol extracts of six native date varieties grown in Saravan were screened for their antioxidant activities and total phenol contents.

MATERIALS AND METHODS

Plant collection

province that has many palm gardens. Most of the plantations and groves of date palm in Saravan are concentrated in MokSokhteh (8000 hectares) of Jalgh district. About 56 cultivars have been identified in Saravan (Agricultural Jihad of Saravan, unpublished data), that their prominent are Mozafati, Rabbi, Zardan, Rengeno, Makili, Halile, Shendeshkan, Sabzo, Popo, Kalegi, Sohrok, Pimazoo, Khoshkij, Kroch, and Baranshahi (Sadegi and Kuhestani, 2014). Some of these cultivars has been shown in Figure 1 (Supplementary Figures). Six cultivars, including Zardan, Mozafati, Halile, Rabbi, Sohrok and Kalegi were kindly provided by Ms. Uosefi from Saravan date garden. Fresh and ripe fruits, without any physical and insect damage and fungal infections, were selected and used for all experiments.

Antioxidant evaluation

Preparation of extracts

The fruits were washed and air-dried. Then, they were further dried at about 50°C for 4 h. After that Date fruits of each variety were separately milled in a heavy-duty grinder to pass 1 to 2 mm screens. A portion of dried date material (40 g) was extracted with ethanol using soaking for 48 h. After filtration and solvent evaporation, extracts were stored in sealed vials at 4°C until biological testing.

Determination of total phenolic contents

Total phenol content was determined by Folin Ciocalteu reagent. A dilute solution of methanolic extract (0.05:1 g/ml) or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (2.5 ml, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (2 ml, 5%). The mixture was allowed to stand for 30 min and the phenols were determined by colorimetry at 765 nm (Wojdylo et al., 2007). The total phenolic content was determined as mg of gallic acid equivalent using an equation obtained from the standard gallic acid calibration graph.

DPPH radical-scavenging activity

The free radical scavenging activity of the plant extracts was performed according to the method of Brand-Williams et al. (1995), 2, 2-diphenyl-1 picryl hydrazyl (DPPH) free radical (Thaipong et al., 2006). 2.0 ml of different extracts with 500, 1000, 2000µgr/ ml range of concentrations, was mixed with DPPH (0.1 mM), after 60 min standing the absorbance of the mixture was measured at 517 nm against methanol as the blank on a UV/visible light spectrophotometer (UNICO UV 2100). Triplicate measurements were made and the radical scavenging activity was calculated by the percentage of DPPH that was scavenged using the following formula:

Reduction (%) = [(AB – AA)/AB] × 100
where *A*B is the absorption of blank sample; *A*A is the absorption of tested extract solution.

All analyses were run in triplicates and standard deviation (SD) was calculated.

Ferric-reducing antioxidant power (FRAP) assay

The antioxidant capacity of plant extracts by iron reduction (FRAP assay) was done according to Benzie and Strain (1996) with some modifications. FRAP reagent was prepared right away before analysis by mixing 25 ml acetate buffer (300 mM), 2.5 ml, 2, 4, 6-tripyridyl-s-triazine (TPTZ) solution (10 mM), and 2.5 ml $FeCl_3.6H_2O$ solution (20 mM). Date extracts (1000 µg/ml) were prepared by different solvent. 200 µg/ml of exteracts reacted with 1.8 ml of the FRAP reagent and was incubated at 37°C for 30 min in the dark condition before being used. Then absorbance of the colored product [ferrous tripyridyltriazine complex] was determined at 595 nm against distilled water blank. $FeSO_4.7H_2O$ (100 to 1000 µM) was used for calibration. Ascorbic acid was used as positive control. Results are expressed as mM Fe^{2+}/mg sample (Wojdylo et al., 2007; Chaouche et al., 2013).

Statistical analysis

All measurements include antioxidant activities and total phenolic content were done in triplicates to test the reproducibility of them. SPSS 15.0 (statistical soft ware) was used for statistical analysis of results. Correlations among obtained data were calculated using Pearson's correlation coefficient (r). The values of $P<0.01$ were considered statistically significant. All results are presented as mean ± standard error.

RESULTS AND DISCUSSION

The level of phenolic compounds in ethanolic extracts measured according to the Folin-Ciocalteu method and antioxidant activities of date varieties is presented in Table 1. The total amount of phenolic contents varied significantly between 8.45 and 4.66 mg gallic acid/g dry weight.

As shown in Table 1, Zardan date exhibited the highest values of total phenolic contents, DPPH and FRAP, whilst Kalegi date exhibited the lowest values of these parameters. The maximum values of phenolic contents, DPPH and FRAP were 8.45, 236.82 (mg catechin equivalents (CEQ)/100 g dry weight) and 24.64, respectively and the minimum values were 0.85 (mg CEQ/100 g dry wei), 1.57 (mg CEQ/100 g dry wei), respectively.

Total phenolic contents and antioxidants activity of the six varieties decreased in the following order: Zardan > Mozafati > Halile > Rabbi > Sohrok > Kalegi. Table 2 shows the mean scores of some physical characteristics of date cultivars that were considered for antioxidant and total phenolic evaluation. Due to variety and growth conditions shape, color, size, weight and moisture contents vary (Haider et al., 2013). Significant differences between the results were likely due to genotopic and environmental differences (namely, fertility, harvesting and pest exposure).

The results of correlation analysis indicated a significant negative relationship between total phenolic contents and antioxidant activities measured by DPPH ($r= -0.974$, $P<0.01$) and positive association by FRAP ($r= 0.975$, $P<0.01$) methods. Visual representation of DPPH radical-scavenging activity and FRAP assay for differnnt varities of dates is as shown in Figure 2.

Antioxidants have various physicochemical properties and then may utilize different mechanisms in different assays and conditions. A number of methods and modifications have been proposed for antioxidant power measurement. Total antioxidant activity, metal chelation, radical scavenging (DPPH) effects and reducing power as well as destructive activities to active oxygen species such as the superoxide anion radical, hydroxyl radical, and hydrogen peroxide are mainly used for this aim (Aksoy et al., 2013). Owing to, a single method could not assay whole antioxidant activity, therefore application and combination of several tests recommended to provide a better description (Prior et al., 2005; Frankel and Mayer, 2000).

Currently, there has been no agreement on one preferred method over others in measuring antioxidant capacities of food materials (Wu et al., 2004). DPPH and FRAP are among the most popular spectrophotometric methods widely used in antioxidant activity assessment. In this study, both were used. Results show that Zardan and Mozafati cultivars had the highest total phenolic contents between other cultivars. These observed differences can be related to cultivar specific inherited characteristics or location conditions including growing condition and soil type, as well as climatic factors, amount of sunlight received, fertilizer practices, disease and pest exposure, processing, storage conditions and handling (Al-Turki et al., 2010).

Moreover, ranking of investigated cultivars for antioxidant power indicated that Zardan has the highest antioxidant effects among other varieties in two assessment methods. The ability of date extract to prevent the generation of free radicals through iron chelating was the major antioxidant mechanism responsible for the observed antioxidant activity as compared to DPPH method. In DPPH, ethanol extract showed less potency than the control butylated hydroxytoluene (BHT, IC_{50}=4.85 ppm), because it may be the DPPH scavenging ability of the extract attributed to its hydrogen donating ability (Ebrahimzadeh et al., 2008).

However, the chemical constituents present in the extract, which are responsible for antioxidant activity, need to be investigated. But it is obvious that the phenolic compounds in the extracts may be responsible for such activity. Various phenolic compounds (e.g. benzoic and cinnamic acid, coumarins, tannins, lignins, lignans, flavonoids, etc) possess a diverse range of beneficial biological functions, including antioxidant activity. Unique structure and high tendency of phenolic compounds for metal chelation and their redox properties allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers and causes antioxidant activity (Bhatt and Negi, 2012; Huda-Faujan et al., 2009; Huang et al., 2009; Morgan et al., 1997). The date fruit pulp is wealthy

in phytochemicals like sterols, phenolics, carotenoids, procyanidins, anthocyanins and flavonoids. The concentrations and ratio of these constituents depend on the stage of fruit picking, type of the fruit, location and soil conditions (Abdelhak et al., 2005; Allaith and Abdul,

Table 1. Some physical properties of six date cultivars grown in Saravan.

Cultivar	Average								
	Fruit Length (cm)	Fruit diameter (cm)	Fruit pulp weight(g)	Seed weight (g)	Total weight	Fruit moisture percentage	Color	Maturity	Other properties
Mozafati	4.04±0.23	2.76±0.07	16.22±2.20	1.26±0.09	17.50±2.25	38.89±3.51	Dark brown	September	The most famous cultivars that accounts for 10% of total Iranian date crop. Long shelf life
Rabbi	4.57±0.39	2.00±0.06	12.91±1.74	0.89±0.11	13.85±1.43	41.32±5.33	Dark brown	September	A cultivar favoured by many Baluchs for its sweetness level and very popular in Jalgh region of Saravan
Zardan	3.49±0.42	2.10± 0.08	8.47±0.74	1.06±0.18	9.54±0.72	45.26±4.96	yellow-brown	August	Medium-sweet date, precocious
Sohrok	3.70±0.61	2.15±0.04	9.70±0.35	0.85±0.12	10.57±0.40	45.49±5.42	Dark brown	September	-
Kalegi	3.01±0.72	2.18±0.09	7.86±0.6	1.00±0.15	8.95±0.51	44.89±3.65	bright brown	September	Tender skin, sweet flavor, small seed
Halile	3.13±0.26	2.54±0.08	12.71±2.68	0.71±0.86	13.46±2.66	41.39±2.98	yellow-brown	November	Serotinous cultivar

Table 2. Antioxidant activity as IC50 (µg/ml) for DPPH assay or Fe^{2+} (mg/g) for FRAP assay of total phenolic content of ethanol extracts of different date varieties.

Cultivar	IC50 (µg/ml) (DPPH)	Fe^{2+}mg/g dry weight (FRAP)	TPCmg gallic acid/g dry weight
Zardan	236.82 ± 11.14	24.64 ± 4.31	8.45 ± 0.52
Mozafati	272.83 ± 10.34	19.94 ± 3.70	8.11 ± 0.48
Halile	308.94 ± 12.46	14.76 ± 3.33	6.36 ± 0.33
Rabbi	426.61 ± 11.73	10.08 ± 4.12	4.66 ± 0.24
Sohrok	450.45 ± 12.23	9.34 ± 2.90	4.317 ± 0.75
Kalegi	579.54 ± 9.32	7.13 ± 1.23	2.980 ± 0.98

2008). There are some reports on the antioxidant activity and presence of polyphenolic compounds in date fruit varieties. Shams Ardekani et al. (2010), Khanavi et al. (2009) and Biglari et al. (2008) investigated antioxidant activity of some date varieties. They demonstrate the potential of Iranian dates as antioxidant functional food ingredients. Results of this work are in agreement with that reported by Khanavi (2009) who found that total phenol contents of dates were 308 and 276 mg/100 g in methanol and water extracts, respectively (Shams Ardekani et al., 2010; Khanavi et al., 2009; Biglari et al., 2008). Methods that used to measure antioxidant activity in this study, are based on electron capture mechanisms, therefore application of other assessment with hydrogen donating, radical scavenging mechanisms are recommended for more investigation.

Conclusion

Generally, various type of date palm have different physicochemical properties including antioxidant activity, dry matter and total phenolics contents. Total phenolics assay may be sufficient to evaluate antioxidant properties in date fruit. In the present study, ethanol extract of Zardan bears comparable phenolics group and antioxidant activity to the standard compounds. Free radicals are often generated as byproducts of biological reactions or from exogenous factors. The involvements of free radicals in the pathogenesis of a large number of diseases are well documented. A potent scavenger of free radicals may serve as a possible preventative intervention for the diseases (Ebrahimzadeh et al., 2008). The suitable antioxidant activity of date palm supports its possible use as a natural antioxidant in food industries and other pharmaceutical preparations. In summary, the present study demonstrates the potential of Iranian dates as antioxidant functional food ingredients. The crude ethanolic extract me-rits further experiments and thus identification of the metabolites responsible for such pharmacological

Figure 1. Some of prominent date palm cultivars of Saravan.

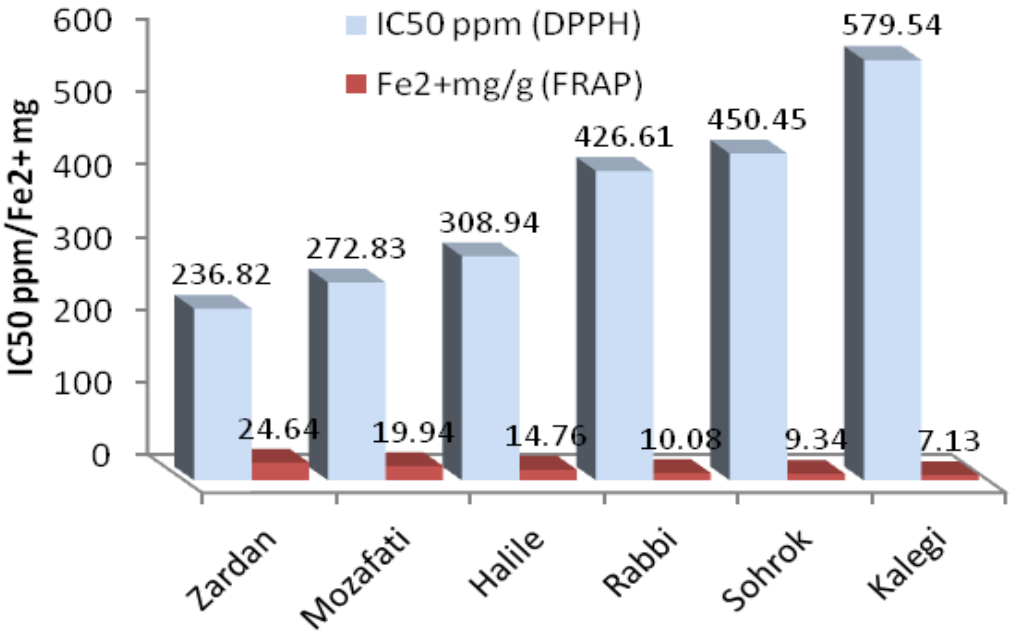

Figure 2. Concentration of diffrent extracts required to reduce the initial DPPH radical by 50%.

activities is suggested.

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Conflict of interest

The authors report no declarations of interest.

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Antimicrobial and antioxidant activities of *Blumea lanceolaria* (Roxb.)

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Medicinal plants are being used for the treatment of several ailments by the local tribes in Mizoram, North East India. The present study was focused to analyze the antibacterial and antioxidant activities of a widely used traditional medicinal plant, *Blumea lanceolaria.* Total phenol content was found to be high in the ethanolic extract of leaf (33.91 mg GAE/g DW) in comparison to root and stem of the plant. Reducing activity was measured by potassium ferricyanide reducing (FRAP) assay and found to be highest in the methanolic extract of root (39.29 mg GAE/g DW). Methanolic extracts of root showed highest reducing activity of superoxide dismutase (9.4 SOD units/ mg protein) and ascorbic acid oxidase (1.52 ascorbic acid oxidase units/mg proteins). A positive correlation was obtained between total phenol content and antioxidant activities of the extracts. The screening of the antibacterial activity of different extracts was conducted by using agar well diffusion test against three human bacterial pathogens. Ethanol extracts of stem, root and leaf showed promising results against *Staphylococcus aureus* with high (10 to 12 mm) inhibition zone as compared to standard antibiotics (2 to 10 mm). This study concludes that *B. lanceolaria* has high antioxidant and antibacterial activities and could be used as a potent plant in the treatment of a variety of ailments.

Key words: *Blumea lanceolaria*, ethno-medicine, antimicrobial activity, phenolic content, antioxidant activity.

INTRODUCTION

Several new antibiotics are being produced annually by pharmacological industries, whereas resistance to these drugs by micro-organisms has also increased gradually. The mortality rates in the hospitals are increasing due to new infection caused by multi-drug resistant bacterial strains (Cohen, 1992). Moreover, the use of synthetic drugs can also have serious side-effects and are ineffective for sustainable disease management (Sydney, 1980; Cunha, 2001). Hence, it is necessary to search the Replacement of synthetic antimicrobials with natural products that can inhibit the resistance mechanisms.

Plant extracts have great potential as antimicrobial compounds and are being used for the treatment of infections caused by resistant microbes (Silvia et al., 2013; Nascimento et al., 2011). About 80% of world population depends exclusively on plants for treating diseases (Khali et al. 2007; Vermani and Garg, 2002). Phenolic compounds improve the quality of plants by hindering oxidative degradation of lipids. Phenols comprise the largest group of plant secondary metabolites and have multiple

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biological effects, including antioxidant property. A correlation between the antioxidant capacity and phenolic content shows the importance of phenolic compounds (Vega-Galvez et al., 2007; Wojdylo et al., 2007). Medicinal herbs contain diverse classes of compounds such as polyphenols, tocophenols, alkaloids, tannins, carotenoids, terpenoids, etc.. Flavanoids and phenolic acids are particularly attractive as they are known to exhibit anticarcinogenic, antiviral, anti-inflammatory and antiallergic properties (Khali et al., 2007; Kraus et al., 1981). Antimicrobial compounds in plant extracts can lead to novel drug discovery against infectious diseases (Salwa et al., 2011).

Ethno-medicine has found a special place in lives of the people living in rural Mizoram. Due to the topography of the state, local people cannot avail all the modern medical facilities (Sharma et al., 2001). Thus, majority of the population is still following traditional methods of treatment to cure various diseases by using medicinal plants. *B. lanceolaria* (Roxb.) is a unique folkloric medicinal plant used by the native of Mizoram and is found in Thailand and Africa as well. A decoction of the leaves is taken orally to treat stomach ulcers, dysentery and wounds (Rai and Lalramnghinglova, 2010). More than 70 constituents have been isolated from the genus *Blumea*, including flavonoids, monoterpenes, sesquiterpenes, acetylenic thiophenes, triterpenoids, xanthenes, diterpenes, and essential oils. Blumealactones A, B, and C isolated from *B. balsamifera* exhibited antitumor activities against Yoshida sarcoma cells in tissue culture (Chen et al., 2009, Yasuo et al. 1988). Two acetylenic thiophenes, 63 and 64, isolated from *Begonia obliqua* showed antifungal activity against *Epidermophyton floccosum* and *Pleurotus ostreatus* (Chen et al., 2009; Ahmed and Alam, 1995). The essential oil of Blumea lanceolaria (Roxb.) Druce was analyzed by Dung et al., (1991) and methyl thymol (95%) was found to be its main constituent. As per the available literature, there is not much experimental evidence with regard to antimicrobial activities, total phenolic content, reducing activity and free radical scavenging activities on *B. lanceolaria*.

The present study was undertaken to evaluate the antimicrobial and antioxidant activities of different plants parts of *B. lanceolaria.* This study also assesses the correlation between *in-vitro* antioxidant activity of plant extracts and their total phenolic content.

MATERIALS AND METHODS

Collection and preparation of samples

B. lanceolaria (Roxb.) was collected from Dampa Tiger Reserve forest, Mamit district (23°42'N 92°26'E) of Mizoram in the month of June, 2014. The fresh leaf, stem and root samples were washed thoroughly 2 to 3 times with distilled water, cut into small pieces of 2 to 3 cm length using sterile blade and dried in hot air oven at 37°C for 72 to 96 h. The dried plant materials were ground to fine powder and stored in air tight dark bottles at room temperature. 10 g of each plant materials were extracted by mixing with distilled water (100 ml), 60% methanol (100 ml) and 95% ethanol (100 ml) in a waterbath at 40°C for 30 min. Extracts were filtered through Whatman No.1 paper filter and collected. Ethanol and methanol extracts were concentrated to dryness in a soxhlet apparatus at 60°C for 30 min and aqueous extracts were freeze dried. All extracts were stored at 4°C until further analysis (Wijeratne et al., 2006; Hatamnia et al., 2013).

Determination of antimicrobial activity

Strains and media

Three human pathogenic micro-organisms – *S. aureus* (MTCC 96), *Pseudomonas aeruginosa* (MTCC 2453) and *Escherichia coli* (MTCC 739) were collected from Microbial Type Culture Collection (MTCC), Chandigarh, India and used to test the antimicrobial activity of the plant extracts. All bacteria strains were grown in Nutrient broth at 37°C.

Antibacterial assay of known antibiotics

The test bacteria from the stock cultures were spread on the surface of the solidified Nutrient agar using sterile L spreader. After drying the agar surface, antibiotic discs of streptomycin (10 µg), tetracycline (30 µg), erythromycin (15 µg), chloramphenicol (30 µg), norfloxacin (10 µg) (Hi Media, Mumbai, India) were placed on the agar plate and incubated at 37°C for 24 h. Antibiotic sensitivity of each bacterial pathogen was evaluated by measuring the inhibition zone (mm) around each antibiotic disc.

Antimicrobial activity of plant extracts

Evaluation of the antibacterial activity of the crude ethanolic, methanolic and aqueous extracts of *B. lanceolaria* parts was determined by the agar well diffusion method of Silvia et al. (2013), Shoba et al., (2014). Inoculum of the bacterial strains (10^8 CFU/ml) was spread using sterile spreaders into 90 mm petri dishes with Mueller–Hinton agar. Wells of 6 mm were cut with help of sterile cork borer and filled with 50 µL of 30 mg/ml of solvent extracts. Empty wells were used as negative control. The Petri dishes were incubated for 3 h at room temperature for complete diffusion of the samples (Das et al., 2010; Moller, 1966). After initial incubation samples were incubated at 37 ± 1°C for 24 h. Antibacterial activity was evaluated by measuring the inhibition zone.

Estimation of total phenolic content

Preparation of standard solution

Gallic acid was used as the standard which represents the phenolic compounds in the plant extracts. Aliquots of 0.25, 0.5, 1.0, 1.5, 2.0 and 2.5 ml from the Gallic acid stock solution (100 mg/ml) were taken in 6 different 10 ml volumetric flask. To each flask, 2.5 ml of 1N Folin-Ciocalteu reagent and 2 ml of 20% sodium carbonate were added. The mixture was allowed to stand for 15 min and the volume was made up with water to get a concentration ranging from 2.5 to 25 µg/ml. The absorbance was measured at 760 nm against reagent blank. A standard calibration curve was prepared by plotting absorbance Vs concentration and it was found to be linear over this concentration range (Singleton and Rossi, 1965).

Preparation of plant extracts

100 µl of extract was taken in a sterile test tube to which 900 µl of double distilled water along with 500 µl of Folin Ciocalteau phenol reagent were added. The sample was shaken continuously and

Figure 1. Antibacterial activity of *B. lanceolaria* plant extracts against human pathogens.

incubated at room temperature for 10 min. Three ml of Sodium carbonate (20% W/V) was added to the sample, mixed and incubated for 10 min with addition of 10 ml of double distilled water. Similar procedure was carried out for the rest of different extracts with different temperature and time. The concentration of total phenol in the test sample was determined by extrapolation of the calibration curve (Singleton and Rossi, 1965).

Measurement of reducing activity

Ferric reducing activity of plant extracts was determined according to Li et al. (2008). 1 ml of different extract solution was mixed with 2.5 ml potassium buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. The reaction was stopped by adding 2.5 ml trichloroacetic acid. Further, 2.5 ml of distilled water and 0.5 ml 0.1% of ferric chloride were added and allowed to incubate for 30 min. at room temperature. Absorbance was measured at 593 nm. The absorbance obtained was expressed as gallic acid equivalent in mg per gm of dry weight (GAE/g) using gallic acid standard curve (Rao et al., 2013).

Estimation of enzymatic antioxidants

Assay of superoxide dismutase

0.5 ml of plant extracts were mixed separately with 1 ml of 125 mM sodium carbonate, 0.4 ml of 25 µM nitro blue tetrazolium (NBT) and 0.2 ml of 0.1 mM EDTA in a test tube. The reaction was initiated by the addition of 0.4 ml of 1 mM Hydroxylamine hydrochloride in the mixture. Absorbance was measured at 560 nm using spectrophotometer (Agilent Technologies, cary 60, USA) at 5 min. interval. SOD activity was expressed as units /mg protein and unit of SOD was expressed as amount of enzyme required for inhibiting the reduction of NBT by 50% (Beauchamp and Fedovich, 1976).

Assay of Ascorbic acid oxidase

0.1 ml of the plant extracts were added to 3 ml solution of ascorbic acid (8.8 mg in 300 ml phosphate buffer, pH 5.6). The change in absorbance was observed at 265 nm and measured at 30 s intervals for 5 min. One enzyme unit was expressed as to 0.01 OD change / minute/mg protein (Oberbacher and Vines, 1963).

Statistical analysis

All the experiments were performed statically in triplicate by using Microsoft Excel XP 2007 and expressed as mean ± SD (standard deviation). The correlation between the antioxidant capacities and total phenolic contents was analyzed using the simple linear regression, and the correlation coefficient (R^2) was calculated by using statistical package for the social sciences (SPSS) software version 16.0. ANOVA (analysis of variance, $P < 0.05$) was also performed using SPSS statistical software package version 16.0. Furthermore, the differences in means were contrasted using Duncan's new multiple range test following ANOVA.

RESULTS

Evaluation of antibacterial activity of plant extracts

The antimicrobial potential of the extracts of root, stem, leaves of *B. lanceolaria* was compared based on their zone of inhibition against the gram positive (*S. aureus*) and gram negative bacteria (*P. aeruginosa* and *E. coli*). Both ethanolic (12.1 mm ± 0.28) and methanolic (8.1 mm ± 0.28) extracts of root showed the highest antibacterial activity against *S. aureus*. Among gram negative bacteria, ethanolic extract showed more inhibition towards *E. coli* (8.1 mm ± 0.28). Ethanol and methanol extracts of leaf of *B. lanceolaria* showed the highest antibacterial activity against *S. aureus* strain. Highest zone of inhibition was achieved in case of ethanol extract (12 mm ± 0.00). Among gram negative bacteria, ethanolic extract of leaf showed more inhibition towards *P. aeruginosa* (7 mm ± 0.0) zone of inhibition. Methanolic extract of leaf too showed more zone of inhibition towards *P. aeruginosa* among gram negative bacteria (3.6 mm ± 0.28). Methanolic extract did not show any inhibition toward *E. coli* Figure 1. Ethanol and methanol extracts of stem showed the same results as obtained with extracts from leaf and root, showing highest antibacterial activity against *S. aureus*. Highest zone of inhibition was achieved

Table 1. Antibacterial activity of *B. lanceolaria* against human pathogen.

Test organism	Extracts(mm) ± SD									Antibiotics				
	Root			Leaf			Stem			N	T	E	C	S
	Met	Eth	DW	Met	Eth	DW	Met	Eth	DW					
S. aureus	8.1±0.28^{a}	12.1±0.8^{a}	0.0±0.0	6.0± 0.50^{a}	12.0±0.00^{a}	0.0±0.0	7.0±0.0^{a}	10.1±0.28^{a}	0.0±0.0	7.0±0.0^{a}	10.1±0.28^{a}	3.0±0.0^{a}	8.1±0.28^{a}	2.1± 0.28a
P. aeruginosa	4.0±0.0^{bc}	6.0±0.50^{c}	0.0±0.0	3.6±0.28^{bc}	7.0±0.0^{bc}	0.0±0.0	0.0±0.0^{d}	5.1±0.28^{bd}	0.0±0.0	4.0± 0.50^{bd}	10.1±0.28^{a}	3.1±0.28^{a}	5.1±0.28^{bd}	2.0±0.0^{a}
E. coli	4.0±0.0^{bc}	8.1±0.28^{d}	0.0±0.0	0.0± 0.0^{bd}	6.0±0.50^{bc}	0.0±0.0	6.0±0.5^{e}	9.0± 0.0^{ce}	0.0±0.0	5.1±0.28^{ce}	8.0±0.50^{b}	0.0±0.0^{b}	0.0±0.0^{ce}	2.0±0.5^{a}

The values are the average of three replicates and are expressed as mean ±S.D. Mean (±SD) followed by the same letter(s) in each column are not significantly different at P<0.05 using Duncan's new multiple range test. DW - Aqueous extract, Eth - Ethanol extracts, Met - Methanol extract, N- Norfloxcin, T- Tetracycline, E-Erythromycine, C- Chloramphenical, S- Streptomycin.

in case of ethanol (10.1 mm ± 0.28) and methanol (7 mm ± 0.0) extracts. Among gram negative bacteria, methanolic extract did not show any inhibition toward *P. aeruginosa*. Ethanolic extract showed inhibition towards *P. aeruginosa* with inhibition zone of 5.1 mm ± 0.28. Both ethanolic and methanolic extracts showed more inhibition towards *E. coli* (9 mm ± 0.00 and 6 ± 0.50 mm, respectively) when it comes to gram negative bacteria (Table 1). Aqueous extract from all plant parts did not show any inhibition zone.

Antibiotic susceptibility test

Bacterial pathogens were tested for their antibiotic sensitivity against five standard antibiotics. All pathogens were found to be sensitive particularly against tetracycline, showing highest zone of inhibition (*S. aureus* – 10 mm, *P. aeroginosa* – 10 mm and *E. coli* – 8 mm). Norfloxin and chloramphenicol were also found to inhibit most of the pathogens. *E. coli* alone was found to be resistant against chloramphenicol and erythromycin. Streptomycin showed inhibition against all pathogenic bacteria but also exhibit least inhibition zones (2 mm) for assessing susceptibility (Table 1).

Total phenolic content

The phenolic components were extracted from leaf, stem and root by three different solvents viz. water, methanol and ethanol. The total phenol concentration was expressed as gallic acid equivalent /g/dry wt. Phenolic contents ranged from 21.4 to 33.9 mg gallic acid equivalent/g/dry wt. Ethanol extracts of leaf showed the highest amount of phenolic content (33.91mg/ml/gw) while the lowest content was observed in aqueous extract of stem (21.4 mg/ml/gw) (Table 2).

Measurement of reducing activity

In vitro antioxidant activity of the plant extract is shown in (Table 2). Methanolic extract of root has the highest reducing activity of 39.29 mg equivalent to Gallic acid (GAE)/g while aqueous extract of stem has least reducing activity of 20.58 mg GAE/g DW.

Correlation between antioxidant capacity and total phenolic content

The correlation between antioxidant capacity and total phenolic content of extracts of *B. lanceolaria* is shown in Figure 2. There was a positive linear correlation between antioxidant capacity and total phenolic content with R^2=0.63. This indicate positive linear correlations between total phenolic compounds and antioxidant capacity supported by scatter diagram showing linear path diagonally from bottom left hand corner to the top right. Correlation was plotted by using SPSS 16.0 software.

Estimation of enzymatic antioxidants

Superoxide dismutase and ascorbic acid oxidase are important antioxidant defense in plants. Methanolic extract of root possessed highest SOD and ascorbic acid oxidase activity with 9.4 SOD units/ mg protein and 1.52 ascorbic acid oxidase units/mg proteins as compared to other extracts (Table 3). It is also evident from the tabulated results that ethanolic, methanolic and aqueous extracts of the roots have more SOD and ascorbic acid oxidase activity than other parts. The least activity was observed in aqueous extracts of all the plant parts used in the study.

DISCUSSION

Resistance of bacteria to antimicrobials has become a major concern to clinical and public health sectors. New strategies for antibiotic discovery or new alternatives to control bacterial infection by evading fast become a major concern to clinical and public health sectors. New strategies for antibiotic discovery or new alternatives to control bacterial infection by evading fast evolving resistance

Table 2. *In vitro* antioxidant activity and total phenolic content of *B. lanceolaria* plant extracts.

Solvent	Plant parts	Total phenol Content (mg GAE/g DW)	In vitro antioxidant activity - FRAP method (mg GAE/g DW)
Methanol	Stem	23.18±0.32^{a}	29.00±0.02^{a}
	Root	31.40±0.14^{bc}	39.29±0.05^{bc}
	Leaf	29.2±0.18^{bde}	33.72±0.03^{bc}
Dist. water	Stem	21.40±0.14^{adfg}	20.58±0.05^{bde}
	Root	31.60±0.12^{bcehi}	37.72±0.02^{bcfg}
	Leaf	25.63±0.01^{adfgjk}	31.86±0.15^{adfhi}
Ethanol	Stem	23.96±0.12^{acfhjkm}	21.72±0.07^{bdehjk}
	Root	22.67±0.01^{adfhjlmo}	25.58±0.25^{bdfhjkm}
	Leaf	33.91±0.02^{bcfhilnp}	31.15±0.32^{adfhiln}

The values are the average of three replicates and are expressed as mean ±S.D.
Mean (±SD) followed by the same letter(s) in each column are not significantly different at P<0.05 using Duncan's new multiple range test.

resistance are highly desirable. Plants are known to have therapeutic values to treat various infections and diseases for centuries. Plants can produce complex mixtures of different compounds, some of which are being reported to have high antimicrobial activity against several important clinical pathogens (Vilas, 2013). The antibacterial activity of the tested ethanol and methanol extracts of different parts of *B. lanceolaria* was more effective against the growth of Gram-positive compared to the Gram-negative bacteria. Gram negative bacteria have a lipopolysaccharide outer membrane through which entry of molecules is achieved based on their size and shapes. Most of the compounds present in aqueous and methanolic extracts probably could not pass through the outer membrane to reach their site of action resulting in less inhibition (Fernandez-Agullo et al., 2013; Kavak et al., 2010).

There was significant antibacterial activity in ethanol extract of all the plant parts tested (Table 1). The zone of inhibition measured with ethanolic extract of root and leaf was highest against *S. aureus* which was more than any of the known antibiotics used in this study. However, tetracycline showed more inhibition compared to plant extracts against gram negative bacteria. Though, *B. lanceolaria* ethanol and methanol extracts were showing significant inhibition of gram negative bacteria as compared to few other antibiotics used in the present study. Plants with high phenol content are important for food industry as it can inhibit oxidative degradation of lipid and improve the quality and nutritional value of food (Kahkonen et al., 1999). The highest concentration of phenol was found in ethanolic extracts of root. The concentration of the phenolic compounds is controlled by different factors like environment, development stage, type of solvent used and the degree of polymerization of phenolic (Fratianni et al., 2007; Fernandez-Agullo et al., 2013). Phenolic compounds can stabilize and delocalize the unpaired electron, chelate metal ions, protect against microbial infections and act as a good source of antioxidant (Kahkonen et al., 1999; Boo et al., 2012). In vitro antioxidant activity of methanolic extracts of root *B. lanceoria* has highest reducing activity, suggesting that *B. lanceoria* extracts act as an electron donor which can react with free radicals to stop the radical chain reaction (Huda-Faujan et al., 2009).

Reactive oxygen species (ROS) can lead to many oxidative damages causing degenerative diseases such as atherosclerosis, heart diseases, aging and cancer (Finkel and Holbrook, 2008; Madhavi et al., 1996). Medicinal plants are considered to play a beneficial role in health sector by curing and preventing various ailments and diseases. The health promoting activities of antioxidants from plants can be attributed to reduce the potential effects of ROS. However, synthetic antioxidants such as butylhydroxyanisole and butylhydroxytoluene needs to be replaced with natural antioxidants as they also possess potential health risks (Oyaizu, 1986; Safer and Al-Nughamish, 1999; Li et al., 2008).

Therefore in today's scenario, medicinal plants as a resource of medicine has become more important where oxidative stress is found to be one of the major causes of health hazards (Padmaja et al., 2011). Enzymes such as superoxide dismutase catalyze the dismutation of superoxide (O^{2-}) into oxygen and hydrogen peroxide. Ascorbic acid oxidase is also an important enzyme often regarded responsible for the first line of defense against various oxidative stresses (Nicholas, 1996).

Conclusion

In the present study, methanolic extracts of root was

Table 3. Enzymatic antioxidant activity of the *B. lanceolaria* medicinal plant extracts.

Solvent	Plant parts	SOD units/ mg protein	Ascorbic acid oxidase units/ mg protein
Methanol	Stem	8.2 ± 0.60^{a}	1.05 ± 0.04^{a}
	Root	9.4 ± 0.67^{bc}	1.52 ± 0.09^{ab}
	Leaf	7.6 ± 0.56^{bde}	$0.091 \pm .01^{bcd}$
Dist. water	Stem	4.5 ± 0.21^{bdfg}	0.65 ± 0.05^{abef}
	Root	5.8 ± 0.32^{bdfgi}	0.92 ± 0.10^{abef}
	Leaf	3.3 ± 0.14^{bdfhjk}	0.88 ± 0.06^{abef}
Ethanol	Stem	7.3 ± 0.70^{adehjlm}	1.25 ± 0.14^{abef}
	Root	8.5 ± 0.62^{acfhjlmo}	1.26 ± 0.17^{abef}
	Leaf	6.2 ± 0.66^{bdfhilnp}	0.98 ± 0.08^{abef}

The values are the average of three replicates and are expressed as mean ±S.D. Mean (±SD) followed by the same letter(s) in each column are not significantly different at $P<0.05$ using Duncan's new multiple range test.

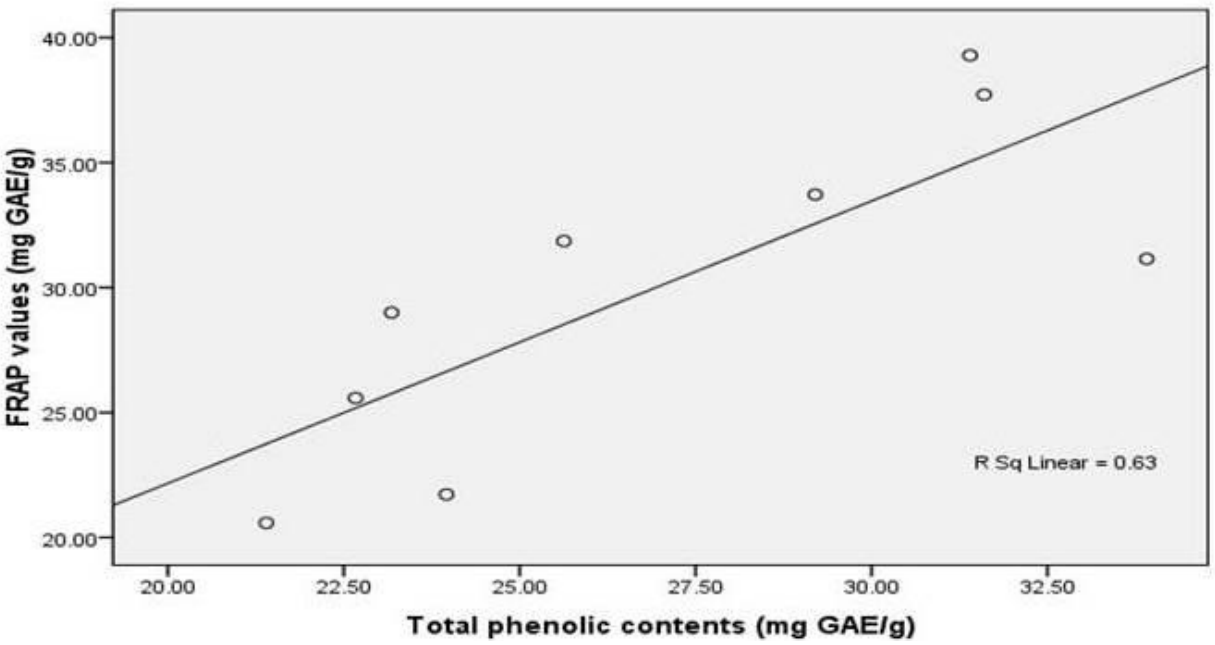

Figure 2. Correlation between FRAP values and total phenolic contents.
GAE: Gallic acid equivalents.

showing highest reducing, superoxide dismutase and ascorbic acid oxidase activities indicating that roots of *B. lanceolaria* are a rich source of antioxidants (Table 3). Phenolic compounds such as flavonoids, tannins and phenolic acids are known to contribute in antioxidant capacities of plants (Cai et al., 2004). The correlation coefficient (R^2) between FRAP values and total phenolic contents for the different parts of medicinal plant extracts was 0.63 (Figure 2). A positive linear correlation between the antioxidant capacities and the total phenolic content of *B. lanceolaria* extracts indicates that phenolic compounds are major contributors of antioxidant activities of the plant. This result was in concordance with previous studies (Cai et al., 2004; Li et al., 2013). The findings of the present study demonstrated the potential of *B. lanceolaria* extracts as rich source of antioxidant and significant antibacterial activity.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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Full Length Research Paper

Pharmacognostic analyses and evaluation of the *in vitro* antimicrobial activity of *Acmella oleracea* (L.) RK Jansen (Jambu) floral extract and fractions

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***Acmella oleracea* (L) RK Jansen is a typical plant species of Northern Brazil, used in gastronomy and in the Amazonian folk medicine of Para State as analgesic, to treat diseases of the mouth and throat. In industry, extracts of this genus have been used in oral hygiene products and in food compositions, as refreshing and flavoring agent, and is also used in cosmetics and toiletry. This paper reports the pharmacognostic characteristics of the herbal drug (flowers) and the *in vitro* antimicrobial effect of its ethanol extract (EEFAO) and fractions on pathogenic microorganisms present both in skin and in gastrointestinal tract of domestic animals. EEFAO Hexane, Chloroform, Ethyl Acetate and Methanol fractions at different concentrations (1000, 500, 250, 125, 62.5, 31.25, 15.62, 7.81 μg/mL) were tested against microorganisms (bacteria and fungus). The phytochemical characterization of *A. oleracea* extract and fractions indicates the presence of, probably, Spilanthol, detected by thin layer chromatography using Dragendorff Reagent. Chloroform fraction inhibited the growth of *Salmonella typhi* at a Minimum Inhibitory Concentration of 31.25 μg/mL and the microscopic analyses of young flowers demonstrates the presence of undifferentiated hypanthium and involucral bracts, cypselas and vascular bundles, structures also observed in other species of this genus. Quality parameters, including phytochemical description, reported in this work allow the identification and standardization of the flowers as herbal drug, whose microscopic description is very useful because it enables its micrographic characterization. The Chloroform fraction of EEFAO can inhibit the growth of *Salmonella typhi* making possible the use of *A. oleraceae* in phytomedicines or conservatives for foods.**

Key words: Antimicrobial, phytotherapy, flowers pharmacognosy, *Salmonella typhi*.

INTRODUCTION

Acmella oleracea (L) RK Jansen, vernacular Jambu, has motivated the interest of researchers because of its therapeutic potential, inducing projects in different areas linked to health sciences, such as medicine, dentistry

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and pharmacy. *A.oleracea* is a perennial, herbaceous flowering shrub common in Northern Brazil, particularly in Para State (Coutinho et al., 2006; Silva and Santos, 2011). Its chemical constitution described in the literature includes the alkamide spilanthol, α and β-amyrinester, stigmasterol, miricilic alcohol glycosides, sitosterol, saponins and triterpenes (Lemos, 2012).

Although, the entire plant is medicinally used, it is mainly in the flowers where the highest amount of Spilanthol (Wongsawatkul, 2008) occurs (Cavalcanti, 2008; Nigrinis et al., 1986), using guinea pigs, confirmed the main biological activity of this plant organ; the local anesthetic effect. The same authors also reported the flavoring, insecticide, bactericide and healing action of the floral extract, both in oral mucosa and on the skin. No scientific report about the microscopic description of *A. oleracea* flowers was found in the surveyed literature. This work reports the result of the pharmacognostic investigation on the *A. oleracea* floral drug and its Ethanol extract. In addition, the *in vitro* antimicrobial activity of EEFAO and its fractions on different bacteria and fungi species were reported.

MATERIALS AND METHODS

Plant materials

Young leaves and flowers of *A. oleracea* (L) RK Jansen were acquired in Marituba, Para State, metropolitan region of Belem (01°21' latitude South and 48°20 longitude West). A voucher is deposited at the Herbarium of the Botany Laboratory, Embrapa Amazonia Oriental, Belem - Para, registered as IAN 188444, in October, 2012.

Macroscopic and microscopic description

Macroscopic characterization of the flowers and leaves was performed with bare eye, according to parameters described by Esau and Silva Provide YEAR (Nigrinis et al., 1986; Esau , 1976). For microscopic description, semi-permanent cuts were prepared using fresh leaves and young flowers. The plant material was sliced transverse in longitudinally by hand. The samples were clarified in 10% aqueous Sodium Hypochlorite and stained with Methylene Blue and Safranin. The cuts were observed using a Nikon optical microscope (Eclipse 50i) equipped with a Motic® camera (Moticam 2300) and the pictures were processed using Motic Image Plus 2.0® software. The photomicrographs obtained at 10X and 40X were analyzed in comparison to literature data.

Preparation of extract

To obtain the Ethanol extract, 3.8 kg of cleaned *A. oleracea* flowers were dried under circulating hot air (Quimis Q317B) at 40°C ± 2°C until constant weight of an aliquot. The dried plant material was grinded in a Wiley knives mill using a sieve of medium mash. The obtained herbal drug weighed 426.1 g, which was macerated in 2.5 L 70% Ethanol in a stainless steel container for seven days. The obtained tincture was then filtered and concentrated under reduced pressure on a rotatory evaporator (800 Fisatom). The aqueous residue was frozen and lyophilized (Freeze dryer L101, LIOTOP). The freeze-dried extract was stored under refrigeration until use.

Pharmacognostic essays

The physic-chemical quality control of the plant drug and its extract EEFAO was performed according to the Brazilian Pharmacopeia, 5th edition, and the following tests were performed: particle size distribution, solids contents, pH, ash content and moisture content, respectively (Silva, 2008).

Fractionation

An aliquot of 4.26 g of EEFAO was fractionated by solid-liquid partition using 5 to 7x 50 ml aliquots of increasing polarity solvents: Hexane, Chloroform, Ethyl Acetate and Methanol. This procedure is established in the routine of Phytochemical Analysis Laboratory, Faculty of Pharmaceutical Sciences, Federal University of Pará. The fractions were concentrated on a rotatory evaporator at low pressure (Fisatom 800).

Chromatographic analyses

The chromatographic profile of the samples was obtained by thin layer chromatography **(**TLC) using silica gel (SIGMA) as stationary phase, mixture of solvents of different polarities as the mobile phase ding according to Wagner and Bladt (2001), to detect Flavonoids, Tannins, Terpene and Alkaloids (ANVISA 2010).

Evaluation of antimicrobial activity

Microorganisms and growth conditions

Bacteria: *Salmonella typhi* ATCC00259, *Enterobacterium faecalis* ATCC29212 and *Staphylococcus aureus* ATCC00577.

Yeast: *Candida albicans* ATCC0175. The Laboratory of Microbiological Control, Faculty of Pharmaceutical Sciences provided the samples of microorganisms. The bacteria grown in Mueller-Hinton broth (Himedia) at 37°C were kept in Mueller Hinton agar plates at 4°C. The yeasts were grown and maintained in broth and Sabouraud Agar (Himedia).

Antimicrobial activity

The tests were performed using broth micro dilutions techniques and MIC of EEFAO and its fractions were determinate as described by Holetz et al. (2012) with modifications. Aliquots of 100 µL of broths and 10 µL of microorganisms adjusted McFarland scale (10^8 Colony Forming Units) were used to evaluate the activity of EEFAO and its fractions at a concentration of 100µg/Ml (Wagner and Bladt, 2001). minimal inhibitory concentration (MIC) was defined as the lowest concentration of the sample, which produces a marked reduction of at least 80% of the tested microorganisms (Wagner and Bladt, 2001).

Statistical evaluation

The data statistical evaluation was performed using Bioestat 5.3, and T Student test with 95% level of confidence.

RESULTS

The macroscopic analysis of *A. oleracea* (L) RK Jansen

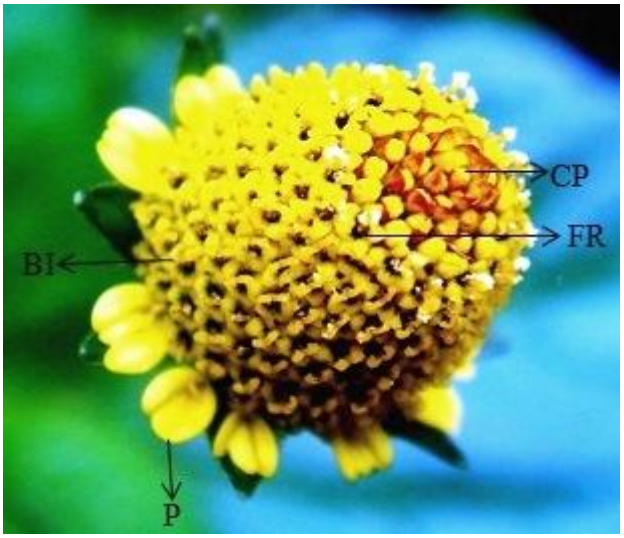

Figure 1. Digital photography of the floral chapter of *A. oleracea* with their P-petals, BI- involucral bracts, FR- Flores the Ray, CP-cispela structures.

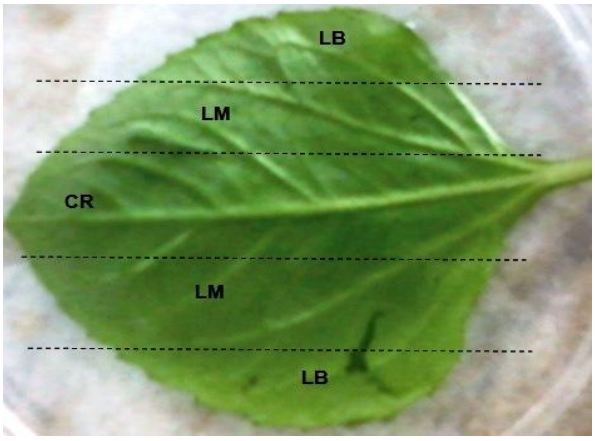

Figure 2. Digital photography of the macroscopic leaf of *A. oleracea* divided in regions of **CR**- Central Rib, **LM** - leaf mesophyll and **LB**- leaf blade.

leaves and fresh flowers was performed on the external surfaces of these organs (Figure 1). The microscopic analysis of *A. oleracea* leaves revealed simple membranous leaves showing wavy uniseriate epidermal cells, bicellular or tricellular trichome with basal cell showing rough cuticle and anomocytic stomata. The ventral mesophyll is well-organized showing two layers of palisade parenchyma and several layers of spongeous parenchyma. The cross-section of central rib shows a concave-convex profile and two vascular bundles are present in addition to the conducting vessels (Figure 2).

The presence of the undifferentiated hypanthium (HP) with various vascular bundles (FV) and differentiated laterally sepals (SP) can be microscopically observed in the longitudinal section of the immature flower buds. The central region of the hypanthium presents uniseriate epidermis, at its ends involucral bracts can be also observed in (BI), peleas (PL) and anthers (AT) (Figures 3 and 4). The pharmacognostic analyses show the following results: the granulometry of the *A. oleracea* herbal drug (dried and grinded flowers). The grinded dried flowers indicates that the sample is a coarse powder since its particles were predominantly retained on the sieve with the highest mash value (1.700 mm) reaching 99.95% of the sample weight (Table 1). The pH of the herbal drug was determined for decoction, after filtration and cooling in a calibrated potentiometer. The result, 5.33, is the middle value of three determinations with standard deviation of ± 0.24, using osmosed water as reference, pH of 6.25 (Table 1).

The determination of total ash present in the sample revealed as a middle value of three experiments 7.07%; standard deviation of ± 0.03% (Table 1). Completing the pharmacognostic analyses, moisture was determined in triplicate yielding, 11.6% standard deviation ± 0.264%. In addition, the middle value of dried residue of the extract is 2.5%; SD ±0.05 (Table 1). All the parameters were determined in triplicate. Chromatographic analyses of EEFAO and its Hexane, Chloroform and Ethyl Acetate fractions where performed using TLC on normal phase silica gel with Hexane/Acetone (80:20) as eluent. The obtained chromatograms, show orange colored bands due to reaction with Dragendorff's reagent, at Rf value of 0,37. The antimicrobial test using EEFAO and its fractions resulted in significant reduction ($p \leq 0.05$) of the colonies number of *Salmonella typhi* ATCC 00259 (Table 2); especially for the chloroform fraction, which inhibited the microorganism growth at a MIC of 31.25 µg/mL of the *Salmonella typhi* ATCC 00259.

DISCUSSION

As described in the literature, genus Acmella is part of the Asteraceae family, consisting of Angiosperm plants (Esau, 1976). The macroscopic description of leaves of this plant species discloses phylliform or membranous aspect of the largely oval leaves, being about 3 to 6 cm long and dark green in color (Coutinho et al., 2006; Silva and Santos, 2011). Mature capitula were macroscopically described as oval, irradiated or discoid; with conical receptacle, golden or pink to reddish paleas and obtuse to acuminate apex; ray flowers when present, are ligulate, pistillate; corolla can be white yellow or orange, with two or three lacinios at the apex. The disc flowers are perfect, tubular, white, yellow or orange corolla, four or five acute lacinia and four to five anthers. Oval or ellipsoid cypselae disc is laterally compressed, sometimes with cortical margin present when mature; pappus absent or 1 to 10 weak bristles, tricosted, ellipsoid cypselae ray are usually

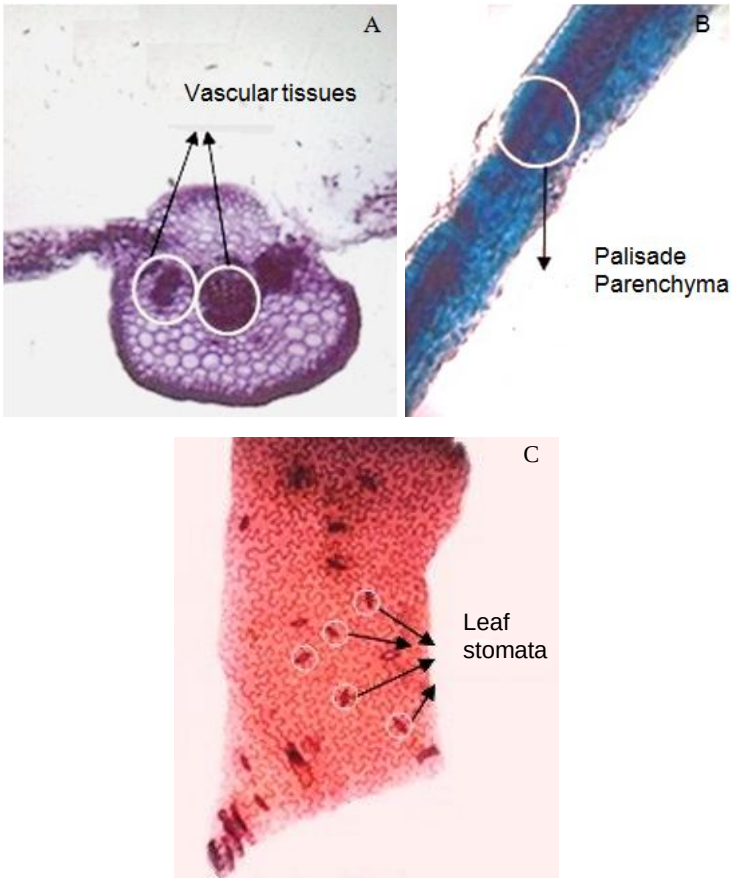

Figure 3. A, B and C - Photomicrographs of structures found leaves *A. oleracea* A - demonstrates the structures where the midrib with vascular tissues. B- Demonstrates the mesophyll with two layers of palisade parenchyma and C demonstrates the abaxial surface (bottom) with bay leaf stomata 40×.

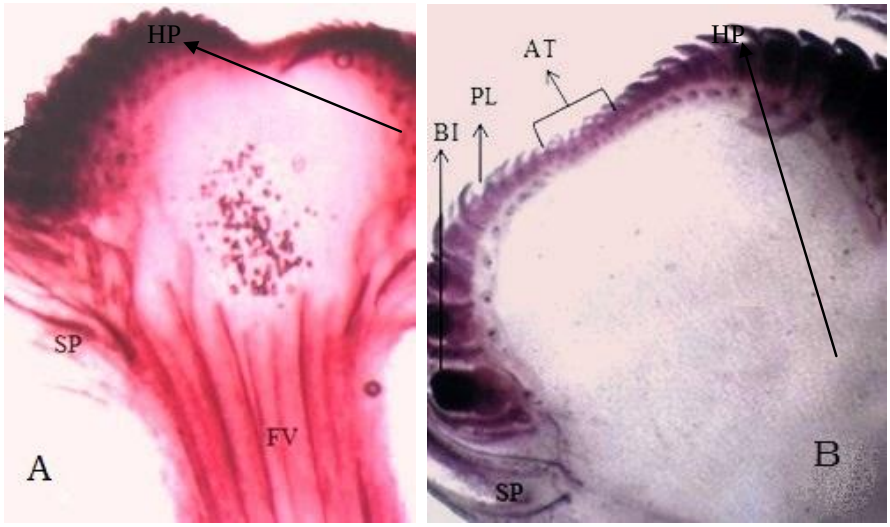

Figure 4. A and B - Photomicrographs of *A. oleracea* flowers: A: highlights the common structures to flowers of the same family with SP-sepals; HP-hypanthium; FV-vascular bundles; and B: BI-Involucral bracts; PL-Paleas and AT- anthers.
Safranin and Methylene Blue 40×.

Table 1. Results of the pharmacognostic tests of herbal drug and extract of *A.oleracea*.

Sample	G±sd	pH±sd	TA± sd	LD ± sd	DR± sd
A. oleracea	99.95%±0,01	5,33±0,24	7,07%±0,03	11,6%± 0,264	2,5%± 0,05

G-Granulometry; TA-Total Ash; LD-Loss on drying; DR- Dried residual; sd- standard deviation p≤0,05.

Table 2. Inhibitory activity of chloroform fraction of EEFAO on *Salmonella typhi* ATCC00259.

Fraction	Microorganism	MIC* (µg/ml)
Chloroform fraction	*Staphylococcus aureus* ATCC 00577	1000
	Enterobacterium faecalis ATCC 29212	1000
	Salmonella typhi ATCC 00259	31.25
	Candida albicans ATCC 0175	1000

MIC*- Minimum Inhibitory Concentration.

usually present in mature cortical margin. Similar structures were found and described in Figure 1 (Coutinho et al., 2006).

No data about the microscopic structure of the flowers of this plant species was found in the literature. However, Flowers of Flowering Plants have similar microscopical structures like hypanthium, which is the region of the flower that receives seeds after differentiation (Figures 3 and 4). Comparing the microscopic structures of the flowers of this plant, species having similar structures were observed in *Acmella brasiliensis* and *Acmella marajoensis* (Holletz et al., 2002; Coutinho et al., 2006), it is possible to infer that *A. oleraceae* also has differentiated hypanthium for seeds accumulation, as well large caliber vascular bundles another to ensure the maintenance of these reproductive structures during prolonged periods.

The analyses of EEFAO show that the results here reported are in accordance with the parameters described in the Brazilian Pharmacopoeia 5th edition (Silva, 2008). In Brazil, the National Agency for Sanitary Surveillance (ANVISA) requires pharmacognostic tests for quality control, such as chromatographic profile by TLC or phytochemical screening, as criterion for notification or registration of traditional herbal medicines and notification of herbal product (Baccarin et al., 2009). This paper reports the TLC analysis, based on the methodology described by Wagner and Bladt (2010) (ANVISA, 2010), highlighting the substance at Rf 0.37, which reacts positively to Dragendorff's reagent. Armond (2007) performed a phytochemical investigation of *A. oleraceae* using the same phytochemical methodology, the author reports about a substance with Rf 0.36, in the same chromatographic condition (ANVISA, 2010), which reacts as Flavonoid with Sulfuric Vanillin and fail to react with Iodine and KOH like some Alkaloids he tested. In the present work the substance with Rf 0.36 reacts to Dragendorff's reagent, as usual for substances containing containing nitrogen, like alkamides. This observation raises the following question, can alkamides react both as N-containing substance with Dragendorff's reagent or as bident structure common in Flavonoids allowing the complexation of a metal ion? (Marques et al., 2012). In fact, the reaction of alkamides with Sulfuric Vanillin may involve a nucleophilic attack of Nitrogen atom on the Aldehyde Carbonyl group of Vanillin.

Among the antimicrobial tests against *Salmonella, typhi* showed the best result. This genus of bacteria is usually found in the gastrointestinal tract of domestic and wild animals, especially birds and reptiles. Numerous Salmonella serotypes are pathogenic for both animals to humans (Anvisa, 2014; Armond, 2007). It is estimated that 36% of dogs are asymptomatic carriers of this bacteria; In witch, the clinical signs of the disease vary depending on the number of infective organisms and the immune status of the animal as well others adverse factors such as intercurrent illnesses. Young animals or elderly one are the most susceptible to the bacteria, increasing the severity of the infection (Armond, 2007).

There is evidence that besides frames of severe enteritis, bacteria can also cause this kind of generalized skin lesions redness, blistering and crusting in immunocompromised patients (Anvisa, 2007). Considering that the antimicrobial susceptibility to plant extracts, the inhibitory action to be considered promising must show a minimum inhibitory concentrations less than or equal to 100 µg/mL. Samples with MIC extracts ranging from 100 to 500 µg/mL are considerate having moderate antimicrobial activity. However, MIC values greater than 1000 µg/mL characterize the samples microbiologically inactive (Wagner and Bladt, 2001). The crude extract of *A. oleraceae* does not show antimicrobial activity as reported by Holetz et al. (2002) (Wagner and Bladt, 2001).

This work reports the antimicrobial activity of the chloroform fraction prepared from the crude extract EEFAO. Prachayasittikul et al. (2009) (Carvalho et al.,

2003) demonstrated that the chloroform fraction of *A. oleracea* was able to inhibit the growth of *Saccharomyces cerevisiae* ATCC 2601 and *Streptococcus pyogenes* II in a MIC 256 μg/mL. In this study *Salmonella typhi* ATCC00259, had their growth inhibited by a MIC of 31.25 μg/mL of chloroform fraction of EEFAO.

Conclusion

Quality parameters (particle size, total ash content, pH, loss on drying and dried residue) described in this work allow the identification and standardization of the herbal drug, the extract obtained from the flowers of *A. oleracea* and its fractions, where the presence of a substance reactive to the Dragendorff' reagent can be detected, probably spilanthol.

The microscopic description of the flowers of this plant species is very useful because it enables the micro-graphic characterization of plant drug (Figures 3 and 4). No records of these data were found in the surveyed literature. The result of antimicrobial activity evaluation indicates that the chloroform fraction of EEFAO is able to reduce the visible growth of *Salmonella typhi*. This observation can justify the use of *A. oleraceae* in the development of therapeutic products or conservatives for foods.

Conflict of interest

All authors declare that they have no conflict of interest.

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Full Length Research Paper

Antitermitic activity of plant essential oils and their major constituentsagainst termite *Heterotermes sulcatus* (Isoptera: Rhinotermitidae)

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Termiticide potential of six plant derived essential oils and their thirteen major active chemical constituents were investigated for their termiticidal activities against the termites, *Heterotermes sulcatus* Mathews 1977 (Isoptera: Rhinotermitidae) using no-choice bioassay method. Responses varied with different plant essential oils and their major chemical constituents at a fixed oil concentration. Among the essential oils tested, strong termiticidal activity was observed with the essential oils of *Pittosporum undulatum*, *Lippia sidoides* and *Lippia gracilis*. Oil was obtained by hydrodistillation for 4 h. Each crude essential oil was subjected to separation over SiO_2 soaked with $AgNO_3$ (15%) column chromatography eluted with n-pentane, n-pentane: CH_2Cl_2 and CH_2Cl_2 . The obtained fractions were analyzed by gas chromatography/flame ionization detector (GC/FID). The major constituent's plant essential oils were identified by GC/MS. The constituents thus identified were tested individually for their termiticidal activities against *H. sulcatus.* Limonene, carvacrol and thymol resulted in 100% termite mortality, after relatively short-time of exposure. However, for the practical use of these components in the field, while agent termiticide, the safety of these compounds in humans and non-target organisms must still be evaluated.

Key words: Subterranean termites, terpenes, alternative control.

INTRODUCTION

By reason of their feeding habits and preferences for relatively undecayed living and dead plant material (Wood 1996), about 10% (Rouland-Lefèvre, 2011) of the 2,882 described species of termites (Constantino, 2014)

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have been reported as pests. They cause significant losses to annual and perennial crops including, palms coconuts, sugar cane, maize, sorghum rice, wheat, groundnuts and cotton (Rouland-Lefèvre, 2011). Among termite species, the most widespread genera in South America is the subterranean *Heterotermes*, being found both in natural and urban environments (Constantino, 2001). Of the six described species of *Heterotermes* in South America, all are pests (Constantino, 2002), because they have been found attacking various crops such as sugar cane (Pizano et al., 1986; Arrigoni et al., 1989), soybeans, groundnut, cotton, maize (Mill, 1992) and rice (Czepack et al., 1998). *H. tenuis* (Hagen) and *H. longiceps* (Snyder) are the most important due to their wide distribution (Constantino, 2002) and financial losses. *H. sulcatus* has been found inside buildings at several urban areas of João Pessoa municipality (Paraiba State), causing an estimated economic loss of US$ 2,101,330.00 (Vasconcellos et al., 2002). Recently, this species was found infesting small crops of cashew trees in Northeastern Brazil (personal observation) and probably causing production losses .

The control of the termite based in overuse of pesti-cides has caused several environmental problems and economic losses because of their high toxicity and low biodegradability (including contamination of soil, water, crops and humans) (Koul et al., 2008). Verma et al. (2009) listed several active ingredients that are used globally as termiticides, as follows: bifenthrin, chlorfenapyr, cypermethrin, fipronil, imidacloprid and permethrin. However, the cryptic lifestyle and the social organization of termites make it difficult to control these species. Thus, it is important to search for new alternative methods for controlling termite pests that are less harmful to the environment.

Essential oils of medicinal and aromatic plants have a wide variety of mixtures of natural organic compounds that are the mainline of defense of plants against herbivores and pathogens, among others functions (Bakkali et al., 2008; Koul et al., 2008). Because of the low molecular weight of their compound, essential oils are highly volatile and so are characterized by a low persistence in the environment (Isman, 2006; Isman et al., 2011). Therefore, products are expected to be environmentally safe, thus providing a good alternative to the use of conventional insecticides for the pests' control. The bioactive compounds of essential oils have demonstrated toxic, repellent and deterrent activities to insects (Aslan et al., 2004; Pavela, 2011), inclusive to some species of termites (Chauhan and Raina, 2006; Zhu et al., 2003). Among their many components, the monoterpenes have attractted the attention of researchers, due to their potential insecticide action (Mondele et al., 2010). Thus, the objective of our study was to evaluate the termiticidal activity of selected plant derived essential oils and their major active chemical components against subterranean termite *H. sulcatus* (Isoptera: Rhinotermitidae).

MATERIALS AND METHODS

Plant and extraction of essential oils

In the present study, essential oils were extracted from the following plants: *Schinus terebinthifolius, Pittosporum undulatum, Lippia sidoides, Lippia gracilis*, *Mentha arvensis* and *Croton cajucara.* All plants were collected in the Northeastern Region Cerrado Biome in Pernambuco State, Brazil, between 1th January and 19th July 2014. Fresh leaves (approximately 350 g) of each plant species were individually subjected to hydrodistillation in a Clevenger type apparatus for 4 h. After extraction using CH_2Cl_2, the essential oils were dried over anhydrous Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. Crude oils were maintained under -40°C until the chemical analysis.

Analysis of essential oils by Gas chromatography (GC)

Each crude essential oil was subjected to separation over SiO_2 soaked with $AgNO_3$ (15%) column chromatography eluted with n-pentane, n-pentane: CH_2Cl_2 and CH_2Cl_2. The obtained fractions were analyzed by gas chromatography/flame ionization detector (GC/FID) and that which displayed purity higher than >95% was analyzed by gas chromatography-mass spectrometry (GC-MS) aiming at the chemical identification. Identification of the each compound was performed by comparison of recorded mass spectra with those available in the system library. GC chromatograms were obtained on a Shimadzu GC-2010 gas chromatograph equipped with an FID-detector and an automatic injector (Shimadzu AOC-20i) using a RtX-5 (5% phenyl, 95% polydimethylsiloxane (Restek, Bellefonte, PA, USA, 30 m × 0.32 mm × 0.25 m film thickness) capillary column. These analyses were performed by injecting 1.0 µl of a 1.0 mg/ml solution of sample material in CH_2Cl_2 in a split mode (1:10) employing helium as the carrier gas (1 ml/min) under the following conditions: injector and detector temperatures of 220 and 250°C, respectively; oven programmed temperature from 40 to 240°C at 3°C/min, holding 5 min at 240°C. GC/MS analysis (70 V and an ion source temperature of 230°C) was conducted on a Shimadzu GC-17A chromatograph interfaced with a MS-QP-5050A mass spectrometer using helium as the carrier gas.

Termites

Subterranean termites, *H. sulcatus* were collected from corrugated cardboard traps of colonies localized in the city of Cristino Castro (PI, Brazil, S08°49'04'', 44°13'27''W). Traps were brought to the laboratory and the termites were transferred to plastic boxes containing moist soil. Only large workers (third instar) were used in

Table 1. Plant species and relative percentages and their principal volatile constituents identified in essential oils.

Plant	Family	Major compound(s) in percentage
Pittosporum undulatum	Pittosporaceae	(±)Limonene 80.8; biclyclogermacrene 5.3; β-pinene 2.8
Lippia gracilis	Verbenaceae	Carvacrol 45.6; o-Cymene 9.5; Y-Terpinene 8.9
Lippia sidoides	Verbenaceae	Thymol 78.5; p- cymene 8.6; (*E*)- caryophyllene 5.6
Mentha arvensis	Lamiaceae	(−)Menthol 40.53; (−)menthone 14.63; (+)menthofuran4.32
Croton cajucara	Euphorbiaceae	Linalool 12.79; α-guaiene 11.50; *epi-β*-santalene 8.70.
Schinus terebinthifolius	Anacardiaceae	β-pinene 30.59; α-Pinene, 28.9; β-phellandrene 6.59

the experiments. Termites were used within four days after field collection.

Termiticidal activity

The no-choice bioassay method of Kang et al. (1990) was employed to evaluate the anti-termitic activity of the plant essential oils and their major chemical constituents. Sample of 3.5 mg of the essential oils dissolved in 600 µl of ethanol were applied to 1 g filter paper samples (Whatman No. 3, 8.5 cm in diameter). A piece of filter paper treated with solvent only was used as a control. After the solvent was removed from the treated filter papers by air-drying at ambient temperature, 100 active termites (90 workers and 10 soldiers) above the third instar were put on each piece of filter paper in a Petri dish (9 cm in diameter × 1.5 cm in height). Drops of water were periodically sprinkled onto the sterilized sand in the dishes to maintain sufficient moisture for the termites. The experimental delineation used was randomized, with six repetitions for each sample. The mortality of termites was evaluated daily for 14 days.

Statistical analyses

Mortality data in termiticidal tests were analyzed using ANOVA. The means were compared by Student-Newman-Keuls test (SNK) with the help of the software SISVAR 4.6. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Chemical composition of essential oils

Essential oils are very complex natural mixtures which can contain about 20 to 60 components at quite different concentrations. They are characterized by two or three major components at fairly high concentrations (20 to 70%) compared to others components present in trace amounts. On the basis of comparisons to GC peak area patterns of authentic chemicals, 18 active plant chemical constituents were identified from the plant essential oils. These active major plant chemical constituents are represented in Table 1. The most abundant compound of *P. undulatum* oil was (+)limonene (80.8%). L. gracilis had carvacrol as the major component (45.6%) which was followed by o-Cymene (9.5%) and Y-Terpinene (8.9%). *L. sidoides* had thymol as the major component (78.5%). Menthol (40.53%) and menthone were detected as the main components in *Mentha piperita.* The major compounds of C*. cajucara* were linalool (12.79%) and α-guaiene (11.50%). β-pinene was the major active constituent identified from *S. terebinthifolius* (30.59%).

Anti-termitic activity of plant essential oils

All the tested essential oils by using the “no-choice” bioassay method at 3.5 mg/g concentration caused high mortality of *H. sulcatus* in the feeding tests, after relatively short-time exposure (14 days). As shown in Table 2, the most efficient essential oils were *P. undulatum*, *L. sidoides* and L. gracilis, and resulted in 100% of mortality in 10 days, indicating strongest toxicity against *H. sulcatus*. The essential oils of *M. piperita* and *S. terebinthifolius* resulted in 100% mortality in 14 days. Among the six essential oils, *C. cajucara* was unable to kill all termites at a dosage of 3.5 mg/g after 14 days and had the lowest toxic effect to termites.

Termiticidal activity of the essential oil constituents

In order to understand the relationship between the constituents of plant essential oils and antitermitic activity, 17 constituents of plant essential oils were tested for antitermitic activity against *H. sulcatus*. Limonene, Biclyclogermacrene, Carvacrol, o-Cymene, y-Terpinene, Thymol, p- cymene, (E)-Caryophyllene, (−) Menthol, (−)

Table 2. Termiticide-activity of six active plant essential oils against *Heterotermes sulcatus* at a 3.5 mg/g dosage for 14 days after treatment.

Essential oil	Termite mortality (%)			
	1 day	5 days	10 days	14 days
Pittosporum undulatum	5.3 ± 2.3^{c}	88.0 ± 5.0^{d}	100 ± 0.0^{d}	-
Origanum vulgare	4.7 ± 5.0^{c}	82.0 ± 5.2^{d}	100 ± 0.0^{d}	-
Lippia gracilis	4.0 ± 0.0^{c}	78 ± 6.1^{d}	100 ± 0.0^{d}	-
Mentha piperita	2.7 ± 2.3^{bc}	40.0 ± 4.0^{c}	73 ± 5.2^{c}	100 ± 0.0^{b}
Croton cajucara	2.7 ± 2.1^{b}	11.0 ± 2.3^{b}	50.3 ± 5.0^{b}	90.0 ± 5.0^{b}
Schinus terebinthifolius	3.3 ± 3.1^{b}	24.0 ± 5.0^{b}	59.5 ± 3.0^{b}	100 ± 0.0^{b}
Control	0.0 ± 0.0^{a}	1.3 ± 1.2^{a}	2.7 ± 1.2^{a}	4.7 ± 1.4^{a}

The means followed by the same letter in the same column do not differ by Student-Newman-Keuls (SNK test) at $P < 0.05$.

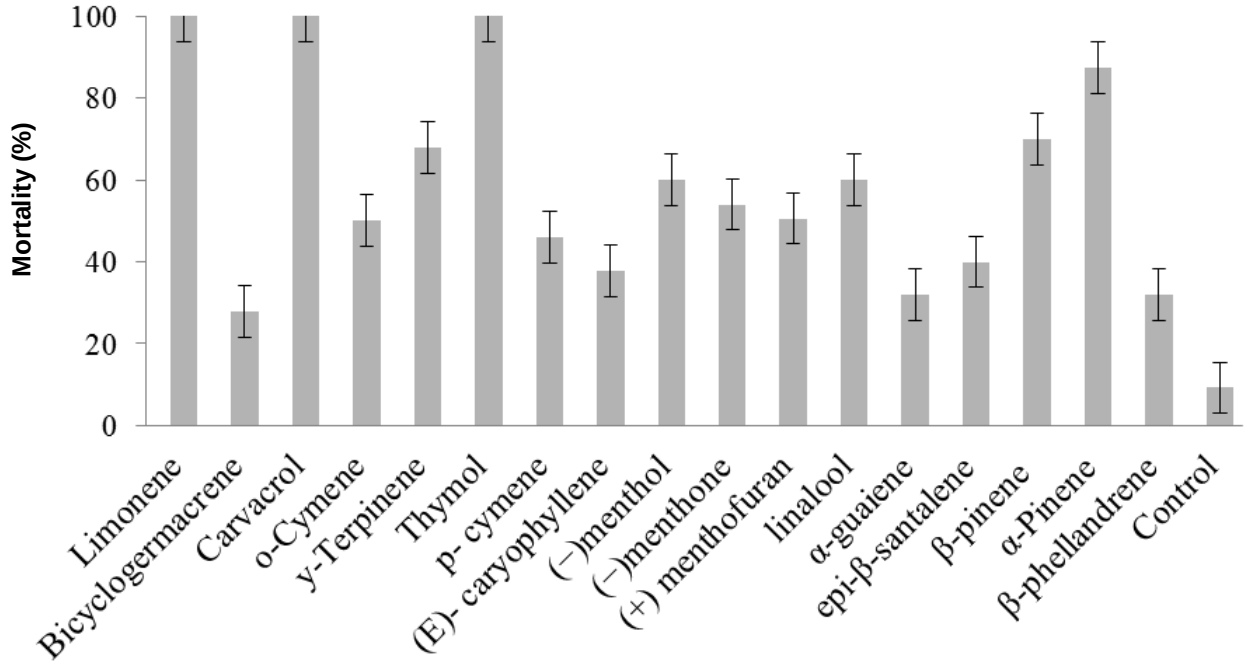

Figure 1. Termiticide-activity of seventeen active essential oil constituents against *Heterotermes sulcatus* at a 3.5 mg/g dosage for14 days after treatment.

Menthone, (+)Menthofuran, Linalool, α-Guaiene, epi-β-santalene, β-Pinene, α-Pinene, and β-Phellandrene at a concentration of 3.5 mg/g showed different percentage of mortality after 14 days. The results are summarized in Figure 1. Among these constituents, limonene, carvacrol and thymol resulted in 100% termite mortality.(−)α-pinene and β-pinene showed 84.5 and 70% termite mortality. The constituents biclyclogermacrene, (E)-caryophyllene, α-guaiene and β-phellandrene showed low termiticidal activity as compared to others tested at the same concentration, with antitermitic activity not higher than 40%.

DISCUSSION

Many phytochemical components are known by their antitermitic, antifeedant (Cärdenas-Marei et al., 2012), or repellent activities against a number of insect pests (Abdelgaleil, 2010; Zahran and Abdelgaleil, 2011). In the present study, all 6 essential oils tested showed antitermitic activity against *H. sulcatus*. *P. undulatum*, *L. sidoides* and L. gracilis exhibited higher toxicities to *H. sulcatus*. The major compounds of these oils (limonene, carvacrol, and thymol) also exhibited higher toxicities to *H. sulcatus* resulted in 100% of termite mortality. This can be explained by structure or mechanism of action of the compounds.

Limonene, carvacrol, and thymol showed symptoms of seizures as a way of toxicity for termites, indicating the presence of bioactive molecules present in plant secondary metabolites. Thus, probably the high mortality that both products caused térmites is related to their mechanism of action in the nervous system of insects. Although little is known regarding the physiological actions of essential oils on insects, various oils or their components cause symptoms that suggest a neurotoxic mode of action (Regnault-Roger et al., 1995; Choi et al., 2007; Ebadollahi et al., 2013). These symptoms include hyperactivity, seizures, and tremors followed by knock down. Such symptoms are very similar to those produced by the insecticides pyrethroids (Isman, 2006).

Among the essential oils components, limonene is known to have insecticidal, antibiotic and antifungal activity (Ibrahim et al*.,* 2001Regnault-Roger et al., 2012). Several studies have shown that monoterpenes of relatively simple structure, such as (±)-limonene causes insect mortality, by inhibiting the enzyme acetylcholinesterase (AchE) (Bruno et al., 1999; Viegas-Jr. et al., 2003). Limonene is a registered active ingredient in 15 pesticide products used as insecticides and insect repellent. Toxicological studies, in recent years, illustrated that carvacrol have very good acute toxicities on various invertebrate pest species including insects, acari, and nematodes (Lei et al., 2010). This compound acts as a positive allosteric modulator for insect binds to γ-aminobutyric acid (GABA) receptors, and can cause inhibitory effect on the insect nervous system (Tong and Coats, 2010). Pandey et al. (2012) also tested the antitermitic activity of seven essen-tial oils and their main components on *Odontotermes assamensis* Holmgren (Isoptera: Termitidae), and found that carvacrol caused 100% mortality of termites in concentration of 2.5 mg/g after 8 days. Carvacrol also proved antitermitic activity to nymphs of the termite *Reticuliter messperatus* Kolbe (Isoptera: Rhinotermitidae) in concentration of 1.5 mg/g after 48 h (Ahn et al*.*, 1998*;* Ebadollahi et al., 2010). Xie et al. (2014) tested the toxicities of forty-two monoterpenes, including eleven monoterpene hydrocarbons and thirty-one oxygenated monoterpenes against *Reticulitermes chinensis* Snyder (Isoptera: Rhinotermitidae) found that (+)-pulegone and carvacrol (ketones and phenols) had the highest toxicity.

Thymol also resulted in 100% termite mortality. This component has shown insecticidal activity against many organisms, such as *Culex quinquefasciatus* Say (Diptera: Culicidae) and *Musca domestica* L. (Diptera: Muscidae) (Pavela, 2011). According to Priestley et al. (2003), thymol binds to GABA receptors associated with chloride channels located on the membrane of postsynaptic neurons and disrupts the functioning of GABA synapses. Lima et al. (2013) also evaluated the biotoxicity of some plant essential oils against the termite *Nasutitermes corniger* (Isoptera: Termitidae) and found that the essential oils of *L. sidoides* containing 44.55% of thymol, proved to be one of the most potent over *N. corniger*, to kill all the termites with 48 h exposure dose 0.27 µg/mg. According to Hu and Coats (2008), thymol presents few risks to the ecosystem because of its rapid dissipation and low level of residues left in the environment.

In the present study, the compounds of monoterpenes hydrocarbons (limonene, α-pinene, β-pinene) and phenols (carvacrol and thymol) were more effective than alcohols (Linalool and menthol) to *H. sulcatus*. This may be explained in a structural as well as pharmacological manner. The termiticidal activity of phenols (thymol and carvacrol) was stronger than that of the monoterpenes alcohol group (linalool). These results are in agreement with those of Seo et al. (2009) who reported that the antitermitic activity of phenols was stronger than that of the alcohol group. Linalool compound was tested against subterranean termite *C. formosanus* in contact toxicity tests in a Petri dish, and caused 59.4% mortality of termites in 7 days in the concentration of 0.5% (Raina et al., 2012). The compound of sesquiterpenes hydrocarbon group (−)- α-pinene was more effective than (−)-β-pinene. These results agree with those of Cheng et al. (2004) who also reported the antitermitic activity of β-pinene was less stronger than α-pinene. This result indicated that the position of the double bond inside or outside of the six-member ring is also very important in the antitermitic activity (Xie et al., 2014).

In conclusion, the results of this study indicate that the essential oils of *P. undulatum, L. sidoides* and *L. gracilis* were the most toxic to *H. sulcatus.* Among the compounds limonene, carvacrol and thymol constitute a

promising alternative to harmful chemicals and persistence in the environment, which are commonly used in termite control. However, for the practical use of these components in the field, while agent termiticide, the safety L. gracilis of these compounds in humans and non-target organisms must still be evaluated.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Full Length Research Paper

Anti-inflammatory and immunoregulatory properties of fractions from *Sargentodoxa cuneata* ethanol extract

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Sargentodoxa cuneata **is widely used in the treatment of rheumatic arthritis in China. This study was conducted to investigate the anti-inflammatory and immunoregulatory activities of the ethanol extracts from *S. cuneata* and analyze the bioactive constituents. The ethyl acetate (ESCe), dichloromethane (ESCd) and aqueous (ESCa) fractions at a dose of 200 mg/kg significantly inhibited mice ear edema. Furthermore, the ethyl acetate (ESCe) and aqueous fractions (ESCa) inhibited rat paw edema, decreased the levels of Malondialdehyde (MDA) and biosynthesis of the prostaglandin (PGE_2), increased superoxide dismutase (SOD) activity, inhibited adjuvant arthritis (AA) rat paw edema, enhanced splenocyte proliferation and macrophage phagocytosis activity. ESCa at a dose of 50 mg/kg significantly suppressed IL-1β and TNF-α levels to 0.101 ng and 15.45 pg in each million macrophages. *S. cuneata* possessed anti-inflammatory and immunoregulatory activities, and phenolic compounds are the important bioactive constituents of this plant.**

Key words: *Sargentodoxa cuneata*, anti-inflammatory, edema, immunoregulatory, total phenolics.

INTRODUCTION

Some bioactive constituents from anti-inflammatory and anti-rheumatic Traditional Chinese Medicine (TCM), such as triptolide from *Tripterygium wilfordii*, and sinomenine from *Sinomenium acutum*, exhibited excellent immunosuppressive and anti-neurodegenerative effects based on inhibition of neuro-inflammation (Jiao et al., 2008; Shukla et al., 2011).

Recently, several anti-rheumatic herbs have become of great interest due to their potentials as possible anti-inflammatory and anti-neuroinflammative agents (Suk, 2012). *S. cuneata* (Family: Sargentodoxaceae, previously attributed to Lardizabalaceae), a plant endemic to China, has been widely used in TCM or ethnic medicine (called as *Hongteng*) for the treatment of rheumatic arthritis, acute appendicitis, amenorrhea and menstrual pains. The ethanol and water extracts could relieve inflammation,

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exhibit immunoregulatory and antioxidant effects, referred to as *ZhongHua BenCao* (1998). Previous phytochemical studies on *S. cuneata* revealed the presences of various kinds of constituents such as anthraquinones, lignans, flavonoids and phenolic compounds (Damu et al., 2003; Tang et al., 2012). But the systematic investigation on the bioactive components present in the plant have rarely been reported, therefore this present study was carried out to investigate the anti-inflammatory and immunoregulatory constituents from *S. cuneata*.

MATERIALS AND METHODS

Materials

Carrageenan was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Folin-Ciocalteu reagent was purchased from Merck Co. (Santa Ana, CA, USA). SOD, MDA and PGE_2 kits were obtained from Jiancheng Biotech (Nanjing, China). The plant was collected in Qin Mountain, Shanxi Province, China in September, 2009 and authenticated as *Sargentodoxa cuneata* Rehd. Et Wils. A voucher specimen (DXT0909) was deposited at the Herbarium of Pharmacognosy laboratory, School of Pharmacy, Jiangsu University.

Extraction

S. cuneata stem powder (80 g) was extracted with 80% ethanol under reflux for 2 h (800 mL ×2). The extract was evaporated, and further portioned successively with petroleum ether, dichloro-methane and ethyl acetate. The residual aqueous fraction was subjected to a macro resin column (4.0 cm×35 cm), eluting with distilled water (5-fold) and 95% ethanol (5-fold). The solvents were removed on a rotary evaporator or a lyophilizer to obtain petroleum ether fraction (ESCp, 0.11 g), dichloromethane fraction (ESCd, 0.77 g), ethyl acetate fraction (ESCe, 2.09 g) and aqueous fraction (ESCa, 4.77 g). All samples were stored at -20°C until further use.

Animals

Healthy adult male Sprague-Dawley (SD) rats (180 to 200 g) and Kunming mice (18 to 22 g) of either sex were used for the experiment. The animals were obtained from the animal center of Jiangsu University.

Acute toxicity in mice

Groups of 6 mice received orally doses of 0.5, 1.0 and 2.0 g/kg of ESCp, ESCd, ESCe and ESCa, respectively, and 1.0, 2.0 and 4.0 g/kg of ESC, while the control group received only 1% Tween 80 (25 mL/kg). The groups were observed for 48 h and at the end of this period LD_{50} values were calculated for each group (Franzotti et al., 2000).

Xylene-induced ear edema in mice

Kunming mice of either sex were randomly divided into 12 groups of eight animals per group. Groups 1 to 5 were treated orally with ESC, ESCp, ESCd, ESCe, ESCa at a dose of 100 mg/kg respectively, while groups 6 to 10 were treated with a dose of 200 mg/kg of ESC, ESCp, ESCd, ESCe, ESCa, respectively. Group 11 received the vehicle control (1% Tween 80, 25 mL/kg), while group 12 was treated with the standard drug indomethacin at 25 mg/kg orally. One hour after administration, edema was induced by applying 50 µL of xylene to the inner surface of the right ear. The mice were sacrificed 30 min after induction of edema by cervical luxation and yield discs (ID 6 mm) was taken from each ear. The anti-inflammatory activity was tested and expressed as percentage inhibition of ear edema of treated mice compared with controls (Yang et al., 2008).

Carrageenan-induced paw edema in rats

Male SD rats were randomly divided into six groups (six rats per group), group 1 was treated with the vehicle control (1% Tween 80, 10 mL/kg), group 2 positive control (indomethacin, 10 mg/kg), while groups 3-6 were treated orally with ESCe and ESCa at doses of 50 and 100 mg/kg, respectively (Yang et al., 2008). One hour after the oral administration of the extracts, 100 µL of 1% carrageenan was injected subcutaneously into the planar tissue of the right hind paws. Paw volumes were measured using a plethysmometer at 0, 1, 2, 3 and 4 h after administration. After 4 h, the animals were sacrificed and the right hind paws were dissected rinsed in ice-cold normal saline, and placed in 0.5% trichloroacetic acid at 4°C. The homogenates were centrifuged at 3000 rmp for 5 min. The supernatant was obtained and stored at -80°C for the MDA and PGE_2 assays. The blood and liver were likewise obtained from the animals; the serum was separated by centrifugation at 3000 rpm for 10 min and utilized for the estimation of MDA and SOD assays.

Determination of MDA and PGE_2 in paw

The lipid peroxidation product malondialdehyde (MDA) in the hind paw was estimated by determination of the level of thiobarbituric acid reactive substances (TBARS) at 532 nm, according to the reported method of Ohishi et al. (1985). MDA content was expressed as absorbance/g of tissue. PGE_2 was determined by ELISA according to the instructions in the manuals of the kit.

Determination of SOD and MDA in serum and liver

MDA level and SOD activity in serum and liver tissues were assayed according to the manufacturer's instructions in the assay kit.

Adjuvant arthritis induction

AA was induced by intradermal injection into the right hind paws with complete Freund's Adjuvant as previously reported by Nair et al. (2010). Male SD rats were divided into 7 groups (eight rats per group) as follows: group 1; normal control (1% Tween 80, 10 mg/kg), group 2; arthritis control, group 3; positive control (tripterygium glycosides, TG, 10 mg/kg), groups 4 to 7 were treated with ESCe and ESCa at doses of 25 and 50 mg/kg by gastric probe to AA rats from thirteenth day to twenty forth day. Paw volumes were measured with a plethysmometer at 0, 4, 8, 12 and 16 days after the administration.

Splenocyte viability assay by MTT method

Splenocytes were isolated aseptically from rats and suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum

(FBS). Splenocytes (1×10^5cells/well) was incubated with lipopolysaccharide (LPS) (6 mg/L) for 44 h at 37°C, 5% CO_2. After incubation, 50 µg/well of methylthiazol-2-yl-2, 5-diphenyl tetrazolium bromide (MTT) was added to the cell suspension for 4 h, and the resulting solution was centrifuged to remove the supernatant. The insoluble formazan was dissolved in 100 µl of Dimethylsulfoxide (DMSO) for 30 min. The optical density of the cultured wells was then measured using an ELISA reader at 570 nm. Results were expressed as ratio of optical density values between treatment and blank control cells.

Macrophage pinocytosis assay

Peritoneal macrophages was collected from rats peritoneal cavity, centrifuged, washed with D-Hanks (5% FBS), and suspended in RPMI 1640 with 10% FBS at 5×10^6 cells/ml. After attachment, the cells were seeded with RPMI 1640 (100 µl/well) and further incubated for 24 h at 37°C in a 5% CO_2 incubator. After incubation, 0.075% of neutral red dye was added (100 ml/well) and further incubated for another 30 min. The cells were washed with D-Hanks and lysed, and the optical density was read at 570 nm on a Bio-Rad microplate reader.

Determination of IL-1β and TNF-α in peritoneal macrophages

The macrophages (1 ml/well) with RPMI 1640 were incubated for 2 h at 37°C in a 5% CO_2 incubator. The cells were washed with D-Hanks solution to gain monolayer macrophages. The cells were incubated for another 48 h in 1 ml of RPMI 1640 including LPS (5 mg/L). The supernatant after centrifugation was evaluated for IL- 1β and TNF-α levels according to the ELISA manufacturer's instructions.

Determination of total phenolic content

The total phenolic contents were determined by the Folin-Ciocalteu colorimetric method (Beyhan et al., 2010). Folin-Ciocalteu reagent was used and a standard calibration curve: Y= 0.1174X+0.0139 (R^2=0.9996) was prepared using different concentrations of gallic acid. The measurement was carried out in triplicate and the results were expressed as mg gallic acid equivalents (GAE) per gram of extract (GAE/g).

Statistical analysis

Results were expressed as mean ± SD. Statistical differences between control and treated group were tested using a one-way analysis of variance (ANOVA). Following ANOVA analyses, LSD post hoc tests were used.

RESULTS

Acute toxicity evaluation and dosage determination

LD_{50} of ESC was above 4.0 g/kg in mice, compared to 12 g/kg of water extract injected intraperitoneally. LD_{50} values of ESCp, ESCd, ESCe, ESCa were 0.8, 1.0, 1.0 and 1.3 g/kg, respectively. A series of dosages under 200 mg/kg were chosen in the further activity evaluation.

Inhibition of xylene-induced ear edema in mice

As shown in Figure 1, all tested samples inhibited xylene induced ear edema in a dose dependent manner. ESCd, ESCe and ESCa significantly ($p < 0.05$, $p < 0.01$) inhibited the ear edema at a dosage of 200 mg/kg. The inhibition rate was found to be 50.3, 61.8 and 69.2% in ESCd, ESCe, ESCa, respectively, while the standard drug indomethacin showed 39% inhibition.

Inhibition of carrageenan-induced rat paw edema

Table 1 shows the effect of various extracts on carrageenan-induced rat paw edema. ESCe and ESCa significantly inhibited rat paw edema within the first 4 h of administration ($p < 0.05$, $p < 0.01$). However, ESCa had a higher suppression on edema than ESCe, with ESCa having the highest peak of 68.6% (100 mg/kg) observed at 2 h, similar to the maximum inhibitory effect displayed by indomethacin (69.2%) at 3 h.

Determination of PGE_2, MDA and SOD levels

As shown in Table 2, the level of PGE_2 in the hind paw was found to be significantly reduced by 26.4% in ESCe and 48.4% in ESCa treated models at the dosage of 50 mg/kg. MDA levels in hind paw, serum and liver, decreased dose-dependently and significantly ($p < 0.05$, $p < 0.01$) in ESCe and ESCa treated groups (50 and 100 mg/kg), while SOD levels were significantly increased in the serum and liver tissue of ESCe and ESCa treated groups.

Inhibition on AA rat paw edema

The edema of untreated AA model group as observed in Table 3 was increased from day 12 to day 24, and gradually decreased from day 24 to day 28. A similar pattern was observed in animals treated with ESCe and ESCa, however, there was a significant inhibition on paw edema of the treated groups when compared to that of the untreated model group (Table 3).

Splenocyte viability and macrophages pinocytosis activity

There was a significant increase in living splenocyte and the pinocytosis activity of macrophages in ESCe and ESCa treated group. The activity was found to be displayed in a dose-dependent manner, with the highest dose stimulating the maximum increase of living splenocyte and pinocytosis activity of macrophages (Figure 2). In general, ESCe and ESCa were more sensitive to the

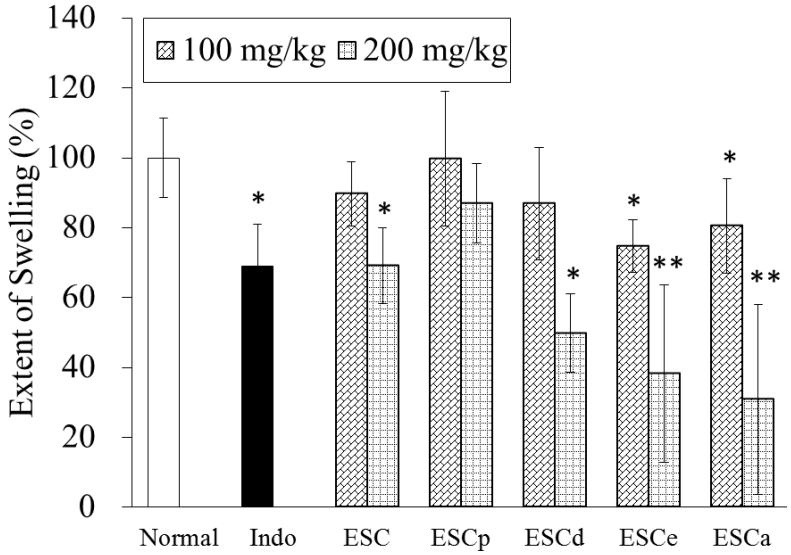

Figure 1. Inhibition of extracts of *S. cuneata* on xylene-induced ear edema in mice.
*p<0.05, **p <0.01.

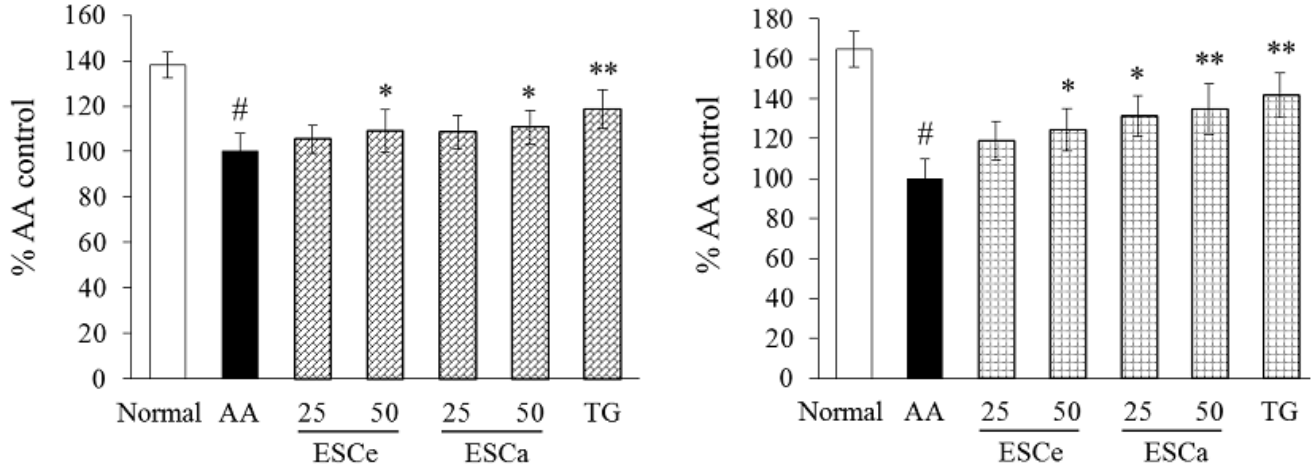

Figure 2. Effect on spleen lymphocytes viability (2A) and peritoneal macrophages pinocytosis (2B).
#p <0.05 vs normal group. *p<0.05, **p <0.01 vs. AA group.

the macrophage phagocytosis activity than splenocyte growth. ESCa (50 mg/kg) exhibited a 34.8% growth in enhancing macrophages pinocytosis ability, which is almost similar to the effect observed in the positive control treated group (41.7%).

IL-1β, TNF-α level of peritoneal macrophages

From the result (Figure 3), it was observed that ESCa at a dose of 50 mg/kg and the positive control (TG) exhibited significant inhibition of the IL-1β and TNF-α expression in macrophages ($p < 0.05$, $p < 0.01$). ESCa inhibited the IL-1β and TNF-α levels to 0.101 ng and 15.45 pg in each million macrophages when compared to higher levels of 0.225 ng/million macrophages (IL-1β) and 19.87 pg/million macrophages (TNF-α) observed in the untreated model group.

Analysis of bioactive constituents of *S. cuneata* extracts

In order to find the relationship between the activity and

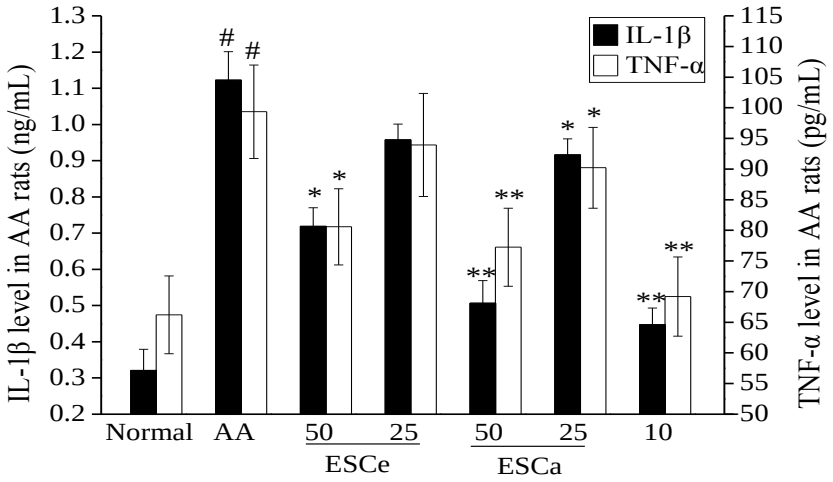

Figure 3. Effect on IL-1β (■) and TNF-α (□) levels in macrophage supernatants. $^{\#}p$ <0.05 vs normal group, *p<0.05, **p <0.01 vs. model group.

Table 1. Effects of ESCe and ESCa on the carrageenan-induced paw edema of rats.

Group	Dose (mg/kg)	Degree of swelling (mL) and Inhibition (%)			
		T_{1h}	T_{2h}	T_{3h}	T_{4h}
Control	-	0.812±0.265	1.427±0.370	0.995±0.382	0.905±0.350
Indo	10	0.437±0.125* (46.2)	0.262±0.067** (67.8)	0.250±0.066** (69.2)	0.257±0.047** (68.4)
ESCe	50	0.482±0.126 (40.7)	0.488±0.169* (39.9)	0.513±0.123* (36.8)	0.430±0.114* (47.0)
	100	0.488±0.092 (39.9)	0.555±0.169* (31.7)	0.722±0.233 (11.1)	0.663±0.138 (18.3)
ESCa -	50	0.403±0.152** (50.3)	0.412±0.075** (49.3)	0.352±0.188** (56.7)	0.37±0.093** (54.4)
	100	0.330±0.165** (59.4)	0.255±0.052** (68.6)	0.407±0.107** (49.9)	0.267±0.058** (45.6)

Values are expressed as mean±SEM (n=6). Indo: indomethacin. *p<0.05, **p<0.01 vs control (one way ANOVA followed by Dunnett's multiple comparison test).

Table 2. Effects of ESCe and ESCa on PGE_2, MDA and SOD levels in rats treated with carrageenan.

Group	Dose (mg/kg)	PGE_2	MDA			SOD	
		Hindpaw (ng/g)	Hindpaw (nmol/g)	Serum (nmol/mL)	Liver (nmol/mgprot)	Serum (U/mL)	Liver (U/mgprot)
Control	-	1.718±0.092	61.95±1.19	11.14±0.31	2.07±0.14	34.70±10.14	7.99±1.70
Indo	10	0.857±0.074**	32.81±7.74**	6.27±0.52**	0.94±0.10**	74.21±3.16**	15.44±0.11**
ESCe	50	1.265±0.087*	45.71±7.10**	9.70±0.15**	1.67±0.15*	47.66±2.26	12.03±0.20*
	100	2.376±0.090**	36.03±4.37**	9.40±0.60*	1.19±0.07**	62.29±2.20**	13.60±0.15**
ESCa -	50	0.887±0.072**	38.60±4.32**	9.45±0.60*	1.53±0.10**	51.84±1.31*	12.37±0.17*
	100	1.352±0.075**	31.70±7.40**	8.06±0.98**	1.12±0.06**	64.38±1.92**	14.09±0.15**

Values are expressed as mean±SEM (n=6). *p<0.05, **p<0.01 vs control. Indo: indomethacin.

Table 3. Effect of ESCe and ESCa on paw swelling of AA rats.

Group	Dose (mg/kg)	Degree of swelling (ml) and Inhibition (%)				
		12d	16d	20d	24d	28d
Normal	-	0.10±0.06	0.10±0.07	0.09±0.05	0.10±0.08	0.09±0.06
Model	-	0.50±0.08$^{\#}$	0.97±0.12$^{\#}$	1.25±0.08$^{\#}$	1.38±0.09$^{\#}$	0.96±0.12$^{\#}$
ESCe	25	0.51±0.04	0.92±0.13	1.19±0.10	1.26±0.08	0.81±0.05
-	50	0.49±0.07	0.88±0.09	1.15±0.11	1.21±0.14*	0.79±0.14*
ESCa	25	0.49±0.07	0.89±0.04	1.14±0.07	1.25±0.12	0.78±0.14
-	50	0.51±0.09	0.86±0.13	1.12±0.07	1.20±0.06*	0.72±0.06*
TG	10	0.52±0.07	0.79±0.11	0.87±0.06	0.94±0.06*	0.56±0.07*

Note: $^{\#}p$ <0.05 vs normal group. $^{*}p$<0.05 vs AA group.

total phenolic content of the fractions, Folin-Ciocalteu colorimetric method was used to determine total phenolic content. ESCa was found to contain the highest amount of total phenols (449.83 mg/kg), while ESCe, ESCd, ESC, and ESCp contain 346.76, 238.59, 242.84, and 63.69 mg /kg of total phenolic contents, respectively.

DISCUSSION

The results obtained in this study provided scientific basis for the traditional usage of *S. cuneata* as an anti-inflammatory agent, with ESCa and ESCe having high contents of phenols and showing markedly anti-inflammatory activity. This study preliminarily clarifies possible mechanisms mediating the anti-inflammatory action of *S. cuneata,* including the suppression of MDA and PGE_2 production, and SOD activity reinforcement in different tissues. Interestingly, after 3 h of administration, the edema observed in 100 mg/kg treated group was found to be more visible than that of the 50 mg/kg treated group, which correlated to the levels PGE_2 contents in hind paws (Table 1 and 2).

This implied that PGE_2 could be an important inflamematory biomarker induced during swelling. ESCa and ESCe exhibited significant suppression on MDA levels, which was probably due to the increase in SOD activities. These were probably the mechanisms responsible for anti-inflammatory activity of *S. cuneata*. The immunoregulatory activities were also evaluated for a comprehensive explanation of its traditional use. Splenocyte proliferation is an important indicator reflecting the organism's immune function, and macrophage cells could affect immune response and pro-inflammatory factor's release. ESCe and ESCa could enhance splenocyte proliferation and macrophage phagocytosis, yet inhibit the IL-1β and TNF-α expression, thus implying that ESCe and ESCa possesses good immunoregulatory activity. This partly confirms the basis for the traditional usage of *S. cuneata* in the treatment of rheumatic arthritis. The immunosuppressive positive control TG was found to enhance the splenocyte proliferation and macrophage phagocytosis, which was contrary to the previous report (Li et al., 2000). This disparities might owe to the lower dosage of 10 mg/kg used.

In other to ascertain the bioactive constituents responsible for the observed activity in *S. cuneata*, we analyzed the total phenolic content in each fraction. Our findings suggested that total phenolic content was positively correlated to the anti-inflammatory activity of each fraction, suggesting that the phenolic compounds could be the bioactive constituents present in the plant.

Conclusion

Further investigation will be carried out to isolate and elucidate the bioactive constituents of *S. cuneata* contributing to its remarkable anti-inflammatory and immunoregulatory activities, and to evaluate its potential as a possible anti-neurodegenerative agent based on inhibition of neuro-inflammation.

ACKNOWLEDGEMENTS

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Conflict of Interest

Authors have no conflict of interest.

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