Qualitative and Quantitative Phenolic Compounds Analysis of *Dicranopteris linearis* Different Fractional Polarities Leaves Extract

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Abstract: *Dicranopteris linearis* occupies in an open ground that contains poor soils and often colonizing disturbed space that partly shaded area. It has been known for various traditional values including medicinal, edible food, soil erosion protection, pen and furniture. Even though the plants possess both economic and medicinal value, they still form the neglected group of a plant. The present study was carried out to characterize the phenolic compounds in *D. linearis* leaves extract in different fractional polarities qualitative and quantitatively. Dried leaves of *D. linearis* were successfully extracted by using water extraction before separated by petroleum ether, ethyl acetate and butanol fractions. All the fractional extracts have been analysed by using GCTOF-MS and HPLC. The result from GCTOF-MS analysis of fractional extracts showed 38 compounds found in petroleum ether, ethyl acetate and butanol extracts. However, only four phenolic compounds were identified through HPLC analysis in ethyl acetate and butanol extracts which were 2-Methoxy-4-vinylphenol, Vanillin, 4-Hydroxybenzaldehyde and 4-Hydroxybenzohydrazide. The results revealed that *D. linearis* contained 699.83 ± 6.26 µg GAE /g DW of total phenolic acid whereas individual phenolic acids were predominantly caffeic acid (0.44 ± 0.01 µg/g DW) and ferulic acid (0.22 ± 0.00 µg/g DW) in ethyl acetate and caffeic acid (0.10 ± 0.00 µg/g DW) and 2-Coumaric acid (0.44 ± 0.00 µg/g DW) in butanol extracts. In the present study, the plant extracts demonstrated the highest phenolic compound detected in ethyl acetate and butanol compared to petroleum ether extract.

Keywords: Phenolic compound, phenolic acid, *Dicranopteris linearis*, HPLC, GCTOF-MS, total phenolic content, fern.

INTRODUCTION

Dicranopteris linearis (Burm.f.) Underw. (family: Gleicheniaceae) commonly called forked fern or *paku resam* is pioneer succession species under critical conditions such as landslides, road cutting and degraded forest lands [1]. *D. linearis* also consider a problematic issue to forest management and improvement of the public function of the forest [2]. As described by [3], *D. linearis* contained long rhizome that is brown, hairy, creeping and spreading along the ground or climbing on other vegetation and often forming deep thickets up to 3 m or more. While the stems grow from the rhizome with greenish-brown. Primary rachis-branches usually twice forked and nearly equal. The leafy branches can grow up to 10 m or more when supported by the tree. While the ultimate branches grow up to 30 cm or more and 5 to 8 cm wide. The ultimate segments of the leaves linear in shape, entire apex rounded up to 4 mm broad, firm, prominent vein [3]. In Southeast Asia, *D. linearis* is traditionally used to control fever and treat wounds and ulcers [4] which also support medicinal properties such as antibacterial, antifungal, hepatoprotective, expectorant, anti-inflammatory antioxidant, anthelmintic and analgesic activities [5]. Meanwhile, in Malaysia, *D. linearis* was used in old folk medicine for various skin disease treatment [6]. The active fraction of *D. linearis* is rich in polyphenolic substances exhibited substantial antioxidant activities, induced cellular repair and contributed positively to fibroblast proliferation and migration *in vitro* [6]. On top of that, from an ecological perspective, *D. linearis* has potential as an ecological indicator for urban climate changes at a different altitude that are commonly caused by the phenomenon of urban heat island (UHI) [7]. The litter of *D. linearis* reported could stabilize the organic source of soil organic matter while burning of plant biomass could transform organic matter to black carbon fraction [1]. This plant might also have allelopathic effects by preventing the growth of the other plants [8]. As tested on Malaysian broad-leaves weeds and common grass, the study revealed that *D. linearis* strongly reduced the

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weed density in its vicinity [9]. On the other hand, plant phenolic compounds have been widely reported to be substances inhibitory of seed germination and plant growth by affecting photosynthesis, protein synthesis, mineral uptake, chlorophyll content, membrane permeability, and water utilization in plants [10]. Phenolics include simple phenols, phenolic acids (benzoic and cinnamic acid derivatives), coumarin, flavonoids, stilbenes, hydrolyzable and condensed tannins, lignans and lignins that commonly occurred as secondary metabolites [11]. This category and cinnamic acid derivatives are commonly produced by higher plants. Phenolic acids with carboxyl and hydroxyl groups are capable of binding with starch and other polysaccharides through hydrogen bonds, chelation or covalent bond, forming bridges or cross-link [12]. Phenolic compounds arise from the shikimic and acetic acid (polyketide) metabolic pathways in plants which are one of the categories of many secondary metabolites [13]. Therefore, the research aimed to characterize the phenolic compounds in *D. linearis* leaves extract in different fractional polarities qualitative and quantitatively.

MATERIALS AND METHODS

Sample Preparation

The leaves of the *Dicranopteris linearis* (paku resam) were removed with a secateurs and a random 1000 g fresh weight sample was immediately frozen at -20 °C. The leave samples were freeze-dried for three days, after which the samples were ground into a fine powder and stored at -20 °C until further analysis.

Extraction of Phenolic Compounds

The extraction procedure essentially followed the methods described by [14, 15] with the following modification. For each sample, 10 g of powdered freeze-dried material was mixed with 100 ml of distilled water, and then the sample was incubated in an oven at about 60°C for 30 min before allowed to stand overnight in darkness at room temperature before the clear supernatants re-extracted with different solvents polarities; petroleum ether, ethyl acetate and butanol.

Separation of Phenolic Compounds

The phenolic compounds were isolated from *D. linearis* water extract according to the method of [15]. The crude extraction (clear supernatant) was extracted using petroleum ether, ethyl acetate and butanol using a funnel separator. For further analysis, the final concentration was resuspended with 5 ml of methanol in tubes then capped and sealed with parafilm to exclude oxygen and immediately stored at -20°C until subsequent analysis. 50 µL of the re-dissolved sample was then transferred in a vial for further analysis by gas chromatography-time of flight mass spectrometry (GCTOF-MS), total phenolic content (TPC) and high-performance liquid chromatography (HPLC).

Determination of GCTOF-MS Analysis

The composition of leaf extracts from *D. linearis* species was qualitatively performed by GCTOF-MS (Agilent 7890 system) equipped with a capillary column (30 m x 0.25 mm, 0.25 µm) based on the method reported by [16]. Split-less injection of a 1.0 µL sample was performed with a purge time of 1.0 min. The solvent delay was set at 4 min. The carrier gas used was helium at the flow rate of 1.0 mL min^{-1}. The column temperature was initially maintained at 80°C for 2 min, then programmed at 5°C min^{-1} to 80 °C min^{-1} and then at 10 °C min^{-1} to 250 °C. The inlet temperature and detector sets were 220 °C and 340 °C respectively. The time-of-flight mass spectrometer was operated at 1 spectrum/s acquiring the mass range m/z 50-1000. The identification of the peaks was based on mass spectra based on >90 % similarity index with the National Institute of Standards and Technology library (NIST 14) and by comparison with published data.

Determination of Total Phenolic Content

TPC was determined by the Folin-Ciocalteau assay as reported by [17]. 90 µL of Folin-Ciocalteu reagent was diluted in deionised water (20% v/v) and then placed in each well of a flat-bottomed 96 well clear microplate. After that, 1.0 mg/g DW of the sample that was diluted with distilled water (1000 µg/mL) was added and incubated at room temperature for 5 minutes. Next, 90 µL of sodium carbonate in deionised water (7.5% w/v) was mixed and incubated for two hours at room temperature. The absorbance of extracts and standard was then read at λmax = 725 nm against a blank (deionised water without extract or standard) using a TECAN microplate reader.

The total phenolic compound (TPC) was determined using a gallic acid calibration curve whereas the domain for gallic acid (GAE) calibration curves was calculated concerning the dilution factor = 11. Twofold serial dilution (five different concentrations) was performed in 30 mL glass vials using a micropipette, and 1.0 mg/g DW of each concentration (GAE) per gram dry weight sample ± standard error of the mean

(SEM). The TPC concentrations would be expressed in terms of a microgram GAE per 1.0 g dry weight of freeze-dried matter (µg GAE/g DW).

HPLC Analysis of Individual Phenolic Compounds

The HPLC analysis of phenolic acids was performed on an Agilent 1200 series rapid resolution LC system (Agilent Technologies, Palo Alto, CA, USA) comprised of a binary pump with autosampler injector, micro vacuum degassers, thermostatted column compartment and a diode array detector (DAD) [18]. The column used was a Zorbax Eclipse XDB-C_{18} end-capped 5 µm, 4.6x150 mm reverse phase column (Agilent Technologies, USA). For the analysis, a linear gradient elution was used, with the two mobile phases consisting of 1% formic acid in water/ acetonitrile 90:10 v/v (phase A) and acetonitrile (phase B) using the following gradient: 0-20 min, linear gradient from 0% B to 40% B; 20-25 min, linear gradient from 40% B to 60% B; 25.10-35 min, linear gradient from 100% B to 100% B and 35.10-40 min, isocratic of 0% B. The temperature of the column was set at 25°C. The injection volume was 20 µL, and the flow rate was set at 1.0 mL min^{-1}. Phenolic acid standards of Caffeic acid, Ferulic acid, trans-p Coumaric acid, 2-Coumaric acid, 4-Coumaric acid, Hydroxybenzoic acid and Vanillic acid were purchased from Sigma-Aldrich. The individual phenolic acids were detected at the wavelength of maximum absorption of the phenolic acids in the mobile phase at 280 nm by using photodiode array detection. Individual phenolic acid concentrations were calculated by comparing their relative proportions, as reflected by integrated HPLC peak areas. The individual phenolic acid compound concentrations would be expressed in terms of a microgram per 1.0 g dry weight of freeze-dried matter (µg/g DW).

RESULTS AND DISCUSSION

GCTOF-MS Analysis of Volatile Compounds

The GCTOF-MS analysis from the leaves of *D. linearis* (paku resam) revealed the presence of 38 compounds belonging to different chemical groups, formula and exact mass by referring to the National Institute Standard and Technology (NIST) library data. The data is reported in Table **1** with different extracts identified in petroleum ether, ethyl acetate and butanol. The result was based on >90% similarity with NIST library data. Based on the volatile compounds of water extraction analysis (Table **1**), 4 different phenolic compounds were detected. Three phenolic compounds identified in ethyl acetate extract were 2-Methoxy-4-vinylphenol, Vanillin and 4-Hydroxybenzaldehyde while two phenolic compounds for butanol extract were 2-Methoxy-4-vinylphenol and 4-Hydroxybenzohydrazide. However, none of the phenolic compounds were found in the petroleum ether extract. In order to obtain an optimal extraction few parameters must be observed including the correct choice of solvent that becomes a fundamental of extraction, extraction time whereby increasing the extraction time the quantity of analysis extracted is increased even though there might be a risk on degradation and also technique of extraction (conventional or instrument) [19]. The solvent polarity indicates the electrical charge across the molecule related to the number of л electrons and lone-pair electrons in a molecule to form a group [20]. In the present study, most of the phenolic compounds were detected higher in ethyl acetate and butanol compared to petroleum ether extract. Furthermore, selecting the right solvent affects the amount and rate of the phenolic compounds while long extraction times and high temperatures increase the chance of oxidation of phenolics [21]. Therefore, it is important to choose an efficient extraction procedure to maintain the stability of the phenolic compounds.

Total Phenolic Content Analysis and HPLC Analysis of Individual Phenolic Compound

The total phenolic content (TPC) of *D. linearis* was determined using the Folin-Ciocalteau method as presented in Table **2**. Based on the value of TPC, *D. linearis* contained 699.83±6.26 µg GAE/g DW. The HPLC chromatogram of the water extract of *D. linearis* as in Table **2** indicates that Caffeic acid and 2-Coumaric acid are the two major compounds that exist in water extraction of *D. linearis* leaves even though their concentrations are low (<0.50 µg/g DW). No trace of other phenolic acids was found in petroleum ether extract. The highest result for individual phenolic was Caffeic acid (0.44±0.00 µg/g DW) and 2-Coumaric acid (0.44±0.00 µg/g DW) in ethyl acetate and butanol extract respectively. T*rans-p*-Coumaric acid (0.10±0.00 µg/g DW) was detected as a trace element in butanol extract. The similarity among those fractional extract in this study for *D. linearis* showed that 4-Hydroxybenzoic acid, Vanillic acid, *trans-p*-Coumaric acid and 3-Coumaric acid were not detected. Phenolic compounds are generated by plants in response to environmental stress such as in hilly area, slopes, open ground and when exposed to a number of stress factors such as low air temperature, decreased partial oxygen,

Table 1: Volatile Compounds Profiles in Different Fractional of Water Extract of *D. linearis* Leaves

Fractional extracts	Volatile compounds	Formula	Exact mass
Petroleum ether	1,3-Dioxolane	$C_3H_6O_2$	74.07
	15-Methylhexadecanoic acid methyl ester	$C_{18}H_{36}O_2$	284.48
Ethyl acetate	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150.17
	1,6-Anhydro-β-D-glucopyranose	$C_6H_{10}O_5$	162.14
	Hexanoic acid	$C_6H_{12}O_2$	116.16
	1,3-Dioxolane	$C_3H_6O_2$	74.07
	15-Methylhexadecanoic acid methyl ester	$C_{18}H_{36}O_2$	284.48
	2-Hexenoic acid	$C_6H_{10}O_2$	114.14
	5,6-Epoxy-3-hydroxy-9-apo- β-carotene-9-one	$C_{13}H_{20}O_3$	224.30
	2-Hydroxyethyl salicylate	$C_9H_{10}O_4$	182.17
	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	206.32
	Vanillin	$C_8H_8O_3$	152.14
	4-Hydroxybenzaldehyde	$C_7H_6O_2$	122.12
Butanol	2-Methylbutanoic anhydride	$C_{10}H_{18}O_3$	186.25
	3-Amino-S-triazole	$C_2H_4N_4$	84.08
	2-Methyl-1,4-butanediol	$C_5H_{12}O_2$	104.14
	N-(tert-Butoxycarbonyl)glycine	$C_7H_{13}NO_4$	175.18
	4-Aminobutyric acid	$C_4H_9NO_2$	103.12
	Maltol	$C_6H_6O_3$	126.11
	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150.17
	Furfural	$C_5H_4O_2$	96.08
	3,3-Dimethylacrylic acid	$C_5H_8O_2$	100.11
	4-Hydroxybenzoic acid	$C_7H_6O_3$	138.12
	2-Furanmethanol	$C_5H_6O_2$	98.10
	4-Hydroxybenzohydrazide	$C_7H_8N_2O_2$	152.15
	Phenylacetic acid	$C_8H_8O_2$	138.14
	Ethanesulfonyl fluoride	$C_2H_5FO_2S$	112.11
	2,3-Dihydrothiophene	C_4H_6S	86.15
	5-Methylfurfural	$C_6H_6O_2$	110.11
	5,6-Epoxy-3-hydroxy-9-apo- β-carotene-9-one	$C_{13}H_{20}O_3$	224.30
	Benzoic acid	$C_7H_6O_2$	122.12
	2-Hydroxyhippuric acid	$C_9H_9NO_4$	195.17
	3,3-Dimethylacrylic acid	$C_5H_8O_2$	100.11
	Undecane	$C_{11}H_{24}$	156.31
	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	206.32
	Benzeneacetaldehyde	C_8H_8O	120.15
	5-Hydroxymethylfurfural	$C_6H_6O_3$	126.11
	1,1-Dibutoxybutane	$C_{12}H_{26}O_2$	202.33

Table 2: Total (µg GAE/g DW) and Individual Phenolic Content (µg/g DW) of *D. linearis* in Water Extraction for Different Fractional Polarities Extracts

Total phenolic (µg GAE/g DW)	Fractional extracts	4-Hydroxybenzoic acid (µg/g DW)	Caffeic acid (µg/g DW)	Vanillic acid (µg/g DW)	*trans-p*-Coumaric acid (µg/g DW)	Ferulic acid (µg/g DW)	3-Coumaric acid (µg/g DW)	2-Coumaric acid (µg/g DW)
699.83±6.26	PE	ND	ND	ND	ND	ND	ND	ND
	EA	ND	0.44±0.01	ND	ND	0.22±0.00	ND	ND
	B	ND	0.10±0.00	ND	ND	ND	ND	0.44±0.00

Note: PE (Petroleum ether); EA (Ethyl acetate); B (Butanol); ND (Not detected).

increased UV radiation and unfavourable water regime [22].

CONCLUSION

This study demonstrated that phenolic acid compounds were detected in *D. linearis* leaves in free and bound forms. The different solvent polarities indicated that phenolic acids were esterified with different components. The qualitative and quantitative analysis by GCTOF-MS and HPLC chromatogram revealed that 4 different phenolic compounds were detected. Three phenolic compounds identified in ethyl acetate extract were 2-Methoxy-4-vinylphenol, Vanillin and 4-Hydroxybenzaldehyde while two phenolic compounds from butanol extract were 2-Methoxy-4-vinylphenol and 4-Hydroxybenzohydrazide. The concentrations of the various compounds changed widely. Therefore, it is important to choose efficient extraction procedure to maintain stability of phenolic compound.

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