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The Effect of Extraction Solvent on the Phytochemical Contents and Antioxidant and Acetylcholinesterase Inhibitory Activities of Extracts from the Leaves, Bark and Twig of *Dipterocarpus alatus*

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ABSTRACT

The leaves, bark, and twigs of *Dipterocarpus alatus* have been used to treat many diseases because they contain large amounts of bioactive compounds that can protect against free radical damage. The extraction process is a key step in the utilization of bioactive compounds from plants. Therefore, the aim of this study was to determine the phytochemical compounds, antioxidant capacity and acetylcholinesterase (AChE) inhibitory activity of extracts of *D. alatus* leaves, bark and twig prepared using three extraction solvents with different polarities; methanol (MeOH), ethanol (EtOH) and isopropyl alcohol (IPA). The results revealed that the bark and twig extracts had a strong antioxidant activity, while the leaves had a potential AChE inhibitory activity. The strong antioxidant activity found in MeOH and EtOH extracts was related to their phenolic acid contents. A class of triterpenoid compounds, dipterocarpol and betulonic acid, which were abundant in the IPA extract, and their presence correlated with AChE inhibitory activity. Solvent polarity was therefore a critical extraction parameter affecting phytochemical content and biological activity.

Keywords: *D. alatus*, Solvent extraction, Antioxidant, AChE

Introduction

Dipterocarpus alatus is an Asian folk medicinal plant included in the traditional recipes of several countries.[1] Several parts of *D. alatus* have been shown to possess bioactivities. Methanolic extracts of leaves, bark and twig exhibited high potential of antioxidant activity, which can be attributed to the phenolic content, while the crude oleoresin had a lower antioxidant activity.[2] Similarly, methanolic *D. alatus* leaves, bark and twig extracts showed anti-inflammatory activity in LPS-stimulated RAW 264.7 cells, reducing production of the inflammatory mediators NO and PGE_2.[3] In addition, methanolic extracts of *D. alatus* leaves, bark, twig and wood showed antibacterial activity against methicillin-resistant *Staphylococcus aureus*, where the twig extract had the highest antibacterial activity.[4] An ethanolic extract of *D. alatus* leaves also showed antidepressant-like effects in an unpredictable chronic mild stress mouse model of depression.[5] Oligostilbenoids from *D. alatus* stem wood, such as dipterocarpol A and hopeahainol A, showed high inhibitory activity against acetylcholinesterase (AChE).[6] Vaticaffinol from *D. alatus* branches and twigs showed anti-hyperuricemic and anti-inflammatory effects on mouse kidneys.[7]

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Solvent selection plays an important step in the extraction of desired compounds from raw plant materials. Methanol (MeOH), ethanol (EtOH) and isopropyl alcohol (IPA) are usually used for phytochemical extraction based on their differences in polarity. The aim of this work was to evaluate the phytochemical components and bioactivities of *D. alatus* leaves, bark and twig extracts prepared from different solvents and to describe the variability of phytochemical components and their bioactivity using Principal Component Analysis (PCA).

Materials and Methods

Plant Extraction

The leaves, bark and twig of *Dipterocarpus alatus* were collected in Khon Kaen province, Thailand during December 2020. A voucher specimen (No. PSKKF03682) was identified by Associated Professor Suppachai Tiyaworanant and deposited in Faculty of Pharmaceutical Sciences, Khon Kaen University. The *D. alatus* samples were ground into a dry powder and 100 g of *D. alatus* was soaked in MeOH, EtOH and IPA solvent. Extraction was performed by sonication for 30 min which was controlled at an ultrasonic frequency of 50/60 Hz and 220 Volts by Ultrasonic cleaners (TRU-SWEEP™, NY, USA) and this was repeated three times. The combined organic solvent was evaporated after filtration to obtain MeOH, EtOH and IPA extracts, respectively. All extracts were kept in a -20 °C freezer until use.

Qualitative and Quantitative Analysis of Phytochemicals by HPLC

The dipterocarpol and betulonic acid contents were measurement by HPLC (Agilent 1100 Series) with a Phenomenex column (Luna 5 µm C18 100 Å 150 × 3.9 mm). The mobile phase consisted of solvent A: solvent B a ratio of 8:2 (acetonitrile (solvent A) and 0.05% trifluoroacetic acid (v/v) in purified water (solvent B)) with a flow rate of 1 mL/min. The column temperature was 25°C and the injection volume was 20 µL. The UV-diode array detection wavelength was 210

nm. The presence of dipterocarpol and betulonic acid in *D. alatus* samples was based on the retention time.[8]

The phenolic acid and flavonoid contents, namely gallic acid (GA), protocatechuic acid (PCCA), *p*-hydroxybenzoic acid (*p*-HO), vanillic acid (VA), syringic acid (SyA), chlorogenic acid (ChA), *p*-coumaric acid (*p*-CA) ferulic acid (FA), rutin (RU), and quercetin (QU) were measured using the HPLC method. Briefly, the extract was passed through a Hichrom 5 C18 column of 4.6 × 250 mm. The mobile phase consisted of purified water with 1% acetic acid (v/v) (solvent A) and acetonitrile (solvent B). The system was described by our previous report.[9] The flow rate was 0.8 ml/min, column temperature 40°C and the injection volume was 20 µL. The respective UV-diode array detection wavelengths were 280 nm for hydroxybenzoic acids and 320 nm for hydroxycinnamic acids. The HPLC chromatograms for extracts and standard phenolic compounds were compared.

Determination of Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

Total phenolic content (TPC) was determined using the Folin-Ciocalteu assay. Briefly, Folin-Ciocalteu reagent was diluted 10-fold with purified water before use. The *D. alatus* sample was mixed with Folin-Ciocalteu reagent in 96-well plates. After 5 min, 7% sodium carbonate was added and incubated for 30 min. The absorbance of the mixtures was read at 760 nm by microplate reader (Sunrise Tecan, Grödig, Austria). The total phenolic content is shown as gallic acid equivalents (mg GAE/g extract).[10] Total flavonoid content (TFC) was determined by mixing the *D. alatus* sample was mixed with 2%$AlCl_3$ in a 1:1 ratio into 96-well plates and incubated for 20 min. The absorbance of the mixtures was read at 415 nm by microplate reader. The total flavonoid content is shown as quercetin equivalents (mg QE/g extract).[11]

Antioxidant Capacities

The 200 µM 2,2-diphenyl-2-picrylhydrazyl (DPPH) reagent was prepared in MeOH and mixed with different concentrations of *D. alatus* samples in 96-well plates. After an incubation time of 30 min, the absorbance of the mixtures was read at 490 nm by microplate reader. Trolox was used as a standard and the IC_{50} was calculated.[12] The 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was prepared in potassium persulfate in purified water to generated the radical cation ($ABTS^{\bullet+}$) in dark conditions. The $ABTS^{\bullet+}$ reagent was mixed with different concentrations of *D. alatus* sample in 96-well plates. Then, the plates were incubated for 10 min at room temperature in dark conditions. The absorbance of the mixtures was read at 415 nm by microplate reader. Trolox was used as a standard and the IC_{50} was calculated.[13] The ferric reducing antioxidant power (FRAP) reagent was prepared by mixing 300 mM acetate buffer pH 3.6, 10 mM 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3$ solution in a 10:1:1 ratio. FRAP reagent was mixed with *D. alatus* samples. After 4 min, the absorbance of the mixtures was read at 595 nm by microplate reader. Trolox was used as a standard.[14]

Acetylcholinesterase (AChE) Inhibitory Activity

The acetylcholinesterase (AChE) inhibitory activity of *D. alatus* was determined using Ellman's spectrophotometric assay. Briefly, the *D. alatus* sample was dissolved in 50 mM TRIS/HCl buffer pH 8.0. After that, the sample (100 µg/mL) was mixed with 0.2 units/mL AChE in 96-well plates and incubated for 15 min. Then, 1.5 mM acetylthiocholine iodide (ATCI) and 1 mM 5,5'-dithio-bis(2-nitrobenzoicacid) (DTNB) were added and incubated for 15 min. The absorbance of the mixtures was read at 405 nm by microplate reader. Tacrine (1 µg/mL) was used as a standard.[15]

Statistical Analysis

All experiments were performed in triplicate unless otherwise mentioned and the results are reported as mean ± standard deviation (SD). Analysis of variance (ANOVA) was carried out with SPSS 23.0. Tukey's HSD (Tukey's Honest Significant Difference) test was used to determine significant differences between samples at $p<0.05$. Principal Component Analysis (PCA) was performed using XLSTAT.

Results and Discussion

Antioxidant Capacities

The antioxidant properties of MeOH and EtOH bark extracts were superior to all other extracts, as shown in Table 1. In terms of antioxidant capacity, MeOH and EtOH extracts from bark and twig showed strong antioxidant activity in the DPPH, ABTS and FRAP assays that are correlated with the polarity index of the solvent used for extractions, as shown in Table 1. The IPA extract of leaves, bark and twig showed lower antioxidant activity than the MeOH and EtOH extracts. However, these extracts had 2- to 5-fold lower antioxidant activities than trolox. This result suggests that the most effective solvent for antioxidant extraction were MeOH, EtOH and IPA, respectively. Differences in extraction solvents that affect the concentration, antioxidant activity and bioactive substances in extracts have been reported.[16]

AChE Inhibitory Activities

Regarding AChE inhibitory activity, the EtOH and IPA extracts of the leaves showed high inhibitory levels of 93.21 ± 0.07% and 93.97 ± 0.21%, respectively. In addition, EtOH and IPA extracts of bark and twig had stronger AChE inhibition than MeOH extract. However, extracts of the leaves, bark and twig of *D. alatus* had a lower AChE activity than the positive control as tacrine in Table 1.

Phytochemical Constituents

As shown in Table 2, the highest TPC was found in EtOH bark and MeOH leaves extracts with TPC values of 297.49±0.65 and 220.19±7.58 mg GAE/g extract, respectively. The highest TFC was in MeOH leaves extract (64.72±0.11 mg QE/g extract). Dipterocarpol, a phytochemical component of *D. alatus*, was found in high yield in IPA extracts, especially in IPA twig extract (76.54±2.62 mg/g) and IPA bark extract (34.11±0.14 mg/g). Dipterocarpol has been reported to be an important component of *Dipterocarpus* plant family that has shown anticancer activity in several cancer cell lines.[8,17] Betulonic acid was also extracted in high yield in IPA solvent. It has been reported to have good antiviral activity.[18]

The polyphenols content of the extracts is shown in Table 3. Sinapic acid (SyA) was the highest yielding polyphenol in EtOH bark extract (2519.60±6.99 mg/g extract). Gallic acid (GA) was abundant in all parts of *D. alatus* and all solvent extracts. The highest concentrations of GA was found in the EtOH extract of bark (2483.64±3.74 mg/g extract). The highest relative concentration of phenolic acids was observed in EtOH bark extract, followed by the MeOH twig, MeOH bark and EtOH twig extracts, respectively. Interestingly, the bark contained different phenolic acid components more than the leaves and twig parts of *D. alatus* (leaves and twig). Ethanol and methanol can be considered good solvents for phytochemical extraction of *D. alatus*, and solvent polarity greatly affected TPC and TFC values, which is mainly increased along with the increasing solvent polarity indices.[19]

Pearson's Correlation

The Pearson correlations between the phytochemical content and bioactivity (antioxidant and AChE inhibitory activity) of *D. alatus* leaves, barks and twigs extracted in MeOH, EtOH and IPA are presented in Table 4. In the methanolic extracts, RU and TFC were strongly correlated with antioxidant (DPPH; r = 0.779) and AChE inhibitory (r = 0.981) activities. The phenolic acids; *p*-CA, PCCA, *p*-HO, FA, GA and VA showed a moderate to high correlations with AChE inhibitory activity. In the ethanol extracts, most phenolic acids and TPC were highly correlated with antioxidant capacity (ABTS, DPPH, and FRAP), while dipterocarpol was moderately correlated with antioxidant capacity. In the IPA extracts, betulonic acid and TPC showed a strong correlation with antioxidant capacity. The phenolic acids, PCCA, VA, SyA and GA were also correlated with antioxidant capacity.

Table 1: Antioxidant and AChE Inhibitory Activities of *D. alatus* Extracts

Sample	Solvent	Polarity Index	%Yield	Antioxidant Capacities			AChE inhibitory (%inhibition)
				DPPH; IC_{50} (µg/ml)	ABTS; IC_{50} (µg/ml)	FRAP value (mmol/100g extract)	
	MeOH	5.1	8.66	13.45 ± 0.07^{e}	9.77 ± 0.05^{f}	333.34 ± 14.63^{e}	47.44 ± 0.50^{c}
Leaves	EtOH	4.3	4.12	28.69 ± 0.17^{g}	11.79 ± 0.15^{g}	178.76 ± 2.93^{f}	93.21 ± 0.07^{b}
	IPA	3.9	2.61	71.53 ± 0.35^{h}	21.82 ± 0.11^{h}	119.87 ± 2.27^{f}	93.97 ± 0.21^{b}
	MeOH	5.1	9.65	7.92 ± 0.08^{c}	5.19 ± 0.03^{b}	952.26 ± 35.77^{b}	16.39 ± 0.21^{g}
Bark	EtOH	4.3	4.35	5.68 ± 0.05^{b}	5.62 ± 0.03^{c}	1007.81 ± 32.04^{b}	24.21 ± 0.26^{d}
	IPA	3.9	1.83	10.38 ± 0.02^{d}	7.92 ± 0.07^{d}	391.40 ± 12.14^{d,e}	21.64 ± 0.23^{e}
	MeOH	5.1	7.34	8.24 ± 0.04^{c}	7.71 ± 0.03^{d}	723.93 ± 22.65^{c}	11.53 ± 0.73^{h}
Twig	EtOH	4.3	4.14	8.41 ± 0.04^{c}	9.11 ± 0.04^{e}	447.93 ± 18.39^{d}	19.14 ± 0.30^{f}
	IPA	3.9	1.54	27.08 ± 0.68^{f}	25.10 ± 0.18^{i}	180.43 ± 3.54^{f}	23.42 ± 0.26^{d}
	Trolox			3.74 ± 0.08^{a}	2.77 ± 0.01^{a}	5352.78 ± 51.97^{a}	-
	Tacrine (1 µg/ml)			-	-	-	98.82 ± 0.00^{a}

Note: Letters indicate the significant difference in data between a row in the same columns at $p<0.05$ using one-way ANOVA with Tukey HSD.

Table 2: Phytochemical Contents of *D. alatus* Extracts

Sample	Solvent	TPC (mgGAE/g extract)	TFC (mgQE/g extract)	Dipterocarpol (mg/g)	Betulonic acid (mg/g)
	MeOH	220.19 ± 7.58^{c}	64.72 ± 0.11^{a}	4.87 ± 0.16^{e}	1.81 ± 0.01^{e}
Leaves	EtOH	178.57 ± 4.30^{d,e}	58.09 ± 0.26^{b}	10.60 ± 0.71^{d}	4.58 ± 0.78^{d}
	IPA	115.20 ± 4.26^{f}	42.80 ± 0.70^{d}	18.03 ± 0.89^{c}	5.55 ± 0.34^{c,d}
	MeOH	255.49 ± 4.66^{b}	4.57 ± 0.06^{h}	ND	2.35 ± 0.07^{e}
Bark	EtOH	297.49 ± 0.65^{a}	5.35 ± 0.06^{h}	28.42 ± 0.10^{b}	2.29 ± 0.35^{e}
	IPA	173.01 ± 2.52^{e}	14.82 ± 0.11^{f}	ND	34.11 ± 0.14^{a}
	MeOH	194.01 ± 4.70^{d}	9.11 ± 0.25^{g}	21.25 ± 0.05^{c}	1.31 ± 0.28^{e}
Twig	EtOH	252.45 ± 6.53^{b}	22.00 ± 0.39^{e}	ND	6.44 ± 0.01^{b,c}
	IPA	88.88 ± 2.37^{g}	54.61 ± 0.59^{c}	76.54 ± 2.62^{a}	7.05 ± 0.15^{b}

Note: Letters indicate the significant difference in data between a row in the same columns at $p<0.05$ using one-way ANOVA with Tukey HSD. ND means Not detect

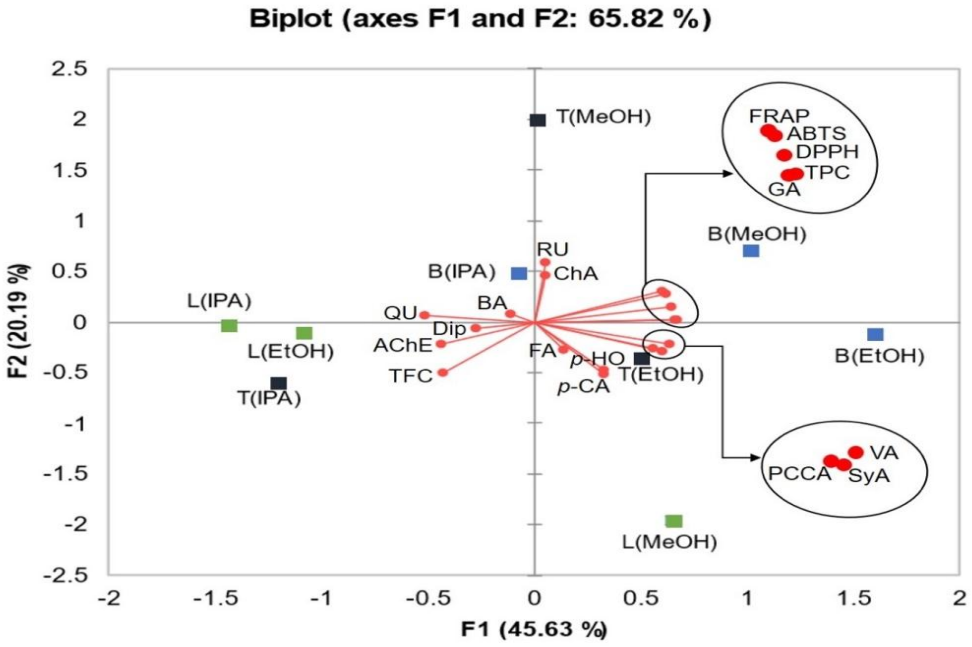

Figure 1: (A) PCA biplot of *D. alatus* Leaves (L), Bark (B) and Twig (T) Extracts on the Effect of Different Solvent Extraction. Symbol ■ and ● were Denoted as Observation and Variables, respectively.

Table 3: Phenolic Acid and Flavonoid Contents of *D. alatus* Extracts

Compound	MeOH			EtOH			IPA		
	Leaves	**Bark**	**Twig**	**Leaves**	**Bark**	**Twig**	**Leaves**	**Bark**	**Twig**
QU	165.88 ± 0.92^{a}	105.54 ± 0.13^{h}	136.51 ± 1.12^{e}	167.27 ± 0.78^{a}	117.47 ± 0.56^{g}	144.45 ± 1.15^{d}	149.49 ± 0.42^{c}	125.32 ± 0.33^{f}	155.60 ± 0.05^{b}
RU	1584.88 ± 2.65^{a}	687.19 ± 5.20^{b}	51.20 ± 0.62^{f}	107.00 ± 2.16^{e}	ND	4.47 ± 0.33^{g}	190.14 ± 1.49^{d}	302.72 ± 0.59^{c}	47.69 ± 0.27^{f}
FA	ND	65.80 ± 4.13^{e}	80.07 ± 3.95^{d}	ND	60.51 ± 1.27^{f}	111.38 ± 1.45^{c}	ND	121.48 ± 1.48^{b}	141.52 ± 0.58^{a}
p-CA	4.86 ± 0.18^{g}	18.29 ± 0.76^{f}	115.33 ± 1.97^{a}	ND	41.06 ± 1.44^{c}	87.16 ± 1.43^{b}	ND	22.20 ± 0.24^{e}	34.03 ± 0.27^{d}
ChA	ND	ND	22.46 ± 2.15^{a}	ND	ND	8.60 ± 0.19^{b}	ND	ND	ND
SyA	257.37 ± 8.1^{i}	2064.95 ± 16.43^{c}	2360.02 ± 26.65^{b}	452.92 ± 1.50^{f}	2519.60 ± 6.99^{a}	1182.34 ± 10.96^{d}	402.31 ± 2.24^{g}	504.52 ± 2.85^{e}	291.53 ± 0.05^{h}
VA	ND	1232.83 ± 37.75^{b}	1247.15 ± 19.54^{b}	90.36 ± 2.24^{e}	1967.82 ± 5.62^{a}	750.30 ± 5.63^{c}	ND	408.77 ± 1.89^{d}	45.87 ± 1.08^{f}
p-HO	87.20 ± 5.22^{c}	ND	414.38 ± 3.04^{a}	27.85 ± 0.88^{e}	181.88 ± 2.14^{b}	185.66 ± 2.17^{b}	ND	0.49 ± 0.08^{f}	68.38 ± 9.30^{d}
PCCA	143.41 ± 0.89^{e}	152.72 ± 1.82^{d}	346.05 ± 3.22^{a}	44.67 ± 0.55^{f}	230.17 ± 0.17^{b}	163.36 ± 1.93^{c}	ND	29.30 ± 0.87^{g}	ND
GA	1711.12 ± 3.60^{d}	2034.29 ± 9.33^{b}	2053.58 ± 14.65^{b}	670.12 ± 3.01^{f}	2483.64 ± 3.74^{a}	1726.45 ± 10.57^{d}	25.98 ± 0.16^{g}	1787.49 ± 2.99^{c}	795.67 ± 1.04^{e}

Note: Letters indicate the significant difference in data between a column in the same rows at $p<0.05$ using one-way ANOVA with Tukey HSD. ND means Not detect. QU: quercetin, RU: rutin, FA: ferulic acid, *p*-CA: p-coumaric acid, ChA: chlorogenic acid, SyA: sinapic acid, VA: vanillic acid, *p*-HO: p-hydroxybenzoic acid, PCCA: protocatechuic acid, GA: gallic acid.

Table 4: Pearson's Correlation Coefficient (*r*) Between Chemical Contents, Antioxidant Capacities and Acetylcholinesterase (AChE) Inhibitory Activity of *D. alatus* Leaves, Bark, Twigs Extracts

Parameter	Methanol				Ethanol				IPA			
	ABTS	DPPH	FRAP	AChE	ABTS	DPPH	FRAP	AChE	ABTS	DPPH	FRAP	AChE
TPC	0.507	0.141	0.443	0.040	0.980	0.972	0.943	-0.902	0.980	0.807	0.865	-0.232
TFC	-0.929	-1.000*	-0.954	0.981	-0.963	-0.954	-0.917	0.930	-0.983	-0.817	-0.874	0.249
Dip	-0.142	0.245	-0.070	-0.415	0.668	0.693	0.764	-0.084	-0.757	-0.417	-0.511	-0.271
BA	0.451	0.078	0.385	0.103	-0.602	-0.628	-0.705	-0.002	0.988	0.963	0.986	-0.558
GA	-0.127	-0.495	-0.199	0.643	0.988	0.982	0.957	-0.882	0.848	0.992	0.972	-0.839
PCCA	-0.884	-0.995	-0.915	0.996	0.975	0.967	0.936	-0.911	0.994	0.949	0.977	-0.519
p-HO	-0.970	-0.989	-0.985	0.947	0.822	0.802	0.736	-0.999*	-0.586	-0.195	-0.299	-0.487
VA	-0.085	-0.458	-0.157	0.610	0.994	0.997*	1.000*	-0.726	0.978	0.976	0.994	-0.603
SyA	-0.206	-0.563	-0.277	0.702	0.994	0.997*	1.000*	-0.730	0.905	0.647	0.724	0.002
ChA	0.077	0.451	0.15	-0.604	-0.062	-0.096	-0.198	-0.552	a	a	a	a
p-CA	-0.849	-0.986	-0.885	1.000*	0.415	0.384	0.286	-0.880	0.066	0.475	0.379	-0.932
FA	-0.243	-0.594	-0.313	0.729	0.489	0.460	0.365	-0.916	0.280	0.654	0.570	-0.988
RU	0.482	0.779	0.544	-0.879	-0.853	-0.835	-0.774	0.995	0.886	0.612	0.693	0.046
QU	-0.445	-0.071	-0.379	-0.110	-1.000*	-0.999*	-0.989	0.806	-0.997	-0.871	-0.919	0.346

Note: * Significant at the 0.05 level (2-tailed). a means cannot be computed because at least one of the variables is constant.

Principal Component Analysis (PCA)

This study used PCA to evaluated and describe the variation in phytochemical contents and their bioactivities. PCA showed F1 and F2 with an eigenvalues greater than 1, which explained 65.82% of the variation in the data (Figure 1). The samples were grouped in F1 and had a strong antioxidant capacity (ABTS, DPPH and FRAP) and high values of TPC and phenolic acids namely VA, PCCA, SyA, and GA. Dipterocarpol (Dip) and betulonic acid (BA) showed a closer relationship with AChE inhibitory activity. IPA leaves, EtOH leaves and IPA twig extracts were the grouped with F2. Based on the cluster analysis, the dataset was divided into 2 clusters. Cluster I, which contained extracts of EtOH bark (B(EtOH)), MeOH bark (B(MeOH)), MeOH leaves (L(MeOH)), EtOH twigs (T(EtOH)), MeOH twigs (T(MeOH)), and IPA bark (B(IPA)) extracts and had strong antioxidant activity and high phenolic acid content. Cluster II consisted of IPA leaves (L(IPA)), IPA twigs (T(IPA)), and EtOH leaves (L(EtOH)) and had high AChE inhibitory activity and high concentrations of Dip, BA, and QU.

Conclusion

The highest antioxidant activity was found in EtOH bark extract. PCA analysis revealed that the high phenolic contents found in EtOH and MeOH extracts had a positive correlation with antioxidant activity. IPA leaves extract showed strong AChE inhibitory activity. The high dipterocarpol and betulonic acid were analyzed in a high yields in IPA extracts and showed a correlation with AChE inhibitory activity. It can be concluded from our study that the high polarity index solvents, MeOH and EtOH were capable of extracting more phenolic compounds than the higher than low polarity index solvent IPA. In contrast, the non-polar compound which is dipterocarpol and betulonic acid compounds were more efficiently extracted with low polarity index IPA than high polarity index MeOH and EtOH. The highest yielding extraction solvents for phenolics, dipterocarpol and betulonic acid from *D. alatus* will assist future investigations of their biological activities.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.\

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