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Phytochemical analysis of *Andrographis paniculata* and *Orthosiphon stamineus* leaf extracts for their antibacterial and antioxidant potential

Malahubban, M.[1,4], Alimon, A.R.[1,2*], Sazili, A.Q.[2], Fakurazi, S.[3] and Zakry, F.A.[5]
[1]Laboratory of Animal Production, Institute of Tropical Agriculture,
[2]Department of Animal Science, Faculty of Agriculture,
[3]Department of Human Anatomy, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[4]Department of Animal Science and Fishery,
[5]Department of Crop Science, Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia Bintulu Campus Sarawak, 97008 Bintulu, Sarawak, Malaysia
[*]Corresponding author email: ralimon@agri.upm.edu.my / masnindah@upm.edu.my
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Abstract. Leaves of *Andrographis paniculata* and *Orthosiphon stamineus* were extracted with water, ethanol, methanol and chloroform to assess their potential as antibacterial and antioxidant agents. High performance liquid chromatography analysis showed that the methanolic extracts of *A. paniculata* and *O. stamineus* leaves gave the highest amounts of andrographolide and rosmarinic acid, respectively. These leaf extracts exhibited antimicrobial and antioxidant activities and, at the highest concentration tested (200 mg/mL), showed greater inhibitory effects against the Gram positive bacteria *Bacillus cereus* and *Staphylococcus aureus* than 10 % acetic acid. *Andrographis paniculata* and *O. stamineus* methanolic and ethanolic leaf extracts also showed the strongest antioxidant activity as compared with the other extracts tested. The bioactive compounds present in these leaf extracts have the potential to be developed into natural antibacterial and antioxidant agents that may have applications in animal and human health.

INTRODUCTION

Since ancient times, medicinal plants have been commonly used to treat diseases and to maintain health. However, the medicinal attributes of such plants have not been well-documented scientifically. Nevertheless, local medicinal plants such as *Andrographis paniculata* and *Orthosiphon stamineus* are used traditionally for healing purposes, and have been commercialized recently. Several medicinal plants, including *A. paniculata* and *O. stamineus*, have been shown to exhibit antimicrobial and anti-oxidative characteristics due to the presence of various phytochemicals such as flavanoids, phenolic diterpenes, phenolic acids, tannins, carotenoids and tocopherols (Akowuah *et al.*, 2004; 2005; 2006; Ebrahimabadi *et al.*, 2010; Roy *et al.*, 2010; Edziri *et al.*, 2011; Zargar *et al.*, 2011). In recent years, there have been many reports on the antibacterial and antioxidant potential of several hundreds of native Malaysian medicinal plants, highlighting their potential to be used to treat specific diseases and promote good health (Dung *et al.*, 2008; Ebrahimabadi *et al.*, 2010; Edziri *et al.*, 2011). Medicinal plants have also been found to play an important role in the development of agro-chemicals to improve crop production (Adebowale & Adedire, 2006; Bajpai *et al.*, 2007; Acda, 2009). Moreover, the frequent use of synthetic and commercial antimicrobial drugs in treatments has led to drug resistance in animal, human and plant pathogens (Bajpai

et al., 2007). The excessive use of synthetic preservative agents and antibiotics such as chloramphenicol in meat products has led to human health concerns.

Lipid peroxidation in food lowers the nutritive value of food and causes the flavor and taste of food to deteriorate. Besides that, it raises health issues as it contributes to aging, heart disease, stroke, emphysema, mutagenesis and carcinogenesis (Barlow, 1990; Bera *et al.*, 2006). Medicinal plants showing antimicrobial and antioxidant activities may have the potential to act as safer substitutes because their molecular structures are different from those of microbes and chemical-based pharmaceuticals; therefore, their mode of action is likely to be different (Bera *et al.*, 2006). Moreover, it is necessary to suppress lipid peroxidation to ensure that the food is safe for consumption and its flavour maintained (Bera *et al.*, 2006).

Andrographis paniculata Nees (Acanthaceae), known in Malaysia as "Hempedu Bumi," is used traditionally as a remedy for fever. The aerial parts of the plant are commonly used to treat cold, hypertension, diabetes, cancer, malaria and snake bite (Perry, 1980). Andrographolides are active components found in the aerial part of this plant. Akowuah *et al.* (2006) successfully identified and quantified two major bioactive compounds in *A. paniculata*, namely andrographolide and 14-deoxy-11, 12-didehydroandrographolide. Andrographolide and 14-deoxy-11, 12-didehydroandrographolide have been characterized as bitter and colourless crystalline diterpene lactone, and as colourless needles, respectively.

Orthosiphon stamineus Benth (Lamiaceae) is used widely in Southeast Asia for the treatment of eruptive fever, epilepsy, gallstone, hepatitis, rheumatism, hypertension, syphilis and renal calculus. Known locally as "Misai Kucing" in Malaysia, the plant preparation is commercially available in the form of dried leaves. It is commonly taken as a beverage to improve health and for the treatment for bladder inflammation, gout and diabetes. *Orthosiphon stamineus* contains several chemically active constituents, including those belonging to the phenolic group, of which twenty have been identified. Among them are sinensetin, eupatorin and rosmarinic acid (Akowuah *et al.*, 2004).

Numerous studies on antioxidant properties of *A. paniculata* and *O. stamineus* have been conducted using different assay methods (Akowuah *et al.*, 2004; 2006; Premanath & Devi, 2011). Some reported variations in the findings may be ascribed to different environmental conditions and sample sourcing. In this study, the plants selected were morphologically similar although the chemical composition of the leaves might have been affected by soil fertility, age and genetic variation. The main objective of this study was to determine the antibacterial and antioxidant potential of *A. paniculata* and *O. stamineus* leaf extracts, and to quantify andrographolide and rosmarinic acid present in the respective species.

MATERIALS AND METHODS

Chemicals

HPLC grade methanol, acetonitrile (ACN) and acetone (Merck) were used. Andrographolide and rosmarinic acid (Chroma Dex, USA) were used as reference standards. Ammonical chloroform, chloroform, sulphuric acid (H_2SO_4), methanol, ethyl ether, hydrochloric acid (HCL), magnesium ribbon, ethanol, ferric chloride, acetic anhydride, acetonitrile, phosphoric acid, 0.5 Folline-iocalteu, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, nitroblue tetrazolium, nicotinamide adenine dinucleotide, tris-HCl buffer, phenazine methosulphate, nutrient broth (OXOID, U.K.), muller hinton agar (MHA) and tetracycline (OXOID, U.K.).

Plant materials

Fresh samples of cultivated *A. paniculata* and *O. stamineus* were obtained from the Herbal Farm in Universiti Putra Malaysia. Identities of the plant samples were authenticated by the Gene Bank Centre, Faculty of Agriculture, Universiti Putra Malaysia. The leaves of plants were collected

and oven-dried at 60ºC for 72 h. The dried plants were ground to powder using a Willey mill (Thomas® Willey cutting mill model 4), sieved through a 1 mm screen and stored at 4ºC until further use.

Sample preparation

Powdered *A. paniculata* and *O. stamineus* leaves (50 g) were added to 500 mL of water, methanol, ethanol or chloroform in a water bath (Memmert, Germany) at 40ºC for 8 h to allow liquid adsorption before transferring to an incubator shaker (Protech SI-50D) at 40ºC for another 16 h. The extracts were then filtered (Whatman No. 1) then centrifuged at 3500 (KUBOTA 5100, Japan) for 10 min. The supernatants were then filtered under vacuum through filter paper (Whatman No. 1). The filtrates were evaporated using a rotary evaporator (Buchi B-491) at 40ºC. The concentrated extracts were transferred to glass petri dishes and dried in a drying oven at 40ºC.

Qualitative screening of bioactive compounds

Alkaloids test

The test for alkaloids was carried out on 5 g ground plant material that had been extracted with 10 mL ammonical chloroform and 5 mL chloroform. After filtration, the supernatant was shaken with drops of 0.5 M sulphuric acid. The appearance of a creamy precipitate indicated the presence of alkaloid.

Saponin test

The test for saponin was carried out by adding 15 mL methanol to 5 g of the powdered plant extract. After evaporation, the residue was shaken vigorously with ethyl ether and 5 mL 2N HCL. The appearance of a precipitate indicated the presence of saponin.

Flavanoids test

The alcoholic extract (15 mL) corresponding to 3 g of plant material was treated with a few drops of concentrated HCL and 0.5 g magnesium ribbon. The appearance of a pink-red color indicated the presence of flavanoids.

Tannins test

One gram of the ground plant sample was boiled in 20 mL of 70 % ethanol for 2 min. The mixture was filtered and a portion of the filtrate was diluted with sterile distilled water in a ratio of 1:4. Three drops of 10% ferric chloride solution was then added. The appearance of a blue to black precipitate indicated the presence of tannins.

Steroids and terpenoids tests

Steroids and terpenoids were detected using the Liebermann-Burchard reaction. Plant material (200 mg) was boiled in chloroform before being filtered. The filtrate (2 mL) was added to 2 mL acetic anhydride and 50% concentrated H_2SO_4. A blue-green ring indicated the presence of steroids and red colour indicated the presence of terpenoids.

High Performance Liquid Chromatography (HPLC) analyses

Preparation of sample and standard solution

Andrographis paniculata and *O. stamineus* leaf extracts were analyzed by HPLC equipped with a photodiode array detector. Five mg of powdered extract was dissolved in 1 mL ethanol (for the ethanolic extract), or chloroform (for the chloroform extract), or methanol (for the methanolic extract), or distilled water (for the water extract), and passed through a syringe filter of 0.45 µm cut-off. Stock solutions of andrographolide and rosmarinic acid (1 mg/mL) were prepared in methanol for each standard. The two analytical standards were further diluted to a concentration of 120 ppm. A compound standard solution prepared by mixing 250 µL of each standard diluted solution was analyzed by HPLC for calibration and retention time reference.

The test extracts were identified by comparing the retention times and UV spectra with those of the standards. A series of dilution were made to obtain concentrations ranging from 10-120 ppm. Sample injections were made in triplicate. The amount of andrographolide and rosmarinic acid in the standardized extract was calculated based on the peak area of the

chromatogram of the extract with that of the calibration standard. Linear regression plots were obtained using the Waters Empower 2® software. The results were expressed as mg andrographolide or rosmarinic acid per gram of *A. paniculata* and *O. stamineus* powdered extract.

Chromatographic conditions

Qualitative and quantitative determinations of the major constituents of *A. paniculata* and *O. stamineus* leaf extracts were performed on a Waters Delta 600 HPLC System® with a Model 600 controller (Waters® MA, USA). The system was equipped with a 996 photodiode array detector connected to a computer running Waters Empower 2® software. An AC–18 column (Phenomenex, Luna 5 µm, 250 x 4.6mm, i.d) guarded by a C-18 security guard cartridge (4 x 3.0 mm, i.d), maintained at room temperature was used as the stationary phase. Andrographolide in samples and in the reference standard was separated by a mobile phase comprising solvent A: 50% water, solvent B: 20% acetonitrile, solvent C: 30% methanol. Standards and samples of rosmarinic acid were separated by a mobile phase comprising solvent A: 0.1% aqueous phosphoric acid and solvent B: acetonitrile. The initial conditions were 85% A and 15% B, with a linear gradient reaching 25% B at t = 12 min. This was maintained for 10 min after which the program returned to the initial solvent composition at t = 25 min, and continued thereafter for another 10 min. The flow rate was 1.0 ml/min and the injection volumes for samples and standards were 10 µL. Chromatographic peaks were detected at 230 nm for andrographolide and at 340 nm for rosmarinic acid.

Antibacterial potential of *Andrographis paniculata* and *Orthosiphon stamineus* leaf extracts prepared with different solvents

Test bacteria

Two Gram-positive bacteria, viz. *Bacillus cereus* and *Staphylococcus aureus*, and two Gram-negative bacteria, viz. *Escherichia coli* and *Salmonella enterica*, were obtained from the Microbial Culture Collection of Laboratory of Bacteriology of Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang, Selangor, Malaysia. The bacterial cultures were subcultured and purified from stocks before use.

Disc diffusion assay

The antibacterial potential of *A. paniculata* and *O. stamineus* extracts was assessed from their reaction against four bacterial cultures using the Kirby-Bauer disc diffusion method (Bauer *et al.*, 1966; Zaidan *et al.*, 2005). Colonies picked from fresh pure culture plates were suspended in 5 ml sterile nutrient broth (OXOID, U.K.) and adjusted to a turbidity of 0.5 McFarland standard. Standardized bacterial suspensions were aseptically swabbed on to the surface of sterile Mueller Hinton Agar (MHA) plates. Twenty-five µL of each plant extract (50 mg/mL, 100 mg/mL and 200 mg/mL) were aseptically transferred to 6 mm sterilized paper discs to give a final concentration of 1.25, 2.5, and 5 mg/disc, respectively. The tests were replicated three times.

The paper discs treated with herbal extracts were aseptically placed in petri plates containing Mueller-Hinton Agar (MHA) seeded with the respective test bacteria. The treated MHA plates were incubated in an upright position at 37ºC. The diameters of inhibition zones were measured after incubation for 24 h and the antibacterial properties were evaluated as follows:

Resistant : < 7 mm inhibition zone

Inhibition : Intermediate inhibition: 7-10 mm
Moderate inhibition: 11-15 mm
Strong inhibition: 16-20 mm

The test bacteria were also assayed for their sensitivity to the antibiotics (OXOID, U.K.) tetracycline (30 µg per disc), and 25 µL of 10 % acetic acid. Data were subjected for one-way analysis of variance (ANOVA) and when differences were found among treatments, Duncan's test was used to compare the mean differences using SPSS software (IBM SPSS version 21), with 0.05 % level of significance.

Antioxidant potential of *Andrographis paniculata* and *Orthosiphon stamineus* leaf extracts prepared with different solvents

According to Wong *et al.* (2006), the antioxidant activity of the plant extracts basically depends on the composition of the extracts, hydrophobic or hydrophilic nature of the antioxidants, type of solvent used for extraction process, method of extraction, temperature and conditions of the test systems. Therefore, it is necessary to use more than one method for evaluation of antioxidant activity of plant extracts to encompass various mechanisms of antioxidant actions. The present study employed three tests to determine antioxidant activity of *A. paniculata* and *O. stamineus* leaf extracts as follows:

Determination of total phenolic content

Total phenolic content was estimated using the Folin-Ciocalteu colorimetric method described previously (Lu *et al.*, 2011) with a slight modification. The sample extracts (0.2 mL) at appropriate dilutions were oxidized for 4 min with 1 mL of 0.5 M Folin-Ciocalteu reagent before the reaction was neutralized with 1 mL saturated sodium carbonate (75 g/L). After 2 h incubation at room temperature, the absorbance of the resulting blue colour was measured at 760 nm on a spectrophotometer (Spectronic® 20 Genesys™). Quantification was performed against a standard curve of gallic acid. Results were expressed as mg equivalent to gallic acid (GAE) per 100 g of dry weight.

Determination of free radical-scavenging activity

The antioxidant activity of each extract was measured using the stable radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) as a standard reagent. This was determined as described by Hatano *et al.* (1988) with minor modification. Briefly, stock solutions of the extract were prepared in ethanol. Ascorbic acid was taken as standard. Different concentrations of extract and a positive control of ascorbic acid in ethanol solution (1 mL) were added to 3 ml of 0.004% ethanolic DPPH free radical solution. An equal amount of ethanol was used as a blank. After incubation at room temperature for 30 min in the dark, the absorbance was measured at 517 nm using a UV spectrophotometer (Spectronic® 20 Genesys™). Scavenging activity (%) was calculated using the following formula:

$$\text{DPPH radical scavenging (\%)} = [(Ac - As)\ Ac] \times 100$$

Where Ac is the absorbance of the control and As is the absorbance of the test sample after 30 min.

Determination of superoxide radical scavenging activity

The superoxide scavenging ability of the test samples was assessed according to the method of Ahn *et al.* (2012) with slight modifications. The reaction mixture comprised nitroblue tetrazolium (0.1 mM) and nicotinamide adenine dinucleotide (0.1 mM) with or without the test sample in a total volume of 1 ml of Tris- HCL buffer (0.02 M, pH 8.3). The reaction was started by adding 10 µM phenazine methosulphate to the reaction mixture, and the change in the absorbance was recorded at 560 nm every 30 sec for 2 min. The percent inhibition was calculated against a control without the test sample. The results were compared with that of quercetin, with the percentage inhibition of superoxide anion generation calculated as:

$$\text{Scavenging (\%)} = [(Ac - As)\ Ac] \times 100$$

Where Ac is the absorbance of the control and As is the absorbance of the tested sample. Data on phenolic content, free radicle-scavenging activity and superoxide radical scavenging activity were analyzed for one-way analysis of variance (ANOVA) and Duncan's test was used to compare the mean differences using SPSS software (IBM SPSS version 21) at 0.05 % level of significance.

RESULTS AND DISCUSSION

Qualitative screening for bioactive compounds

Analyses on the methanolic extracts of *A. paniculata* and *O. stamineus* leaves indicated the presence of alkaloids, saponins, flavonoids, tannins, terpenoids and steroids (Table 1). This finding was consistent with several reports on the effectiveness of methanol in the extraction of bioactive compounds (Akowuah *et al.*, 2005; 2006; Zargar *et al.*, 2011). The chloroform extracts of both leaves were mainly poor in bioactive compound contents, except for alkaloids and flavonoids in the *O. stamineus* samples. Ethanolic extracts of *O. stamineus* leaves also showed the presence of all the bioactive compounds tested for (Table 1). The discrepancies in the levels of bioactive compounds in the two plant extracts could be related to their polarity differences (Akowuah *et al.*, 2005).

Analysis of andrographolide in *A. paniculata* leaf extracts by HPLC

Figure 1 shows the chromatograms obtained for HPLC analyses of the water, ethanol, methanol and chloroform extracts of *A. paniculata* leaves. The peak of andrographolide (AP) was identified by comparison of the retention time with that of the AP reference sample. AP quantification exhibited good linearity in the range from 10 to 120 ppm on the HPLC calibration curve ($y = 479942x - 175328$; $R^2 = 0.998$).

Andrographis paniculata leaves extracted with methanol gave the highest peak as compared to samples obtained with the other extraction solvents. Extraction with methanol thus yielded the highest amount of AP (73.40 mg/g), followed by ethanol (35.85 mg/g), water (26.62 mg/g) and chloroform (15.63 mg/g) (Table 2). These observations are similar to that reported by Akowuah *et al.* (2006).

Analysis of rosmarinic acid in *O. stamineus* leaf extracts by HPLC

The HPLC chromatogram of the *O. stamineus* leaf extract is shown in Figure 2. Rosmarinic acid (RA) was identified in the water, ethanol, methanol leaf extracts, but it was not detected after extraction with chloroform. The RA peak was confirmed by comparison with the retention times of the reference standard. Various concentrations of the calibration standard exhibited a good

Table 1. Qualitative analysis of bioactive compounds in *Andrographis paniculata and Orthosiphon stamineus* leaf extracts prepared in different solvents

Sample	Bioactive Compounds					
	Alkaloids	Saponins	Flavanoids	Tannins	Terpenoids	Steroids
Water Extract						
A. paniculata	+	+	–	+	+	+
O. stamineus	–	–	+	+	+	+
Methanol Extract						
A. paniculata	+	+	+	+	+	+
O. stamineus	+	+	+	+	+	+
Chloroform Extract						
A. paniculata	–	–	–	–	–	–
O. stamineus	+	–	+	–	–	–
EthanolExtract						
A. paniculata	–	+	+	–	+	+
O. stamineus	+	+	+	+	+	+

Note: present (+); absent (-).

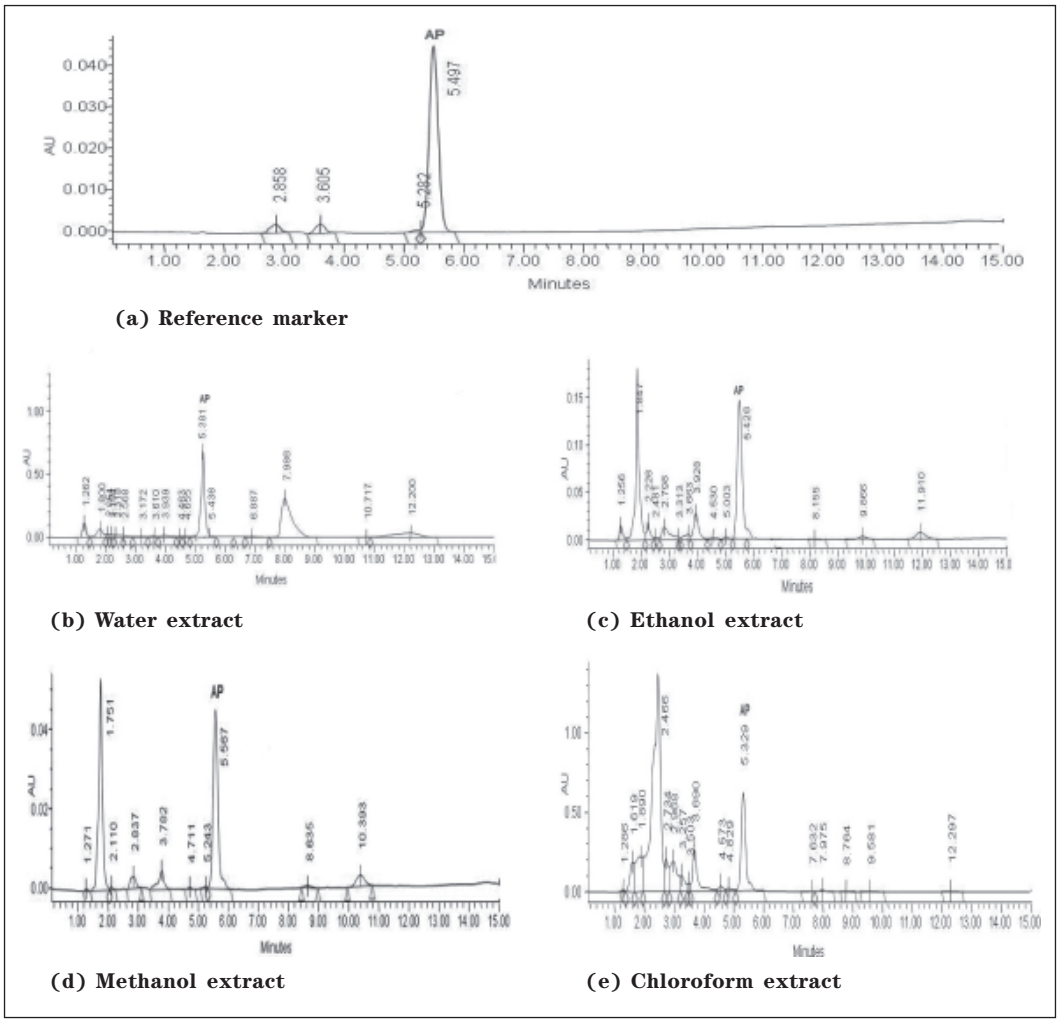

Figure 1. HPLC chromatograms (absorbance at 230 nm) of andrographolide (AP) from *Andrographis paniculata* leaves extracted with different solvents: (a) reference marker; (b) water extract; (c) ethanol extract; (d) methanol extract; (e) chloroform extract

linear variation with their photometric absorbance (y = 788923x – 84260; R^2= 0.996) from 10 to 120 ppm. As shown in Figure 2 and Table 2, the results indicated that greatest peak was observed in the methanolic extract of *O. stamineus* as compared with extracts using other solvents. Accordingly, the highest recovery of RA (17.23 mg/g) was with methanol, followed by water (8.45 mg/g) and ethanol (1.10 mg/g).

Table 2. HPLC quantification of andrographolide in *A. paniculata* leaf extract and rosmarinic acid in *O. stamineus* leaf extract

Extract solvents	Andrographolide	Rosmarinic acid
	mg/g	
Water	26.62 ± 1.06[c]	8.45 ± 0.02[b]
Ethanol	35.85 ± 0.20[b]	1.10 ± 0.07[c]
Methanol	73.40 ± 0.72[a]	17.32 ± 0.41[a]
Chloroform	15.63 ± 0.72[d]	Not detected

Values in each column bearing the same letter are not significantly different (P>0.05)

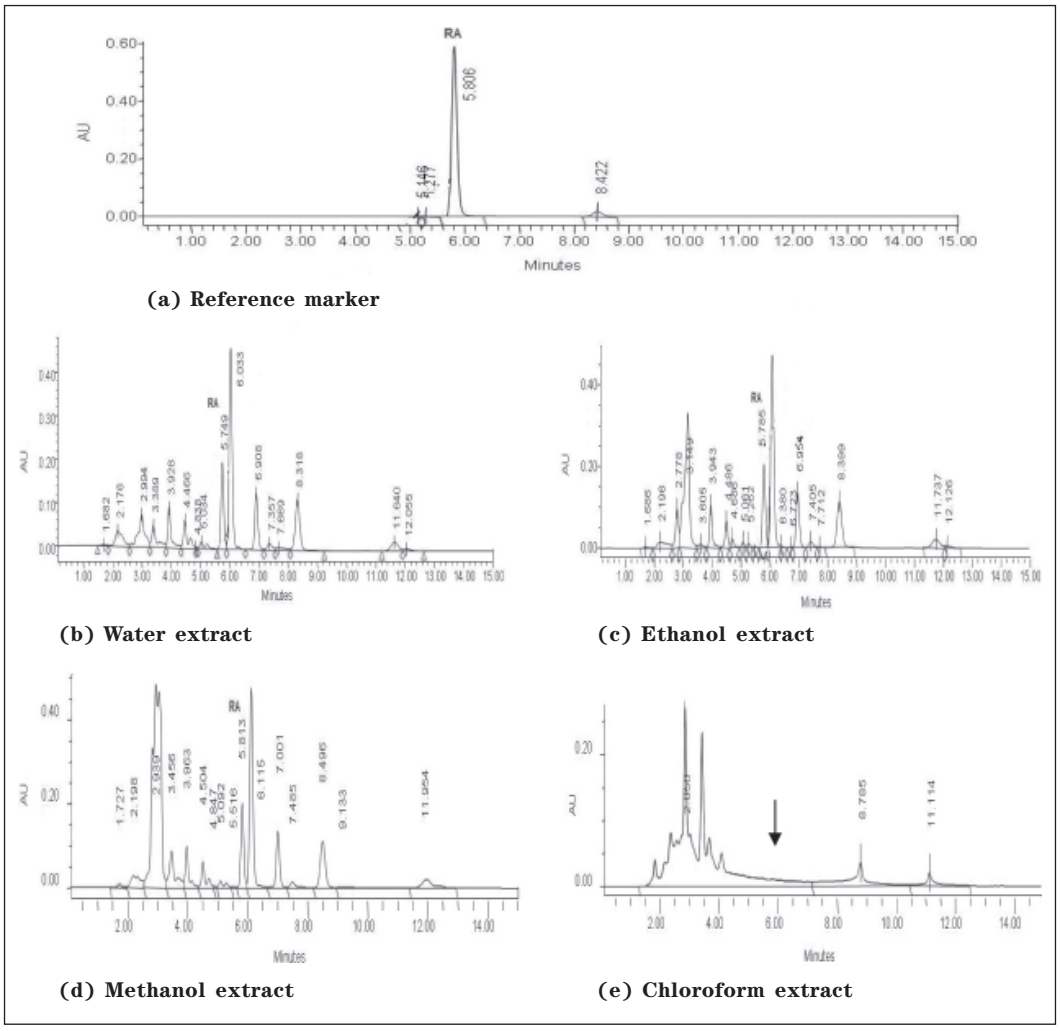

Figure 2. HPLC chromatograms (absorbance at 340 nm) of rosmarinic acid (RA) from *Orthosiphon stamineus* leaves extracted with different solvents: (a) reference marker; (b) water extract; (c) ethanol extract; (d) methanol extract; (e) chloroform extract (the arrow indicates the RA was undetected)

Antibacterial activity of *A. paniculata* and *O. staminues* leaf extracts

Extracts of *A. paniculata* and *O. stamineus* prepared with water, ethanol, methanol, and chloroform were screened for their antibacterial activity against four species of bacteria. The extracts were tested at concentrations of 1.25, 2.50, and 5.00 mg/disc in the disc diffusion assay, which is an effective and rapid method to determine anti-microbial properties (Rovinsky & Cizadlo, 1998). Acetic acid that is commonly used as a food preservative, and tetracycline that is a synthetic antibiotic, respectively served as controls. The diameters of the inhibition zones resulting from the different extracts are presented in Figure 3. In general, all extracts of *A. paniculata* and *O. stamineus* in different solvents exhibited at least some degree of bacterial growth inhibition. Among the treatments and controls, 30 µg/disc of tetracycline (the control) showed the strongest anti-bacterial effect with *S. enterica*, *S. aureus* and *B. cereus*. Among the leaf extracts, the methanolic extract of *A. paniculata* exhibited the strongest inhibitory effects across the bacteria tested, especially at its highest concentration (5.00 mg/disc).

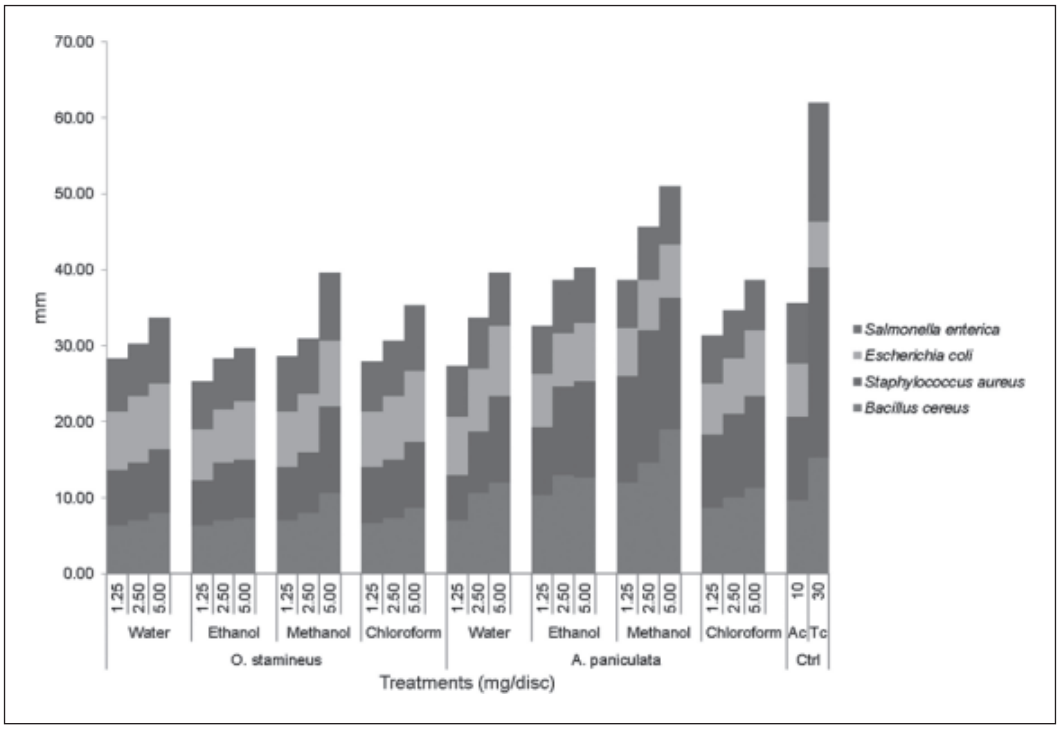

Figure 3. Susceptibility of pathogenic bacteria to *Andrographis paniculata* and *Orthosiphon stamineus* extracts prepared with different solvents. Note: Ctrl: Control; Ac: Acetic acid (10%); Tc: Tetracycline (30 µg/disc). Heights of shaded bars represent inhibition diameters

This extract, although less effective than the tetracycline control, achieved an overall bacterial inhibition effect stronger than that shown by 10% acetic acid.

Methanolic extracts of *A. paniculata* and *O. stamineus* at the highest tested concentration (5.00 mg/disc) in the present study showed the strongest bacterial inhibitory effects as compared with extracts prepared with other solvents. Similar observations have been reported by many researchers for various plant extracts (Negi *et al.*, 2005; Parekh & Chanda, 2007, 2010; Al-Bayati, 2008; Kaushik & Goyal, 2011). The effectiveness of methanolic extracts of *A. paniculata* and *O. stamineus* against bacteria in the present study could be due to their polarity. Antibacterial activities of polar extracts could be attributed to the presence of several types of compounds belonging to different classes, such as flavonoids (Guillen & Manzanos, 1998), and the more polar thermo-labile and/or thermo-stable phenolics (Sokmen *et al.*, 1999). The large amount of andrographolide and rosmarinic acid detected in the methanolic extracts of *A. paniculata* and *O. stamineus*, respectively, as presented in the Table 2, may be significant contributors to the antibacterial effects observed.

The Gram positive bacteria *B. cereus* and *S. aureus* were found to be more susceptible to the highest concentrations (5.00 mg/disc) of methanolic extract of *O. stamineus* and *A. paniculata* extracts of water, ethanol, methanol, and chloroform than the Gram negative bacteria (Figure 3). A report by Al-Bayati (2008) similarly indicated that *B. cereus* and *S. aureus* were more sensitive to plant essential oils and extracts as compared with Gram negative bacteria. The tolerance of Gram-negative bacteria to the herbal extract could be due to the presence of an outer membrane in bacteria that acts as a molecular filter to hydrophilic compounds (Benz & Bauer, 1988; Hammer *et al.*, 2001; Al-Bayati, 2008).

Antioxidant activity of *A. paniculata* and *O. staminues* leaf extracts prepared with different solvents

Total phenolic content

In general, total phenolic contents of *O. stamineus* leaf extracts were higher as compared with similar extracts from *A. paniculata* leaves (Figure 4). The phenolic content of the *O. stamineus* methanolic extract was the highest at 1115.61 mg/100g GAE, followed by the ethanolic extract, the water extract and the chloroform extract. The average phenolic contents of the different *O. stamineus* extracts were significantly different from one another ($p<0.05$). Among the *A. paniculata* leaf extracts, methanolic and ethanolic extracts were again the highest in their phenolic contents, at 264.82 mg/100g GAE and 262.57 mg/100g GAE, respectively, followed by water and chloroform extracts.

DPPH and superoxide scavenging activities

Antioxidant activities of *A. paniculata* and *O. staminues* leaves extracted with different solvents were assessed by determining DPPH free radical scavenging and superoxide scavenging capacity. Figure 5 presents the DPPH scavenging activity of *A. paniculata* and *O. stamineus* extracts in different solvents. In general, *O. stamineus* extracts produced higher DPPH scavenging ability as compared with *A. paniculata* extracts. Among the *O. stamineus* extracts, the methanolic extract of *O. stamineus* showed significantly higher DPPH scavenging activity at 97.5% as compared with the other solvents attempted. Among *A. paniculata* extracts, those prepared from methanol or ethanol (32.7% and 29.9% DPPH, respectively) were significantly higher in DPPH scavenging activity than extracts prepared with water or chloroform (IC_{50} value of 0.059 µg/L ascorbic acid). The chloroform-extract exhibited significantly lower DPPH scavenging activity as compared with water, ethanol and methanol-extracts from both *A. paniculata* and *O. stamineus*.

Results of superoxide scavenging activity of *A. paniculata* and *O. stamineus* leaves extracts are shown in Figure 6. Similarly, as shown in *O. stamineus* leaf extracts yielded higher superoxide scavenging activity as compared with *A. paniculata* extracts. Among the *O. stamineus* leaves extracts, methanolic and

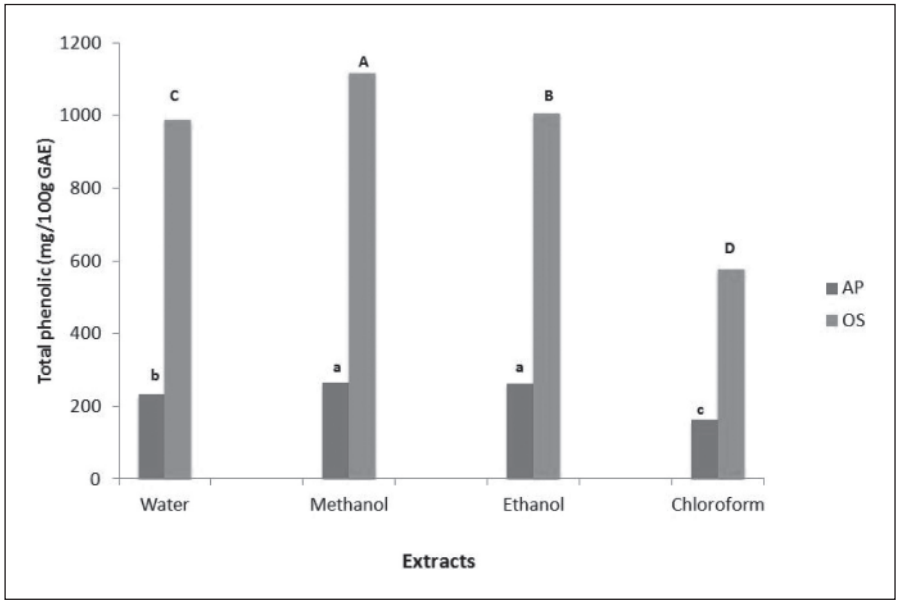

Figure 4. Total phenol contents of *Andrographis paniculata* and *Orthosiphon stamineus* leaf extracts prepared with different solvents. Values in each column bearing the same letter are not significantly different (P>0.05)

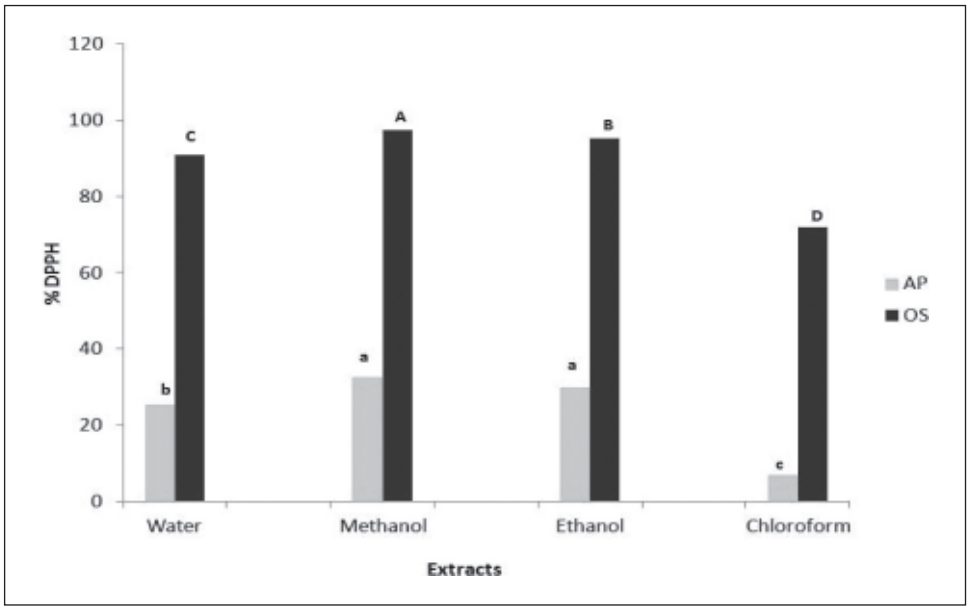

Figure 5. DPPH scavenging activity of *A. paniculata* and *O. stamineus* extracts prepared with different solvents. Values in each column bearing the same letter are not significantly different (P>0.05)

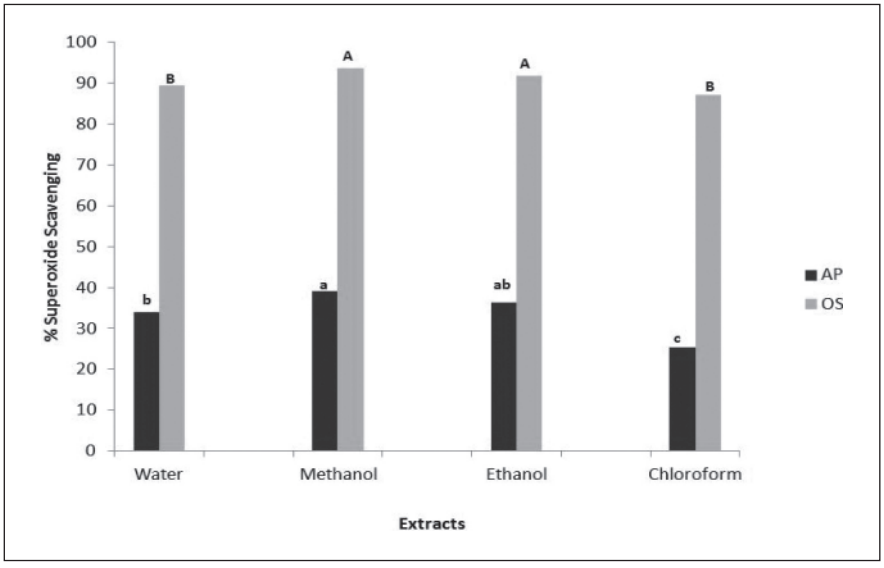

Figure 6. Superoxide scavenging activity of *A. paniculata* and *O. stamineus* extracts prepared with different solvents. Values in each column bearing the same letter are not significantly different (P>0.05)

ethanolic extracts were again significantly higher in superoxide scavenging activity at 93.6% and 91.8% inhibition of superoxide anion generation respectively, as compared to water and chloroform extracts.

Methanolic and ethanolic extracts of *A. paniculata* showed a similar pattern of superoxide scavenging activity the corresponding extracts of *O. stamineus*. The percentage inhibition of superoxide anion

generation in the methanolic and ethanolic extracts were 39.1% and 36.4%, respectively, being significantly higher than the extracts prepared with chloroform (IC_{50} value 0.052 µg/ml Quercetin).

In the present study, methanolic and ethanolic extracts of *A. paniculata* and *O. stamineus* produced higher DPPH and superoxide scavenging activities than extracts prepared with other solvents. As in the case of their anti-bacterial properties, this observation could be explained by the presence of andrographolide and rosmarinic acid present in the leaves of *A. paniculata* and *O. stamineus*, respectively. The anti-oxidant effect of phenolic compounds is mainly due to their redox properties, and is the result of various possible mechanisms: free radical scavenging activity, transition metal-chelating activity and/or singlet oxygen-quenching capacity (Yao & Ren, 2011). Phenolic compounds are also known to play an important role in stabilizing lipid peroxidation and to inhibit various types of oxidizing enzymes (Gallo *et al.*, 2010). Due to the above reasons, herbal plants have been regarded as sources of natural bioactive compounds.

As mentioned above, the total phenolic compounds in *O stamineus* leaves were higher when extracted with methanol as compared with other solvents. These results are consistent with the earlier reports that showed a positive correlation between phenolic content and antioxidant activity (Akowuah *et al.*, 2005; 2006; Tawaha *et al.*, 2007; Premanath & Devi, 2011). Akowuah *et al.* (2005) reported that the methanol and water extracts of *O. stamineus* exhibited high free radical-scavenging properties. The methanol and ethanol extracts of *A. paniculata* recorded the highest phenolic content and also had the highest peroxide scavenging activity. This is in agreement with Akowuah *et al.* (2006) who also found that methanol extracts of *A. paniculata* exhibited high free radical scavenging activity as compared with water extract. The present study found that *A. paniculata* and *O. stamineus* extracted with polar solvents were higher in antioxidant activity rather than non-polar solvents, in agreement with many previous reports (Akowuah *et al.*, 2005; 2006; Tawaha *et al.*, 2007; Premanath & Devi, 2011).

Different solvents of varying polarities were used for the extraction of bio-active compounds, including andrographolide in *A. paniculata*, and rosmarinic acid in *O. stamineus* leaves. The highest yields of these two compounds were obtained by methanol extraction. The methanolic extracts of *A. paniculata* and *O. stamineus* exhibited antimicrobial and antioxidant properties. The inhibitory effects against the Gram positive bacteria *B. cereus* and *S. aureus* shown by methanolic extracts of both plants were superior to that achieved with 10 % acetic acid. Methanolic and ethanolic extracts of *A. paniculata* and *O. stamineus* leaves also produced the strongest antioxidant activities as compared with extracts using other solvent. Such leaf extracts are potential candidates as natural antibacterial and antioxidant agents that can be beneficial to animal and human health.

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