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Towards green extraction: A study on solvent selection and methods for antioxidant activities of *Diplazium esculentum* Retz. and *Stenochlaena palustris*

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Abstract. Green extraction method by combination of stirring method with no presence of heat and the use of aqueous as solvent were highlighted in this study. Various solvents (aqueous, chloroform, ethyl acetate, hexane and methanol) and two extraction methods (stirring and Soxhlet) were used to study their effect on the yield, qualitative phytochemical content, and antioxidant properties of *Diplazium esculentum* Retz. and *Stenochlaena palustris*. Stirring extraction method in aqueous has shown to provide highest yield in both plant species with *D. esculentum* at 8.88% and *S. palustris* at 9.40%. Saponin was also seen present in both aqueous extracts qualitatively. In the case of FRAP (Ferric Reducing Antioxidant Power) assay, aqueous extract of *D. esculentum* (DEA) had the highest value with 687.57 $\pm$ 0.01 μg Fe(II)E/ml, while stirring method in various solvents had shown to produce high antioxidant activities compared to Soxhlet method. This study revealed that aqueous extracts by stirring method is a promising method for extraction of plant materials and at the same time leading towards a green environment.

Introduction.

Plants are known to contain bioactive components which are very beneficial for health. These bioactive components include phenolic, flavonoid, alkaloid and families of terpenoid that contributes to antioxidant properties through various mechanisms [1]. *Diplazium esculentum* Retz. and *Stenochlaena palustris* are two medicinal ferns shown to have antioxidant activities when the extraction was done *via* Soxhlet extraction method in methanol solvent [2,3]. Traditional extraction methods such as Soxhlet extraction, maceration and decoction tend to be time-consuming and requires a large volume of solvent such as methanol and hexane which are harmful and toxic. The methods also involve presence of heat that can cause degradation of bioactive compounds. Hence, the need to reduce the use of hazardous substances, and at the same time reduces the consumption of energy for extraction of plant materials is very crucial.

Green extraction process of plant materials has been a promising approach that aims to save the environment, energy and promote sustainability. This includes the use of green and sustainable solvents that is non-toxic to health, has low environmental impact and provides low cost and energy. Water is potentially considered as a green solvent as it is recognised as safe and environmental-friendly. The use of water as an extraction solvent combined with a suitable extraction method such as microwave assisted extraction and ultrasound-assisted extraction methods have been shown to enhance extraction processes and improve efficiency [4]. However,

there have been very limited studies on the combination of water as extraction solvent and the use of stirring method to extract plant materials. Extraction method by stirring has low cost, uses less energy and no heat is required, thus contributing towards green environment.

This study used various solvents (water/aqueous, chloroform, ethyl acetate, hexane and methanol) and two extraction methods (stirring and Soxhlet) to study their effect on the yield, qualitative phytochemical content, and antioxidant properties of *D. esculentum* Retz. and *S. palustris*. This paper will focus on green extraction process, hence the discussion will highlight on the effect of extraction method by stirring with no presence of heat and the use of aqueous as the extraction solvent.

Materials and Methods.

Reagents and chemicals. All chemicals used were of analytical grade and without further purification. Ascorbic acid, gallic acid, quercetin, sodium acetate buffer, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), Folin–Ciocalteu's phenol reagent, single reagent 2,20-azino-bis(3-ethylbenziazoline-6-sulfonic acid) (ABTS), potassium peroxodisulfate, chloroform, ethyl acetate, hexane and methanol were purchased from Merck, Sigma-Aldrich (Darmstadt, Germany). Free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) powder was from Alfa Aesar, Thermo Fisher Scientific (Heysham, UK).

Plant materials. *Diplazium esculentum* Retz. (Voucher specimen AMB.DE.1) and *Stenochlaena palustris* (Voucher specimen AMB.SP.1) were identified and authenticated by Brunei National Herbarium (BRUN), under Forestry Department, Sg. Liang, Brunei.

Preparation of dried plant powder [5]. Plant powders were prepared following previous method with modifications. Fresh leaves samples of *D. esculentum* Retz. and *S. palustris* were rinsed, cleaned, and dried in an oven at 45°C for 3–4 days. The dried samples were then ground to powder using an electrical blender (Philips HR 2056/90TT, Netherlands) until fine (approximately 1000 μm).

Preparation of plant extracts by stirring method. In a 1:10 ratio of powdered sample and respective solvents (aqueous, chloroform, ethyl acetate, hexane, methanol), the sample was left to stir for 24 hours, and filtered. It was then concentrated using rotary evaporator and stored at 4 ± 1°C until further use.

Preparation of plant extracts by Soxhlet method [5]. 80g of dry powdered sample in 250ml of respective solvents (chloroform, ethyl acetate, hexane and methanol) for approximately 6 hours or until colourless. The extracts were then concentrated using rotary evaporator and stored at 4 ± 1°C until further use.

Preliminary qualitative phytochemical screening of plant extracts [6].

Phytochemical	Method
Saponins	Extracts were diluted with 20ml of distilled water and shaken for 15 minutes. Presence of saponins can be confirmed by formation of foam on top of the solution.
Steroids	To 5ml of extract in a test tube, 2ml of chloroform and 2ml of concentrated H_2SO_4 are added by the walls of the test tube. Presence of steroids confirmed by red colour in lower layer with yellow colour and green fluorescent in the sulfuric acid layer.
Flavonoids	10mg of extract were mixed with a few drops of diluted NaOH. Presence of flavonoids confirmed when a yellow colour disappears or turns colourless with the addition of a few drops of diluted H_2SO_4.
Alkaloids	10mg of extract was dissolved in 2ml of Wagner's solution. Presence of alkaloid is confirmed when appearance of reddish-brown coloured precipitate is observed.
Tannins	10 mg of extract was dissolved in 45% ethanol, and then boiled for 5 minutes before adding 1ml of 15% ferric chloride solution. Presence of tannins confirmed when there is appearance of dark blue or greenish black colour.

DPPH free radical scavenging ability antioxidant assay [7]. 0.1mM solution of DPPH was prepared by dissolving 0.394g of DPPH in 10ml of methanol. 10mg of plant extract/ascorbic acid was diluted in 10ml of solvent as stock solution and as positive standard, respectively. Serial dilutions were prepared in 10, 20, 30, 40, 50, 100, 250, 500 and 750 ug/ml. To 1ml of sample, added was 1ml of DPPH solution. The mixture was left to incubate in the dark for 30 minutes. A colour change from violet of the stable DPPH compound to yellow of the reduced DPPH compound can be seen. After incubation, the absorbance was read at 517.0nm using a UV-Vis spectrophotometer (JENWAY) 6850UV/Vis. Spectrophotometer). Blank sample was prepared by replacing the sample extract with the mother solvent added with DPPH solution. The readings were done in triplicates. DPPH % scavenging ability was calculated using the formula: % inhibition = [A0 – A1] / A0] x 100, where A0 is the absorbance of control and A1 is the absorbance of the extract or standard. IC_{50} value is calculated from the formula obtained in the % inhibition against concentration graph.

Ferric reducing antioxidant power assay [8]. FRAP reagent was prepared by adding 10mM of 2,4,6-Tris(2-pyridyl)-1,3,5-triazine (TPTZ) dissolved in 40mM of hydrochloric acid, 20mM of ferrous chloride ($FeCl_3$) and 300mM of sodium acetate buffer (pH 3.6) in ratio of 1:1:10. Under dark condition, 100ul of sample, 900ul of distilled water and 2ml of FRAP reagent were mixed and left to incubate for 3minutes at room temperature. A colour change into intense blue suggests a reduction of the ferric tripyridyltriazine complex. Using a spectrophotometer, absorbance was read at 593nm. The ferric reducing antioxidant ability was measure using the formula: FRAP value of sample = Absorbance (sample) x FRAP value of standard / absorbance (standard).

ABTS antioxidant assay [9]. ABTS working solution was prepared by 1:1 ABTS to 2.46 mM potassium persulfate solution. The mixture was left in the dark for 16-18hr in room temperature. The mixture was then diluted with ethanol to reach absorbance of 0.700-0.900nm before use. In a test tube, 20ul of sample with 80ul of ethanol was combined and added 2ml of ABTS working solution. A colour change from dark blue to colourless suggest a reaction. The absorbance was read at 734nm. The percentage of inhibition was measured and compared to the standard Gallic acid using the formula: ABTS·+ scavenging effect (%) = ((AB–AA)/ AB) ×100, where AB is absorbance of ABTS radical + ethanol and AA is absorbance of ABTS radical + sample/standard The reading was taken in triplicates.

Determination of total phenolic content (TPC) [10]. 10mg of extract diluted in 10ml of extract solvent; methanol and chloroform, to make concentration of 1mg/ml stock solution. Stock solution was then diluted to prepare concentrations of 500, 250, 100, 50, 10 ppm. A mixture of 0.5ml of each concentration, 2.5ml of 10% Folin-Ciocalteu's reagent in water and 2.5% ml 7% $NaHCO_3$ were prepared. The samples were left to incubate at 45˚C for 45 minutes. After incubation, absorbance was read at 765nm. When phenolic compounds are introduced, the Folin-Ciocalteu's reagent produces a blue colour. The samples were prepared in triplicates. Positive control of the assay was gallic acid. The total phenolic content was calculated using the formula: A = cV / m, where A is the total phenolic content, c is the concentration of gallic acid obtained from the curve (mg/ml), V is the volume of extract in ml and m is the weight of plant extract (g).

Determination of total flavonoid content (TFdC) [10]. 0.5ml of extract or standard solution in a test tube and adding 0.1ml of 10% aluminium chloride followed by 0.1ml of 1M potassium acetate. In the same test tube, 1.5ml of 80% methanol was added followed by 2.8ml of distilled water. They were then incubated for 30 minutes in the dark. Absorbance was read at 415nm. Standard solution was made from Quercetin. The flavonoid content was measured using the formula: A = (c x v) /m, where A is total flavonoid content in mg/g Quercetin equivalent, c is concentration of quercetin in mg/ml obtained from the standard graph equation, v is the volume of extract and m is the mass of extract. Blank was calculated as mean absorbance at 0.0959.

Determination of total flavonol content (TFC) [11]. 1ml of extract or standard solution in a test tube. In the same test tube, 1ml of 2% aluminium chloride was added to each test tube, followed by 3ml of sodium acetate. The mixture was incubated for 2hrs after shaking gently, in room temperature. Absorbance was read at 440nm using spectrophotometer. Blank was calculated as mean absorbance at 0.0032.

Data analysis. The experiments were performed in triplicate. All analyses were performed with Analysis ToolPak extension in Excel 2016 and results presented as mean ± standard deviation. A regression correlation using Pearson's used to observe any correlation between assays. ANOVA test was used to see any significant difference between the type of solvents used for the crude extracts. Statistical analyses were considered significant when $p < 0.05$.

Results and Discussion.

Extraction yields can be used to indicate the effects of extraction in respective solvents. Table 1 shows the percentage yield for all extracts of both *D. esculentum* and *S. palustris* which was conducted using two different methods: 24-hour stirring method (s) with no presence of heat and Soxhlet method. DEA: *D. esculentum* aqueous extract; DEC: *D. esculentum* chloroform extract; DEE: *D. esculentum* ethyl acetate extract; DEH: *D. esculentum* hexane extract; DEM: *D. esculentum* methanol extract; SPA: *S. palustris* aqueous extract; SPC: *S. palustris* chloroform extract; SPE: *S. palustris* ethyl acetate extract; SPH: *S. palustris* hexane extract; SPM: *S. palustris* methanol extract. Interestingly, aqueous extracts by stirring method of both plant species have shown to produce the highest yield compared to other solvents, with 8.88% and 9.40% for *D. esculentum* and *S. palustris* aqueous extracts, respectively. The higher polarity of water and the kinetics of stirring method may have caused higher and faster diffusion or partition rate of the solute to move from the solid matrix into solvent [12]. This reveals that the use of water as a sole extractant with no presence of heat is possible to give a higher yield of aqueous extracts.

Table 1. The percentage yield of D. esculentum (DE) and S. palustris (SP) extracts by stirring and Soxhlet method in different solvents of aqueous (A), chloroform (C), ethyl acetate (E), hexane (H) and methanol (M).

Extract	Yield (%)	Extract	Yield (%)
Stirring method			
DEA	8.88	SPA	9.40
DEC	0.77	SPC	1.17
DEE	1.60	SPE	3.90
DEH	4.70	SPH	1.84
DEM	5.95	SPM	0.78
Soxhlet method			
DEC	3.34	SPC	0.69
DEE	1.33	SPE	0.56
DEH	2.00	SPH	0.54
DEM	0.70	SPM	0.99

Preliminary phytochemical screening on plant extracts were observed qualitatively to indicate the presence of known antioxidants which are saponins, steroids, flavonoids, alkaloids and tannins. There is no aqueous extract for both plant species using Soxhlet method as it is not efficient due to high temperature of 100ºC is required for evaporation. Nevertheless, extraction by Soxhlet method has shown the presence of more phytochemicals qualitatively when compared to extraction by stirring method, as seen in Table 2. However, this does not confirm the complete absence of the phytochemical in that extract and their antioxidant potential, hence, we proceed to the analysis of antioxidant activities and specific phytochemical content for further evidence.

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Table 2. Phytochemical screening of D. esculentum and S. palustris extracts, (+) = present; (-) absent.

Property	Saponins		Steroids		Flavonoids		Alkaloids		Tannins	
Solvents	***D. esculentum***	***S. palustris***	***D. esculentum***	***S. palustris***	***D. esculentum***	***S. palustris***	***D. esculentum***	***S. palustris***	***D. esculentum***	***S. palustris***
Stirring method										
Aqueous	+	+	-	-	-	-	-	-	-	-
Chloroform	+	+	-	-	-	-	-	-	-	-
Ethyl acetate	-	-	-	-	-	-	-	+	-	-
Hexane	-	-	+	+	-	-	-	-	-	-
Methanol	+	+	-	-	-	-	-	+	-	-
Soxhlet method										
Chloroform	+	+	-	+	+	+	+	+	-	-
Ethyl acetate	+	+	+	+	-	+	+	+	-	-
Hexane	-	-	+	-	-	-	-	-	-	-
Methanol	-	+	-	-	+	+	+	+	-	-

In vitro antioxidant activities of plant extracts. There are various colorimetric methods that can be used to analyse *in vitro* antioxidant activities. These methods undergo different reaction mechanisms, hence the need to analyse antioxidant activities of samples using more than one colorimetric method to obtain promising results. There are six different antioxidant assays used in this study, which are 2, 2–diphenyl–1–picrylhydrazyl (DPPH), 2,2'-azino-bis 3-ethylbenzothiazoline-6-sulphonicacid (ABTS), ferric reducing-antioxidant power (FRAP), total phenolic content (TPC), total flavonoids content (TFdC) and total flavonol content (TFC).

Based on the results in Table 3, it is interesting to observe that aqueous extract and stirring method revealed high antioxidant activities and phytochemical content in some assays conducted. This is particularly in FRAP assay whereby aqueous extract of *D. esculentum* (DEA) had the highest FRAP value compared to the other solvents with 687.57 $\pm$ 0.01 µg Fe(II)E/ml, which also correlates with its high TPC value of 29.06 ± 4.21 mg GAE/g showing that phenolic content has relation towards ferric reducing potential. Water is also known to be a good solvent for phenolic acids which could be the reason for the high TPC value in DEA [13].

When comparing between the two extraction methods, stirring method has shown to produce high antioxidant activity and phytochemical content. This can be seen in DPPH assay where DEC (stirring) extract gave the highest IC_{50} value of 42.19 ± 0.407 µg/ml, the highest value in ABTS assay is seen by SPE (stirring) extract of 84.72 ± 0.001 µg GAE/ml, the highest value in FRAP assay is DEA (stirring) extract with 687.57 $\pm$ 0.01 µg Fe(II)E/ml, DEM (stirring) extract gave the highest TPC value of 31.87 ± 5.32 mg GAE/g and DEA at 29.06 ± 4.21 mg GAE/g, and lastly, highest TFC value is by SPC (stirring) extract with 63.77 ± 0.00 mg QE/g. This shows that stirring method without the presence of heat allows better permeability for compounds in plant materials to be extracted into the solvent matrix as compared to Soxhlet method [12].

Conclusion

Extraction method by combination of stirring method with no presence of heat and the use of aqueous as solvent is a promising method towards green environment. This method is non-toxic, has low environmental impact, provides low cost and energy and can prevent the degradation of bioactive compounds as there is no heat involved. Aqueous extracts of *D. esculentum* and *S. palustris* produced the highest yield compared to other solvents, when using the stirring method instead of Soxhlet method. In addition, *D. esculentum* and *S. palustris* have shown to have good antioxidant activities that can be applied into many areas such as functional food and pharmaceutical sectors.

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Table 3 In vitro antioxidant activities and total phenolic (TPC), flavonoid (TFdC) and flavonol (TFC) contents of D. esculentum and S. palustris extracts

Extract	DPPH IC_{50} (µg AAE/ml)	ABTS µg GAE/ml	FRAP µg Fe(II)E/ml	TPC mg GAE/g	TFdC mg QE/g	TFC mg QE/g
			Stirring method			
DEA	953.56 ± 0.112 [14]	42.49 ± 0.001	687.57 ± 0.01	29.06 ± 4.21 [14]	2.74 ± 0.01	51.54 ± 0.22
DEC	42.19 ± 0.407	35.67 ± 0.000	47.46 ± 0.01	13.97 ± 12.89	44.79 ± 0.10	3.67 ± 0.04
DEE	1022.85 ± 0.128	41.72 ± 0.001	31.33 ± 0.00	6.39 ± 7.97	57.10 ± 0.17	5.52 ± 0.04
DEH	187.55 ± 0.296	36.39 ± 0.000	48.55 ± 4.7E-05	0.35 ± 15.40	33.71 ± 0.12	16.47 ± 0.04
DEM	65.98 ± 0.918	48.66 ± 0.001	118.25 ± 0.00	31.87 ± 5.32	40.26 ± 0.10	6.05 ± 0.00
SPA	838.26 ± 0.199 [14]	65.93 ± 0.000	92.70 ± 0.00	27.18 ± 0.46 [14]	12.92 ± 0.19	-
SPC	2387.34 ± 0.276	50.72 ± 0.000	40.16 ± 4.7E-05	6.70 ± 17.06	53.94 ± 0.18	63.77 ± 0.00
SPE	372.41 ± 0.223	84.72 ± 0.001	63.51 ± 0.00	5.98 ± 5.01	46.86 ± 0.24	1.80 ± 0.01
SPH	1238.28 ± 0.235	48.72 ± 0.001	48.52 ± 0.00	24.35 ± 21.21	53.47 ± 0.22	48.56 ± 0.04
SPM	127.16 ± 0.390	47.58 ± 0.000	56.33 ± 0.00	4.81 ± 12.71	64.95 ± 0.18	5.38 ± 0.03
			Soxhlet method			
DEC	2372.43 ± 0.066*[b]	43.63 ± 0.001*[b]	39.04 ± 0.00*[b]	18.77 ± 10.69	28.77 ± 0.12*[b]	2.84 ± 0.01[b]
DEE	61.72 ± 0.264*[b]	46.37 ± 0.001*[b]	142.36 ± 0.00*[b]	14.66 ± 3.77*	60.23 ± 0.21[b]	59.75 ± 0.01*[b]
DEH	476.18 ± 0.413*[b]	48.41 ± 0.001*[b]	37.39 ± 0.00*[b]	20.21 ± 5.77*	24.66 ± 0.32[b]	27.12 ± 0.11[b]
DEM	1890.28 ± 0.081*[b]	51.98 ± 0.001*[b]	80.51 ± 0.00*[b]	5.41 ± 5.48	34.26 ± 0.10[b]	47.98 ± 0.00*[b]
SPC	415.86 ± 0.115*[d]	51.77 ± 0.001*[d]	42.38 ± 0.00*[d]	19.87 ± 3.09*	15.92 ± 0.10*[d]	13.86 ± 0.01*[d]
SPE	433.00 ± 0.245*[d]	46.60 ± 0.001*[d]	104.96 ± 0.00*[d]	26.21 ± 6.47*	60.05 ± 0.13[d]	4.06 ± 0.03[d]
SPH	275.34 ± 0.086[d]	46.91 ± 0.000*[d]	34.97 ± 0.00*[d]	30.64 ± 5.88	48.35 ± 0.08[d]	18.36 ± 0.00*[d]
SPM	777.10 ± 0.144[d]	47.28 ± 0.001[d]	110.55 ± 0.00*[d]	13.85 ± 13.16	90.35 ± 0.17*[d]	38.31 ± 0.02[d]
Standard	Ascorbic acid 14.04 ± 0.141	Gallic acid 100ug/ml 173.90 ± 0.023	Quercetin (100ug/ml) 100.15 ± 0.00	-	-	-

Conflict of interest.
There is no conflict of interests.

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