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Antioxidant and antibacterial properties of Malaysian ferns used traditionally against infection

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ABSTRACT

Stenochlaena palustris, Drynaria quercifolia and Dicranopteris linearis are three Malaysian ferns used traditionally against infections. Due to the lack of scientific evidence to support the ethnopharmacological use, the present work focus on evaluating the antioxidant and antibacterial properties of these plants. The plant extracts were evaluated for their antioxidant activities using total phenols content, DPPH and FRAP assays, while the antibacterial assays were evaluated by disc diffusion and dilution methods. The methanol extracts of S. palustris and D. linearis were found to have significant antioxidant properties and were able to inhibit the growth of almost all bacteria tested at minimum inhibitory concentrations of 0.5 – 1 mg/ml. The antioxidant and antibacterial effects of S. palustris and D. linearis support their use in traditional medicine for the treatment of infection and wound injuries.

Keywords: *Stenoclaena palustris*; *Dicranopteris linearis*; Anti-infective ferns; Antioxidant; Antibacterial

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INTRODUCTION

Infectious diseases are still a major public health threat to the world today although tremendous progress has been attained in human medicine. Morbidity and mortality as a result of infection due to bacteria, fungi, viruses and parasites continue to be in a rise, particularly in developing countries, where effective control measures and newer drugs are unavailable [1]. Adding on to the problem is the development of resistance disease strain due to over-prescription and misuse of anti-infective drugs as well as patient's non-compliance to complete the full course of treatment [2]. As such, there is an urgent need to develop new, effective and inexpensive anti-infective agents to address this rising issue.

In the course of investigating the antimicrobial properties of indigenous plants, we came across three fern species used traditionally for the treatment of infection, namely, *Stenochlaena palustris*, *Drynaria quercifolia* and *Dicranopteris linearis*. *S. palustris* is known locally as "paku miding". The red young fronds of the fern are eaten as vegetables, while the juice is used to treat fever [3]. *D. quercifolia* is used traditionally to treat cough, tuberculosis, typhoid fever, cholera, chronic jaundice and headache [4-6], while *D. linearis*, known as 'resam' locally, is used as poultices and the decoction is used to treat fever, external wound, ulcers and boils [3, 7]. Although these plants were reputed for their anti-infective properties in traditional medicinal systems, only a limited number of studies have

been carried out to evaluate their biological and pharmacological properties. Hence, the present study aimed to investigate the antioxidant and antibacterial effects of the various leaf extracts of *S. palustris*, *D. quercifolia* and *D. linearis.*

MATERIALS AND METHODS

Plant materials

Stenochlaena palustris (Burm.) Bedd. (Blechnaceae) was collected from Sungai Petani, Kedah. *Drynaria quercefolia* (L.) J. Sm. (Polypodiaceae) was collected from the campus of Universiti Sains Malaysia, Penang, while *Dicranopteris linearis* (Burm.) Underwood. (Gleicheniaceae) was collected from Ipoh, Perak. The identity of these plants was authenticated by Ms Maliga Gnasan at the Penang Botanic Gardens where the voucher specimens were deposited (*Stenochlaena palustris*, specimen no. 1645; *Drynaria quercifolia*, specimen no. 1646; *Dicranopteris linearis*, specimen no. 2787).

Chemicals and bacteria culture

Hexane, dichloromethane (DCM), and methanol (MeOH) used for the extraction of plant materials were of analytical grade (Merck, Germany). The reagents used for the antioxidant assays were: Folin-ciocalteu, sodium carbonate, 2, 2-α-diphenyl-1-picrylhydrazil (DPPH), Trolox and 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) purchased from Sigma-Aldrich (UK), respectively; gallic acid and ferric chloride obtained from Merck (Germany); acetic acid and hydrochloric acid were purchased from Qrec (Thailand). For the antibacterial assays, Mueller Hinton (MH) agar, MH broth and blank disc from Oxoid (UK) were used. Bacteria strains *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus subtilis* (ATCC 19569) were purchased commercially.

Extraction procedure

Plant materials which were freshly collected were washed with clean water to remove soil and debris. The leaves were then separated from the stems and kept frozen at -20 °C prior to freeze drying and grinding. Approximately 100 g of freeze-dried powdered leaves of each fern was successively extracted with 1.5 L of hexane, followed by DCM and thereafter with MeOH. Ultrasound-assisted extraction was carried out twice with each solvent for 20 min in a B-5510 ultrasonic cleaner (Branson Ultrasonics Corporation, USA) operating at 42 kHz and 135W. The individual extracts were filtered and evaporated to dryness under reduced pressure at low temperature (<35 °C) prior to carrying out biological assays.

Antioxidant studies

Total phenols content assay

Total phenolic content of the fern extracts was estimated according to the method described by Singleton & Rossi [8]. In brief, 150 µl aliquot of each extract (1 mg/ml) was diluted with 2.4 ml of water followed by the addition of 1 ml of 0.2 N Folin–Ciocalteu reagent. After 5 min, 300 µl aliquot of saturated Na_2CO_3 solution was added and the absorbance at 725nm was obtained after 2 h. Results expressed in µmol gallic acid equivalents per g of extract (µmol GAE/g) was determined from the calibration curve of 0.02 – 0.5 mg/ml.

1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging capacity assay

Free radical scavenging capacity of the fern extracts was measured from the bleaching of purple methanol solution of DPPH [9]. 190 µl of 0.2 mM methanolic DPPH was allowed to react with 10 µl extract (1 mg/ml) in a 96-well microtitre plate. After an incubation period of 30 min in the dark, the capacity of each sample to inhibit DPPH radicals was determined from the calibration curve of Trolox (25 – 800 µM). The calibration curve of the standard was constructed by plotting % inhibition versus concentration, where % inhibition = $(OD_{blank} - OD_{sample})/ OD_{blank}$ x 100. Results were expressed in µmol trolox equivalents per g of extract (µmol TE/g).

Ferric reducing antioxidant potential (FRAP) assay

This assay was carried out according to the procedure described by Benzie and Strain [10] with slight modification. The FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-*s*-triazine (TPTZ) solution in 40 mM HCl and 20 mM iron (III) chloride solutions in the proportion of 10:1:1 (v/v). The conglomerate which was freshly prepared was warmed to 37 °C in a water bath. A 20 µl aliquot of each sample was added to 150 µl of the FRAP reagent and after 30 min, the absorbance was recorded at 593 nm. Standard solutions

of Trolox (25 – 800 µM) were used to construct the calibration curve. Results were expressed in µmol of trolox equivalents per g of extract (µmol TE/g).

Antibacterial Studies

Disc diffusion assay

Disc-agar diffusion assay was conducted according to the method described by Hudzicki [11]. Bacteria culture which was standardized with 0.5 McFarland solution was spread onto the surface of the Muller Hinton agar. Filter-paper disc (6 mm diameter) which was impregnated with 10 µL of the fern extract (10 mg/ml) was then placed on the agar and allowed to incubate at 37 °C for 24 h. Following that, diameter of the clear zone as a result of bacteria growth inhibition was measured. Streptomycin was used as the positive control, while the solvent was used as the vehicle control.

Broth dilution assay

The minimum inhibitory concentration (MIC) of the extracts was assessed using the method described by Eloff [12] with slight modification. The extracts in test concentrations of 16-1000 µg/ml were incubated with standardized bacterial suspension in a 96-well microtiter plate. The final concentration of DMSO in each well was not >1%. Vancomycin was used as the positive control while DMSO was used as the vehicle control. Following an incubation time of 24 h at 37 °C, 50 µL of *p*-iodonitrotetrazolium *(p*-INT) (0.2 mg/ml) was added to the wells. The colour of each well was observed after 1 h. Purple solution was an indication of bacterial growth while colourless solution was interpreted as no growth. MIC was regarded as the lowest concentration of the plant extracts that prevent visible growth of bacteria.

Statistical Analysis

All the antioxidant assays were carried out in two independent triplicate and results are shown as mean ± SEM. The differences between the antioxidant activities of the extracts were analysed by one-way analysis of variance (ANOVA) at 95% confidence level (p = 0.05) using SigmaStat, version 3.1 (Systat Software Inc, Chicago, USA).

RESULTS AND DISCUSSION

Table 1: Antioxidant activities of three medicinal ferns

	Sample	DPPH assay (µmol TE/g)	FRAP assay (µmol TE/g)	TPC assay (µmol GAE/g)
S. palustris	Hexane	227.1 ± 17.97	194.125 ± 18.2	230.3 ± 12.7
	DCM	66.18 ± 0.4	133.72 ± 15.5	94.1 ± 42.4
	Methanol	2156.33 ± 60.0*	1455.5 ± 66.8*	1419.0 ± 65.5*
D. quercifolia	Hexane	60.28 ± 0.3	33.15 ± 3.3	<117
	DCM	<25	149.07 ± 18.9	117.6 ± 9.0
	Methanol	115.1 ± 4.2	114.05 ± 16.8	262.2 ± 16.4
D. linearis	Hexane	146.84 ± 3.0	569 ± 47.7	134.2 ± 12.9
	DCM	50.74 ± 5.5	111.58 ± 13.5	205.7 ± 12.9
	Methanol	1068.72 ± 117.3*	830.38 ± 3.8*	656.6 ± 50.0*

*Results are mean values of 3 replicates ± SEM. **

The antioxidant effects of the three ferns species were evaluated by total phenols, DPPH and FRAP assays. Total phenols and FRAP assays evaluate the antioxidant reductive capacity of the samples, while DPPH measures the free radical scavenging capacity. Although all three assays have different reaction mechanism, they provided comparable results in terms of ranking the order of potency for almost all the extracts (Table 1). While the hexane and DCM extracts of *S. palustris* and *D. linearis* were found to have weak to moderate antioxidant activities, the MeOH extracts of both the plants showed significant antioxidant effects ($p < 0.05$). Between the two plants, *S. palustris* were found to be two times more potent than *D. linearis* in terms of the metal ions reducing capacity as well as the free radical scavenging activities. In contrast, all extracts of *D. quercifolia* were inactive.

The mean difference is statistically significant ($p < 0.05$) antioxidant effects of the MeOH extracts of *S. palustris* and *D. linearis* were considered remarkable compared to the antioxidant activities of 25 edible tropical plants reported by Wong et al. [13].

The antibacterial properties of the three fern species were evaluated by disc diffusion and dilution methods. The non-polar extracts of all the plants did not show antibacterial effect but the MeOH extract of *S. palustris* was found to inhibit the growth of all four bacteria tested, i.e. *B. subtilis*, *S. aureus*, *P. aeruginosa* and *E. coli* at MICs of 0.5 – 1 mg/ml, while the MeOH extract of *D. linearis* was effective against three bacteria, i.e. *S. aureus*, *P. aeruginosa* and *E. coli* at MICs of 0.5 – 1 mg/ml (Table 2). *D. quercifolia* did not exhibit any inhibitory effect on the bacteria tested. Although the antibacterial effect of the MeOH extracts of *S. palustris* and *D. linearis* were much lower than the commercial antibiotics, their activities were found to be comparatively higher than many of the antibacterial medicinal plant extracts reported, where MIC were between 0.4 to 250 mg/ml [14-16]. From the results, it is interesting to note that the MeOH extract of *S. palustris* and *D. linearis* which were high in antioxidant activities were active against most of the bacterial strain tested, while the hexane and DCM extracts which were low in antioxidant activities were also inactive towards the bacteria. This observation seemed to suggest the association between the antioxidant effects of these two ferns and their antibacterial properties but further investigation is needed in order to confirm their relationships.

There are increasing amount of evidences of late, concerning the role of antioxidants in the treatment of infections. During the course of an infection, free radicals are generated from inflammatory cells as a result of invading pathogens. Over production of free radicals and reactive oxygen species lead to oxidative stress which are potentially damaging to biological molecules such as DNA, proteins, lipids and body tissues [17]. Antioxidants play an important role to protect and prevent these damages by scavenging free radicals and regulating the redox environment in the infected area. On the other hand, many antioxidants such as flavonoids and tannins were also found to have bactericidal effects due to their membrane modulating capacity which influences a large number of membrane-dependant cellular processes of the invading microorganism, such as cell signalling, cell cycle, cell proliferation and mitochondrial functionality [18]. In addition, antioxidants are also essential for promoting reepithelialisation and recovery of tissues in healing skin wounds [19, 20].

Table 2: Antibacterial activity of three medicinal ferns

Plant species	Extract (100 µg/ disc)	Disc diffusion assay				MIC, mg/ml			
		B. subtilis	*S.aureus*	*P. aeruginosa*	*E. coli*	*B. subtilis*	*S.aureus*	*P. aeruginosa*	*E. coli*
S. palustris	Hexane	-	-	-	-	>1	>1	>1	>1
	DCM	-	-	-	-	>1	>1	>1	>1
	MeOH	+	+	++	++	0.5	0.5	0.5	1.0
D. quercifolia	Hexane	-	-	-	-	>1	>1	>1	>1
	DCM	-	-	-	-	>1	>1	>1	>1
	MeOH	-	-	-	-	>1	>1	>1	>1
D. linearis	Hexane	-	-	-	-	>1	>1	>1	>1
	DCM	-	-	-	-	>1	>1	>1	>1
	MeOH	-	+	++	+	>1	0.5	1.0	1.0
Streptomycin, 30 µg/ disc		+++	+++	++++	++++				
Vancomycin						<0.06	<0.06	<0.06	<0.06

Note: Clear zone indication: -: none/ 6 mm; +: 7-8 mm; ++: 9-10 mm; +++: 10-15 mm; ++++: >15 mm

The present study confirms the findings reported by Chai et al. [21] and Lai & Lim [22] concerning the antioxidant properties of *S. palustris* and *D. linearis*, respectively. The antibacterial effect of *S. palustris* against *B. subtilis*, *S. aureus*, *P. aeruginosa* and *E. coli* is reported for the first time. The leaf extracts of *D. quercifolia* were found to be weak in antioxidant and antibacterial effects. Since this plant has been used traditionally for the treatment of cough, tuberculosis, typhoid fever and cholera, the bacterial strains used in this study may not be suitable to correlate with its ethnomedical use. To the best of our knowledge, study on the antioxidant and antibacterial effects of the leaves of *D. quercifolia* has not been reported before. Concerning the antibacterial effects of *D. linearis*, our findings seemed to contradict with the findings of Zakaria et al. [23] where the authors reported its weak inhibitory effects on *S. aureus* and *E. coli*. This contradiction may be attributed to the differences in sample preparation and extraction procedures. In the study conducted by Zakaria et al. [23], leaves of *D. linearis* were air dried at room temperature for two weeks prior to maceration in a single solvent system, i.e. chloroform, methanol and water, separately, for 3 days. Drying plant materials without first inhibiting enzymatic reactions may result in changes of plant metabolites, while prolonged drying of plant in humid environment and exposure to ultraviolet ray may also accelerate the degradation of bioactive compounds [24]. In the present study, *D. linearis* leaves were frozen immediately upon collection and lyophilized prior to extracting sequentially with hexane, DCM and followed by MeOH using the ultrasound-assisted extraction method. The drying process not only minimizes the loss of bioactive compounds, sequential extraction to remove the non active apolar substances prior to extraction with MeOH had also resulted in

the concentration of bioactive polar constituents in the MeOH extract. Hence, this could be the reason why antibacterial activity recorded in this study is much higher compared to that reported by Zakaria et al. [23].

In conclusion, the findings obtained in this study correspond well with the ethnopharmacological use of *S. palustris* and *D. linearis* for the treatment of bacterial infection. The fact that MeOH extract of these plants possess both antioxidant and antibacterial effects suggests that the same chemical constituents in *S. palustris* and *D. linearis* may play multiple roles in combating pathogenic infections. Further work to isolate and characterise the bioactive chemical constituents is currently ongoing in out laboratory.

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