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Conference Paper *in* International Journal of Pharma and Bio Sciences · December 2018

DOI: 10.22376/ijpbs/9.SP01/Dec/2018.1-273

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PRELIMINARY INVESTIGATIONS FOR ANTIOXIDANT PROPERTIES OF FERNS SPECIES COLLECTED IN LONG BANGA, SARAWAK

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ABSTRACT

Ferns are traditionally consumed as vegetables and used to prevent or cure various ailments as they have a few medicinal properties including antioxidant activity. However, little is known on ferns in Long Banga, Sarawak such as *Calymmodon clavifer, Hymenophyllum acanthoides, and Oleandra pistillaris* especially on their medicinal properties.Thus, the study study is carried out to evaluate the antioxidant activity of crude extracts of *Calymmodon clavifer, Hymenophyllum acanthoides* and *Oleandra pistillaris* collected in Long Banga, Sarawak. All crude methanolic extracst were subjected to 1,1-diphenyl-2-picrylhydrazyl (DPPH) antioxidant assay. Total phenolic and total flavanoid content were also determined for phytochemical analysis. DPPH antioxidant test of all extracts showed that *H. acanthoides* gave the significant EC_{50} value 0.030 mg/ml in comparison to the EC_{50} value of the standard used, Trolox 0.035 mg/ml. Furthermore, phytochemical analysis showed higher total phenolic and total flavonoid content in the crude extract of *H. acanthoides* with the values of 304.81 ± 0.47 mg gallic acid equivalent (GAE)/g and 231.09 ± 0.91 mg catechin equivalents (CE)/g, respectively supporting the high antioxidant activity of *H. acanthoides* from DPPH test. Therefore, ferns collected in Long Banga, Sarawak shows promising potential as antioxidant agents to be used as alternative approach in therapeutic applications or preventions.

KEYWORDS:
Hymenophyllum acanthoides, DPPH, EC_{50}, flavonoid, phenolic, Long Banga

INTRODUCTION:

Ferns and lycophytes are a member of vascular plant groups and represented with at least 11,000 species [1]. Accroding to the latest unpublished record, Sarawak is home to at least 1,012 species of ferns and lycophytes (Meekiong Kalu, pers. comm), many of which have been utilized as traditional remedies for various ailments. Ferns are traditionally used to treat gastric and renal infections; they are also valued as antibacterial, diuretics, painkillers and even as anti-inflammatory agents [2]. Recently, various studies have been carried out to evaluate the medicinal properties of ferns and several studies reported on the antioxidant activities of some Malaysian ferns [3,4,5,6].

Calymmodon is a genus that belonged to family Polypodiaceae which is also treated as a grammitid fern. The distribution of this genus is ranging from Sri Lanka to Polynesia. In Borneo, this genus could be found until up to 3,400 m a.s.l. In state of Sabah, there were 12 species of *Calymmodon* recorded in Mount Kinabalu, with *Calymmodon innominatus* and *Calymmodon kinabaluensis* are endemics to Kinabalu [7,8]. Besides, Mount Kinabalu, there was also a record of *Calymmodon pallidivirens* could be collected from Mount Alab in Sabah [7]. Meanwhile, in Sarawak, there were seven species of this genus recorded in Mount

Mulu. A species that endemic to Borneo, *Calymmodon borneensis* was recorded for both mountains. *Calymmodon clavifer* that was used as a material in this study was previously recorded in Mount Jaya, Papua. This species distribution is ranging between Sumatra to New Guinea [8]. The previous record of *C. clavifer* in Borneo was from Mount Kinabalu and Tawau.

Oleandra is a genus of fern which name was derived from an angiosperm species of *Nerium oleander* from family Apocynaceae [9]. *Oleandra* is classified as a member of Oleandraceae family, the description of this genus by Smith *et al.* (2006) include blades simple; leaves articulate, abscising cleanly upon senescence from pronounced phyllopodia; sori indusiate, indusia round-reniform; spores reniform,monolete; $x = 41$ as characters [10]. The variability of *Oleandra* species can be found in the rhizome, although in herbarium collection rhizome part is seldom preserved. The distribution of this genus is pantropical however, some Malesian species could be found in Continental Asia to Australia or the Pacific [9]. There are nine *Oleandra* species described by Hovenkamp & Ho (2012), with only two species previously recorded from Borneo; *Oleandra coriacea* and *O. sibbaldi*. The former species is noted as endemic to Borneo, it previously found in Brunei, East Kalimantan (Indonesia), and Sarawak (Malaysia) [9]. There are at least three specimens of Oleandra kept in Sandakan Herbarium (SAN), they are; *O. cumingii*, *O. neriiformis*, *O. undulata*, and *O. pistillaris*.

Genus *Hymenophyllum* is noted as the largest genus of Hymenophyllaceae family, its distribution is throughout temperate region with about 250 species, and the habitat of this genus is mostly epiphytic and sometimes epillithic [7]. Other than *Hymenophyllum*, there are eight other genera classified under Hymenophyllaceae [7]. The members of this filmy fern family are identifed by their single cell thick laminae. Nurul Hafiza (2014), had collected specimen of Hymenophyllacaea from Mount Ulu Kali, Pahang [11]. Their sample that included three genera; *Cephalomanes*, *Hymenophyllum*, and *Trichomanes* were used to investigate leaf photosynthetic charecteristics. *Hymenophyllum acanthoides* was recently found in Trus Madi Range in Sabah [12]. Other than *Hymenophyllum*, there are eight other genera classified under Hymenophyllaceae [7].

However, information regarding benefits and bioactivity of most fern species which might have the potential to become sources of novel medicines is still limited and not clear [13]. Several ferns have been reported to have phenolic compounds and flavanoid components that exhibit antioxidant, antibacterial, anti-tumour and anti-inflammatory activities [2]. Little is known about the antioxidant activities of *Calymmodon clavifer* (Hook.) T. Moore, *Hymenophyllum acanthoides* (v. d. Bosch) Rosenst., and *Oleandra pistillaris* (Sw.) C. Chr. Hence, this study is carried out to evaluate the antioxidant activities of crude extrats of *Calymmodon clavifer* (Hook.) T. Moore, *Hymenophyllum acanthoides* (v. d. Bosch) Rosenst., and *Oleandra pistillaris* (Sw.) C. Chr. collected in Long Banga, Sarawak.

MATERIALS AND METHODS:

Collection and identification of plants

Wild plants of *Calymmodon clavifer* (Hook.) T. Moore, *Hymenophyllum acanthoides* (v. d. Bosch) Rosenst., and *Oleandra pistillaris* (Sw.) C. Chr. were collected during the Heart of Borneo (HoB) Scientific Expedition in Long Banga, Sarawak (Figure 1). The collection site represented a transition zone between upland mixed dipterocarp forest and lower montane forest. The altitude of collection site ranged from 1,173 to 1,203 m a.s.l.

The collected plants were cross-matched with the authentic voucher specimens in Herbarium of the Sabah Forestry Department (SAN). The identification process was also influenced by a reliable book (see Beaman & Edwards, 2007) [15]. Additionally, the identity of these plants was confirmed by fern para-taxonomist, Mr. Markus Gubilil of SAN.

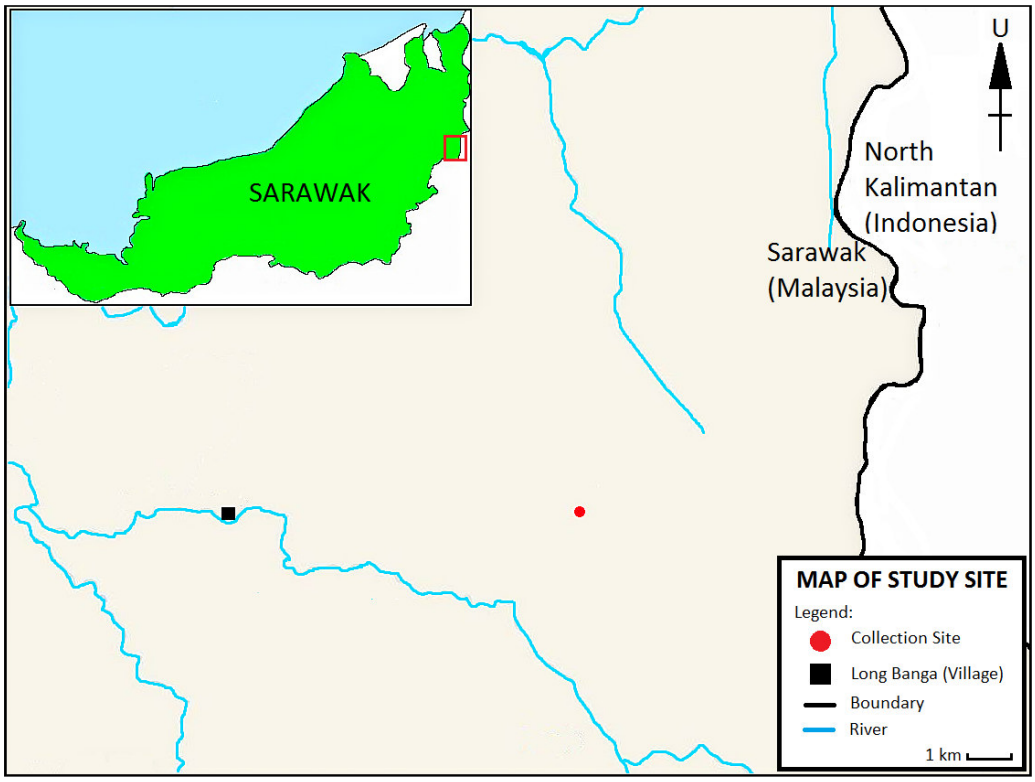

Figure 1
Map showing the location of Long Banga and collection site. Inset: Map of Sarawak (not drawn to scale) showing the study area. (Source: Mohd. Aminur Faiz Suis).

Taxonomic descriptions

Calymmodon clavifer (Hook.) T. Moore (Figure 2) is an epiphytic fern with ascending rhizomes and found among bryophytes on tree trunks. Fronds sessile, densly tufted and pinnatifid. Sori solitary and oblong on each pinna, and protected by a fold of the lobe. *Hymenophyllum acanthoides* (v. d. Bosch) Rosenst. (Figure 3) is a filmy epiphytic fern, and often forms mats on tree trunk and branches. Rhizomes long and thin. Fronds vary in size and margin sharply toothed and conspiciously crisped. Sori at apices of short acroscopic segments, often in apical part of fronds. *Oleandra pistillaris* (Sw.) C. Chr. (Figure 4) is a large terrestial fern with thick and long-creeping rhizomes. Fronds simple and oblanceolate. The margin sub-entire and a little wavy. Sori in one irregular row on each side of the midrib.

Figure 2
***Calymmodon clavifer.* (Source: Mohd. Aminur Faiz Suis).**

Figure 3
Hymenophyllum acanthoides. **(Source: Mohd. Aminur Faiz Suis)**

Figure 4
Oleandra pistillaris. **(Source: Mohd. Aminur Faiz Suis).**

Plant samples collection, preparation and extraction

Samples collected were cleaned up and divided into different parts (stem, leaves, root and flower). Samples were carefully examined to remove old, insect damaged, fungus-infested and twigs. Then, samples were cut into pieces and dried using air dried at control temperature. After they reach its constant weight, all of them were grinded into powdery form using blender. Samples were soaked three times with 100% (v/v) methanol (Fisher) solvents for overnight (24 hours) in ratio of (1:10); which is 1g samples to 10 ml of methanol. The combined methanolic extracts were filtered using Whatman paper no.1 and evaporated under vacuum using rotary evaporator. Extract powder were kept in 4^0C. Upon test, the extract will be dissolved back using methanol (Fisher) in 100 mg/ml concentration (Harborne, 1998).

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

Free radical scavenging activity of extract samples were determined by measuring the radical scavenging ability using the stable radical DPPH reagent (Mensor *et al.,* 2001). Crude methanolic extracts ranging from 0.6 to 500 µg/mL were prepared with methanol or 10% DMSO-methanol (v/v). The reaction mixtures in the

96-well plates consisted of sample (100 µL) and DPPH radical (100 µL, 0.2 mM) dissolved in methanol. The mixture was stirred and left to stand for 15 min in dark. Then the absorbance was measured at 517 nm against a blank. All determinations were performed in triplicates.

The percentage scavenging effect was calculated as:

$$\text{Scavenging rate} = [1 - (A_1 - A_2) / A_0] \times 100\%$$

where A_0 is the absorbance of the control (without sample) and A_1 is the absorbance in the presence of the sample, A_2 is the absorbance of sample without DPPH radical. The scavenging ability of the samples was expressed as EC_{50} value, which is the effective concentration at which 50% of DPPH radicals were scavenged. The EC_{50} values were calculated from the relationship curve of scavenging activities (%) versus concentrations of respective sample. The lowest EC_{50} indicates the strongest ability of the extracts to act as DPPH scavengers.

Statistical Analysis

All anaysis were carried out in triplicates and the result was presented as mean ± standard deviation. The data was statistically analyzed by using one-way ANOVA with significance value of $p<0.05$ followed by least significance difference (LSD) test. All statistical analysis were performed using SPSS version 22.

RESULTS AND DISCUSSION:

Wild plants of *Calymmodon clavifer*, *Hymenophyllum acanthoides* and *Oleandra pistillaris* were collected in Long Banga, Sarawak and methanolic extracts of the plants were prepared based on the method by Harborne (1998). There were two samples of *Calymmodon clavifer* used in the experiment in which *Calymmodon clavifer* 1 was collected at 800 m a.s.l while *Calymmodon clavifer* 2 was collected at 1000-1200 m a.s.l. Based on the sample extraction, the highest percentage yield obtained based on the methanol extraction method was *Calymmodon clavifer* 2 with 27% while the lowest percentage yield was *Calymmodon clavifer* 1 with 3% as shown in Table 1.

Table 1
Extraction yield in percentage (w/w)

Sample	Weight (g)	Percentage yield (%)
Calymmodon clavifer 1	1.0	3.0
Calymmodon clavifer 2	3.1	27.0
Hymenophyllum acanthoides	2.4	24.8
Oleandra pistillaris	10.0	7.0

The potential antioxidant activities of the crude extracts of fern samples collected in Long Banga was tested by using DPPH scavenging assay and the result is expressed as EC_{50} value or defined as the total antioxidant needed to reduce the initial DPPH radical concentration by 50% [14]. Based on the DPPH scavenging assay in Table 2, the EC_{50} values obtained for the crude extracts of *Calymmodon clavifer* 1, *Calymmodon clavifer* 2, *Hymenophyllum acanthoides* and *Oleandra pistillaris,* and even for Trolox as the positive control were appeared to be less than 1 mg/ml. The EC_{50} value for Trolox was 0.035 mg/ml. Our results showed that the crude extract of *Hymenophyllum acanthoides* had the lowest EC_{50} value with 0.030 mg/ml while the crude extract of *Calymmodon clavifer* 1 had the highest EC_{50} value with 0.894 mg/ml. Hence, the results indicate that the crude extracts of *Hymenophyllum acanthoides* to have the highest antioxidant activity compared to the other crude extracts of ferns collected in Long Banga, Sarawak. The low EC_{50} value of the crude extract of *Hymenophyllum acanthoides* indicates high antioxidant activity similar to several ferns that have been reported to show not only high total phenolic content but are also potent antioxidants such as *C. latebrosa, C. barometz, D. quercifolia, B. orientale* and *D. linearis* [16].

Table 2
Scavenging activity of *Calymmodon clavifer* 1, *Calymmodon clavifer* 2, *Hymenophyllum acanthoides* and *Oleandra pistillaris* crude extracts on DPPH radicals

Sample	DPPH (mg/ml)[a]
Calymmodon clavifer 1	0.894
Calymmodon clavifer 2	0.497
Hymenophyllum acanthoides	0.030
Oleandra pistillaris	0.446
Trolox *	0.035

Notes: Data are mean ± standard deviation (n=3). *: Positive control
C. clavifer 1 was collected at 800 m a.s.l, *C. clavifer* 2 was collected at 1000-1200 m a.s.l.
[a] : DPPH free radical scavenging activity was expressed as EC_{50} (mg/ml)

Based on Table 3, crude extract of *Hymenophyllum acanthoides* showed the highest total phenolics (304.81 mg GAE/g) and total flavanoids (231.09 mg CE/g) when compared to the other crude extracts. The total phenolic content of the crude extract of *Hymenophyllum acanthoides* was found to be higher. Several medicinal ferns have been reported to have high phenolic content including *A. aureum*, *A. nidus, B. orientale, C. barometz* and *D. linearis* [17] while *S. palustris* was reported to have high flavanoid content [4]. The high total phenolics and total flavanoids content found in the crude extract of *Hymenophyllum acanthoides* suggests the possible contribution of the total phenolics and total flavanoids in the high antioxidant activity of *Hymenophyllum acanthoides.* Plants contain various free radical scavenging molecules including phenolics compounds and flavanoids which have antioxidant activity. Several studies have reported on significant correlation between total phenolic content with DPPH activities [18,19].

Table 3
Total phenolics and total lfavanoids of *Calymmodon clavifer* 1, *Calymmodon clavifer* 2, *Hymenophyllum acanthoides* and *Oleandra pistillaris* crude extracts

Sample	Total Phenolics [a]	Total Flavanoids [b]
Calymmodon clavifer 1	40.32 ± 0.82^{c}	22.76 ± 1.14^{c}
Calymmodon clavifer 2	49.08 ± 0.71^{d}	27.61 ± 1.6^{d}
Hymenophyllum acanthoides	304.81 ± 0.47^{e}	231.09 ± 0.91^{e}
Oleandra pistillaris	169.23 ± 0.23^{f}	9.42 ± 1.39^{f}

Notes: Data are mean ± standard deviation (n=3), different letters (within columns) are significantly different at $P < 0.05$.
C. clavifer 1 was collected at 800 m a.s.l, *C. clavifer* 2 was collected at 1000-1200 m a.s.l.
[a] :Total phenolic content was expressed as mg gallic acid equivalent in 1g of dried sample (mg GAE/g).
[b] :Total flavonoid content was expressed as mg catechin equivalent to 1g of dried sample (mg CE/g)

CONCLUSION:

The preliminary study on biological activities of fern and fern allies collected in Sarawak further revealed their importances as natural remedies. *Hymenophyllum acanthoides* was proven to have high antioxidant activity and high phytochemical content. As the sample are limited, further investigations on various biological assays and identifications of active compound(s) should be conducted in future studies on ferns collected in Long Banga especially on *Hymenophyllum acanthoides*. The ferns collected in Long Banga shows promising potential as antioxidant agents to be used as alternative approach in therapeutic applications or preventions

ACKNOWLEDGEMENT:

The authors feel gratitude to every opportunity given from Universiti Malaysia Sabah, Jabatan Perhutanan Sarawak, Jabatan Perhutanan Sabah and all members in Long Banga Scientific Expedition 2016.

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