

## ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF A MALAYSIAN MEDICINAL PLANT *ANISOPHYLLEA DISTICHA* (JACK) BAILL. AND QUANTIFICATION OF ITS PHENOLIC CONSTITUENTS

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### Abstract

The ethanolic extracts of different parts of *Anisophyllea disticha* were tested for antimicrobial capability using disc diffusion assay. The result revealed positive growth inhibitory activity, but was considered weak for all extracts against Gram positive bacteria. Leaf extract displayed the highest zone of inhibition with values of  $10.67 \pm 0.58$  and  $8.67 \pm 0.58$  mm against *Staphylococcus epidermidis* and *S. aureus*, respectively. On the other hand, stem showed the highest amount of total phenolic content (TPC) ( $27.73 \pm 4.10$  mg GAE/g extract) and similar trend was also observed in antioxidant potential in terms of DPPH radical scavenging activity ( $66.20 \pm 11.74\%$ ) and ferric reducing antioxidant power assay (FRAP) ( $203.63 \pm 16.03$  mg Fe(II)/g extract). High positive correlation was found between TPC and antioxidant activities, suggesting that phenolic compounds contribute to the antioxidant power of plant extracts. HPLC analysis identified and quantified gallic acid, *p*-coumaric, ferulic acid and quercetin in the extracts.

### Introduction

*Anisophyllea disticha* locally known as Raja berangkat, is a tropical shrub that grows normally in primary or old secondary lowlands as well as hill forests in Malaysia, Indonesia, Singapore, Brunei and Philippines (Singh 2016). This plant belonging to Anisophylleaceae is offering various benefits especially in primary health care. Local people utilize its leaves and stem in curing diarrhea, dysentery and fever either by eating raw or soaking in liquid to prepare the infusion. Patients with jaundice make use of decoction of leaves of *A. disticha* by routinely bathing to cure the disease. According to Quattrocchi (2012) crushed young leaves of *A. disticha* which are able to heal cuts and wounds while its fresh fruits are eaten for septic stings by bees and hornets. Ong (2004) stated that drinking the decoction of roots in combination with onion and black cumin (*Nigella sativa*) are able to relieve weariness and body aches. Apart from that, the boiled roots are used to revitalize the birth canal during post parturition, treat infertility in women and refresh men's body (Ong 2004, Suhajito *et al.* 2014). Furthermore, the root of *A. disticha* is used as tonic to keep person healthy and acts as anti-aging (Chian 2019).

Native people have been using herbal products in treating diarrhea, fever, redness and swelling since ancient times, whereby in modern clinical practice, these symptoms can be associated with bacteria causing diseases (Keller and Nugraha 2011). Numerous therapeutically active plant-derived constituents were discovered from the research of traditional cures and folk knowledge of plants. The previous research findings have shown the antimicrobial potential of different parts of *A. laurina* against bacterial strains and fungi using agar well diffusion and micro-dilution methods (Onivogui *et al.* 2016).

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So far few pharmacological investigations on *A. disticha* have been carried out despite its high utilization by the folklore. Therefore, the present study aimed to evaluate the antimicrobial activity, total phenolic content (TPC), the antioxidant activities of different parts of *A. disticha* in terms of DPPH radical scavenging activities and ferric reducing antioxidant power assay (FRAP) and to analyse its polyphenols by high performance liquid chromatography (HPLC).

### Materials and Methods

*A. disticha* collected from Kenyir Lake, Terengganu, Malaysia was authenticated and a voucher specimen (PIIUM 0003-2) has been deposited at Kulliyyah of Pharmacy, International Islamic University Malaysia. Different parts of *A. disticha*, namely leaf, stem and root were washed with clean water followed by drying in oven at 40°C for a week. The dried samples were ground to fine powder by electric grinder and extracted in Soxhlet (Gerhardt, Germany) apparatus using 250 ml ethanol in dark for 12 hrs. The extracts were then evaporated to dryness by rotary evaporator at 60°C.

The antimicrobial activity of leaf, stem and root extracts of *A. disticha* was determined against Gram positive bacteria *S. epidermidis* (ATCC 12228) and *S. aureus* (ATCC 25923) and Gram negative bacteria *Enterobacter aerogenes* (ATCC 13048) and *Escherichia coli* (ATCC 25922). Antimicrobial activity of extracts was evaluated by using disc diffusion assay following the method by Bauer *et al.* (1966).

Total phenolic content was analysed using the Folin-Ciocalteu assay following the method of Zheng and Wang (2001), with some alterations. For this, 58 µl of diluted extracts (1-5 mg/ml) were oxidized with 968 µl Folin-Ciocalteu reagent (diluted 1 : 10) and the reaction solution was neutralized with 774 µl of 5% sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) solution. The absorbance was measured at 760 nm with a microplate reader spectrophotometer (Infinite M200 Pro) after incubation for 60 min in dark condition. Gallic acid was used as the standard to prepare the calibration curve ( $y = 4.5222x$ ,  $R^2 = 0.9987$ ) in the linear range of 0.02-0.20 mg/ml. The results are expressed as mg GAE/g extract sample.

The free radical scavenging activity of the extracts was evaluated by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay according to Liu *et al.* (2008) with some modifications. A volume of 40 µl of ethanolic extracts (0.2 - 1.0 mg/ml) was mixed with 160 µl of 0.079 mg/ml DPPH in methanol. After 15 min of incubation in the dark at room temperature, the absorbance at 517 nm was measured against blank. Gallic acid was used as standard and the scavenging activity was estimated based on the percentage of DPPH radical scavenged.

The reducing power was determined according to the procedure described by Benzie and Strain (1996), with a slight adaptation for ferric reducing antioxidant power assay (FRAP). Here, 80 µl of each of the appropriately diluted samples (0.2 - 1.0 mg/ml) along with 1 ml of deionized water were mixed with 600 µl freshly prepared FRAP reagent and the mixture was incubated at 37°C for 30 min. The absorbance was measured at 539 nm using a microplate reader spectrophotometer (Infinite M200 Pro). FRAP working solution with deionized water instead of a sample was used as blank. The reducing power was calculated from ferrous sulfate calibration curve ( $y = 3.0636x$ ,  $R^2 = 0.998$ ) with linear range of 0.1 - 1.0 mg/ml. The results have been expressed as mg Fe(II)/g sample extract.

Phenolic compounds were determined by HPLC according to method described by Tasioula-Margari and Tsalolatidou (2015) with slight adaptations. The mobile phase used consisted of methanol:acetonitrile (5:50) (Solvent A) and 1% formic acid in water (45) (Solvent B). The quantification of each compound was determined according to the peak area measurements. The

contents of constituents under study were expressed in  $\mu\text{g/g}$  extract as a mean value  $\pm$  standard deviation of three independent analyses.

All experiments were performed in triplicate and the results have been presented as mean  $\pm$  standard deviation. Experimental data were analyzed using Excel (Microsoft Inc.) and Statistical Package for Social Science (SPSS) version 20.0 software. Analysis of variance (ANOVA) and Duncan's multiple range test were used to determine the significance of differences among the means, and  $p < 0.05$  was considered significant.

### Results and Discussion

Table 1 demonstrates the antibacterial activity of the ethanolic extracts from different parts of *A. disticha*. It is evident from the Table that the test samples inhibited the growth of tested bacteria with varying degree. The present study showed statistically significant difference in antibacterial activity of leaf and root extracts against *S. epidermidis* with diameter of zone of inhibition of  $10.67 \pm 0.58$  mm and  $9.00 \pm 0.00$  mm, respectively, while no significant difference was observed between stem ( $9.67 \pm 0.58$  mm) and leaf as well as root extracts (Table 1). Furthermore, statistically insignificant difference was recorded among inhibitory activity of leaf ( $8.67 \pm 0.58$  mm), stem ( $8.33 \pm 0.58$  mm) and root ( $7.67 \pm 0.58$  mm) against *S. aureus*. According to Prabuseenivasan *et al.* (2006), the positive result was indicated by the zone of inhibition with diameter of more than 7 mm. The present study showed that different parts of *A. disticha* inhibited bacterial growth but their effectiveness is considered weak. The result of antimicrobial potential of *A. disticha* was in line with the previous study by Keller and Nugraha (2011) who demonstrated the sensitivity of *S. aureus* to methanolic extracts of leaf and stem of *A. disticha* with a rate of 25% or less than control. It was also observed that the tested plant parts failed to inhibit Gram-negative bacteria namely *E. aerogenes* and *E. coli*. As reported by Yilmaz *et al.* (2008), Gram-positive bacteria are more vulnerable to antibiotics as they possess only outer peptidoglycan layer which does not work effectively as permeability barrier. On the other hand, the cell walls of Gram-negative bacteria are impermeable to lipophilic solutes as they comprise of an outer phospholipidic membrane bearing the structural lipopolysaccharide components (Sulaiman *et al.* 2011). The variations of the results could be attributed by various intrinsic and extrinsic influences such as climatic and environmental factors, strain differences, extraction procedure and the approaches used to access antimicrobial potential (Kaur and Arora 2009).

**Table 1. Antibacterial activity of different parts of *A. disticha* against selected pathogens.**

Extracts	Diameter of zone of inhibition (mm)			
	<i>S. epidermidis</i>	<i>S. aureus</i>	<i>E. aerogenes</i>	<i>E. coli</i>
Leaf	$10.67 \pm 0.58^a$	$8.67 \pm 0.58^a$	-	-
Stem	$9.67 \pm 0.58^{ab}$	$8.33 \pm 0.58^a$	-	-
Root	$9.00 \pm 0.00^b$	$7.67 \pm 0.58^a$	-	-
Gentamycin (+)	$28.33 \pm 0.58$	$21.67 \pm 0.58$	$28.33 \pm 0.58$	$21.00 \pm 0.00$
Methanol (-)	-	-	-	-

The values were expressed as mean  $\pm$  standard deviation ( $n = 3$ ). Values in the same column with different superscript letter were considered as significantly different ( $p < 0.05$ ); - no activity.

Results of the amount of total phenolic compounds present in different parts of *A. disticha* showed that at sample concentration of 3 mg/ml, the stem extract exhibited the highest amount of phenolic compounds followed by root and leaf with mean values of  $27.73 \pm 4.10$  mg GAE/g

extract,  $19.50 \pm 0.87$  mg GAE/g extract and  $12.19 \pm 1.77$  mg GAE/g extract, respectively (Table 2). Total phenolic contents showed significant differences ( $p < 0.05$ ) among different plant parts. Until today, no study has been reported for quantitative phenolic content of *A. disticha*.

Results presented in Table 2 showed that at sample concentration of 0.6 mg/ml, the stem of *A. disticha* extract exhibited the highest antioxidant activity ( $66.20 \pm 11.74\%$ ), followed by root ( $56.66 \pm 15.00\%$ ) and leaf extract ( $31.06 \pm 12.57\%$ ). The scavenging capability of stem was significantly different ( $p < 0.05$ ) compared to leaf extract while no significant differences ( $p > 0.05$ ) were detected between the root and other parts of plant extract (Table 2). Siddhuraju *et al.* (2002) stated that the presence of high proportions of tannins (proanthocyanidins), flavonols and xanthenes in stem bark fractions might contribute to high scavenging efficiency toward DPPH radicals. The antioxidant activity in terms of FRAP assay whereby at sample concentration of 0.6 mg/ml, the stem extract exhibited significant ( $p < 0.05$ ) total mean value of  $203.63 \pm 16.03$  mg Fe(II)/g extracts compared to leaf ( $87.15 \pm 22.29$  mg Fe(II)/g extracts) whereas there were no significant differences ( $p > 0.05$ ) between the stem and root ( $200.06 \pm 44.56$  mg Fe(II)/g extracts). The order of plant parts in terms of ferric reducing activity is consistent with that of TPC and DPPH free radical scavenging activity. On the whole, the stem bark extract exhibited a much greater antioxidant activities and phenolic content compared to leaf and root. These results are in agreement with the previous findings reported by Onivogui *et al.* (2017) who reported that the reducing power of ethanolic stem extract of *A. laurina* was higher compared to leaf extract with a value of  $36060.9 \mu\text{mol TE}/100$  g extract.

**Table 2. Total phenolic content and antioxidant activities of *A. disticha*.**

Sample	Total phenolic content (mg GAE/g extract)	Antioxidant activities	
		Scavenging activity (% inhibition)	Reducing power (mg Fe(II)/g extract)
Leaf	$12.19 \pm 1.77^a$	$31.06 \pm 12.57^a$	$87.15 \pm 22.29^a$
Stem	$27.73 \pm 4.10^c$	$66.20 \pm 11.74^b$	$203.63 \pm 16.03^b$
Root	$19.50 \pm 0.87^b$	$56.66 \pm 15.00^{ab}$	$200.06 \pm 44.56^b$

The values were expressed as mean  $\pm$  standard deviation ( $n = 3$ ). Values in the same column with different superscript letter were considered as significantly different ( $p < 0.05$ ).

Positive and significant correlations were found between TPC and DPPH free radical scavenging activity ( $r = 0.815$ ) and FRAP assay ( $r = 0.800$ ). The findings support the hypothesis that phenolic compounds, on the basis of the Folin-Ciocalteu assay, contribute to the total antioxidant capacity of plant in terms of DPPH scavenging activity and ferric reducing antioxidant power, where the greater TPC of *A. disticha* resulted in higher antioxidant activities. This claim was in line with the study conducted by Maizura *et al.* (2011) who reported significant and linear relationship between phenolic content and DPPH assay ( $r = 0.86$ ,  $R^2 = 0.7328$ ) and between TPC and FRAP assay ( $r = 0.91$ ,  $R^2 = 0.8220$ ) of *Polygonum minus* (kesum), *Zingiber officinale* (ginger) and *Curcuma longa* (turmeric) extracts.

The content of individual phenolic compound in *A. disticha* as evident from HPLC analysis presented in Fig. 1 showed that gallic acid was the most abundant phenolic compounds in all plant parts. The stem extract displayed the highest gallic acid content followed by root and leaf extracts with mean value of  $319.69 \pm 6.69 \mu\text{g/g}$  extract,  $212.12 \pm 1.44 \mu\text{g/g}$  extract and  $80.47 \pm 0.80 \mu\text{g/g}$  extract, respectively. The presence of gallic acid was also identified in root bark of *A. dichostyla* (Khallouki *et al.* 2007).

The content of *p*-coumaric acid was significantly different ( $p < 0.05$ ) among the plant parts with values of  $39.75 \pm 1.25 \mu\text{g/g}$  extract,  $33.27 \pm 0.98 \mu\text{g/g}$  extract and  $30.23 \pm 0.61 \mu\text{g/g}$  extract for stem, root and leaf, respectively (Fig. 1). The study of Pawar and Dasgupta (2016) reported that *p*-coumaric acid is associated with its antioxidant capability with various physiological actions.

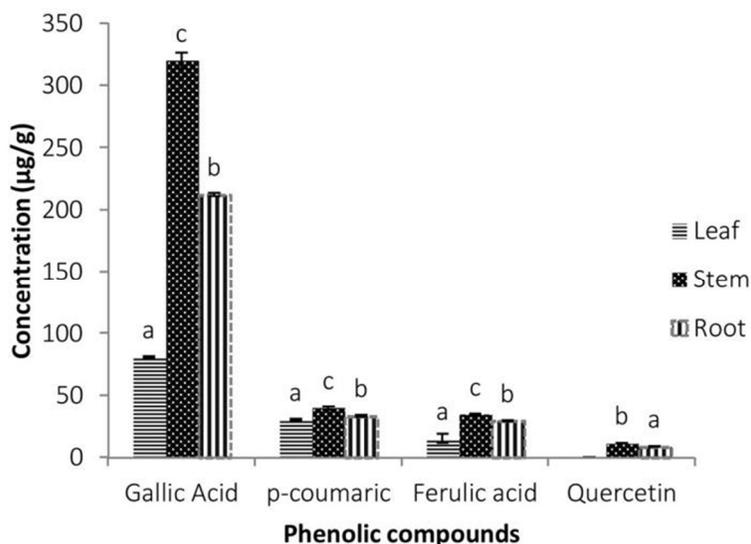


Fig. 1. Quantification of phenolic compounds in different parts of *A. disticha*. The values were expressed as mean  $\pm$  standard deviation ( $n = 3$ ). Different letters indicate significant difference at the level of  $p < 0.05$  between plant parts.

HPLC analysis of the plant under study revealed that the amount of ferulic acid present in *A. disticha* was  $34.51 \pm 0.47 \mu\text{g/g}$  extract in stem,  $29.20 \pm 0.51 \mu\text{g/g}$  extract in root and  $15.08 \pm 3.61 \mu\text{g/g}$  extract in leaf (Fig. 1). Previous study has indicated that ferulic acid exert numerous health benefits particularly as antioxidant, antimicrobial and anticancer activities (Mussatto *et al.* 2007). In the present study, quercetin was found in the lowest concentration of  $11.31 \pm 0.10 \mu\text{g/g}$  extract and  $8.51 \pm 0.28 \mu\text{g/g}$  extract in stem and root, respectively and was not detected in leaf sample of *A. disticha* (Fig. 1). Quercetin has been reported to confer various health benefits including antioxidant, antiviral, anticancer, anti-inflammatory, cardiovascular properties as well as able to reduce mouth sores and asthma (Kumar *et al.* 2017).

From the present investigations it may be concluded that ethanolic extracts of *A. disticha* exhibited positive result for bacterial growth inhibitory activity for all plant parts, but their effectiveness was considered weak against Gram positive bacteria. Stem extract showed the highest TPC and potent free radical scavenging and reducing activities. The results also demonstrated significant positive correlation between TPC and antioxidant activities, suggesting that phenolic compounds contribute to oxidative properties of *A. disticha*. The presence of several phenolic compounds supports the traditional uses of this plant for different biological activities and suggests that *A. disticha* have potential as source of natural antimicrobial and antioxidants agents.

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