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Phenolic Profiling with Antioxidant Activity of Thai Traditional Medicines: Phikud Bencha Thien, Kot and Keson Remedies

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ABSTRACT

Phikud Bencha Thien (BT), Phikud Bencha Kot (BK), and Phikud Bencha Keson (BKS) are three of the most well-known compositions in Thai traditional medicine and are included in a wide range of Thai polyherbal formulas. Each individual formulation has potential as a rich source of natural antioxidants, especially polyphenolic compounds. The present study determined the total phenolic content, total flavonoid content, and antioxidant capacity of water and methanol extracts of these three remedies as well as identifying the phenolic acid and flavonoid compounds present in these remedies using HPLC. Antioxidant activity was determined using DPPH and ABTS reduction assays, the ferric reducing antioxidant power (FRAP) assay, and the oxygen radical absorbance capacity (ORAC) assay. Principal Component Analysis and Hierarchical Cluster Analysis were used to classify relationships between the extract contents and antioxidant activity. Methanol extracts had the highest total phenolic and total flavonoid contents. Antioxidant activities in each assay varied between methanol and water extracts. The BKS extract exhibited significantly stronger antioxidant activity than the BT and BK extracts in all antioxidant models. Caffeic acid was found in high amounts in BT and BK extracts, whereas gallic acid was found as the major constituent of BKS extract. Multivariate analysis revealed that methanol and water extracts of BKS displayed the highest antioxidant capacities and had the highest phytochemical contents.

***Keywords*:** phikud, Thai traditional medicine, antioxidant, phenolics, principal component analysis

Introduction

The concepts and theories of Thai traditional medicine (TTM) are associated with and influenced by Buddhism and Ayurvedic medicine and are integrated with Thai culture and lifestyle. The main precept of treatment is the balancing and harmonization of the four body elements: earth, water, wind, and fire, using herbal medicines, massage, and health maintenance practices.[1] Poor dietary habits, exposure to air pollution and chronic stress are the types of factors that can cause an imbalance in the four elements and lead to illness. While there is a broad array of Thai medicinal plants and herbal formulations that have been utilized as treatments, there are three major sets of crude drugs that are normally included in each formulation. These sets of crude drugs are Phikud Bencha Thien (BT), Phikud Bencha Kot (BK), and Phikud Bencha Keson (BKS). The word "Phikud" in Thai means the drug formula consists of at least two herbal ingredients and the word "Bencha" means five. Therefore, the BT, BK, and BKS formulations all consist of five herbal ingredients, as shown in Table 1.

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BT, BK, and BKS are unique herbal formulations prepared from the root, seed, and flower parts of medicinal herbs and they are likely to contain a wide variety of natural antioxidants, such as phenolic acids, flavonoids, and tannins, and possess antioxidant activity. Some previous studies have investigated herbal formulae that contain BT, BK, or BKS as ingredients and found in vitro erythrocyte protective effects, anti-inflammatory activity, and blood cholesterol lowering activity.[2,3,4,5,6] However, to our knowledge, there has been no previous study examining the chemical constituents and antioxidant activity of BT, BK and BKS. Phenolic acids and flavonoids from were the main focus of the current study due to their well-known antioxidant activity. The objectives of the current study were to determine the phenolic profile of BT, BK, and BKS extracts using high-performance liquid chromatography (HPLC) and to evaluate their antioxidant activities. It is hoped that the findings from this study will provide scientific evidence to support their use according to Thai traditional medicine concepts and work as a guide for further pharmacological studies of these formulae as health maintenance products.

Materials and methods

Herbal Material and Crude Extraction

The BT, BK and BKS remedies were purchased from Charoensuk Pharma Supply Co., Ltd., Nakhon Pathom, Thailand in March 2021. All extractions were conducted in triplicate. One gram of each remedy was ground (Model 800G Grinder Machine, Jing Gong Yi, China), added to 10 mL of water and boiled for 30 min. The aqueous layers were combined, evaporated, and freeze-dried to obtain the aqueous extracts. One gram of each remedy was mixed with methanol (4 mL) and macerated overnight. The combined organic layers were evaporated to

dryness using a rotary evaporator (Rotavapor® R-100, Buchi, Switzerland) at 40ºC to obtain the methanol extracts. All extracts were kept at -20°C for analysis.

Determination of Total Phenolic and Total Flavonoid Contents

Total phenolic contents (TPC) of the aqueous and methanolic extracts were determined by the Folin Ciocalteu reagent method with some modifications.[7] Briefly, the extract (20 µL) was mixed with 100 µL 10% (v/v) Folin-Ciocalteu's reagent (Merck, Darmstadt, Germany). Then, 80 µL of 7% sodium carbonate (Na_2CO_3) were added and the mixture was incubated for 30 min. The absorbance was read at 760 nm using a microplate reader (Sunrise Tecan, Grödig, Austria). TPC was estimated from a five-point calibration curve standard curve of the gallic acid in the range 0.5 to 50 µg/mL, and the results are expressed as mg gallic acid equivalents per gram of extract (mgGAE /g extract). For total flavonoid content (TFC), the extract (100 µL) was mixed with 2% (w/v) aluminum chloride (100 µL) and incubated for 30 min.[8] The absorbance was measured at 415 nm. Quercetin was used as standard and TFC was expressed as quercetin equivalents (mg QE/g extract).

Determination of Phenolic Acid and Flavonoid Contents by HPLC

HPLC analysis was carried out using a Unisol C18 column (4.6 x 250 mm) with a diode array detector according to our previous report.[9] The detection was set at 280 nm for the hydroxybenzoic acids, gallic acid (GA), protocatechuic acid (PCCA), *p*-hydroxybenzoic acid (*p*-HO), vanillic acid (VA) and syringic acid (SyA); 320 nm for the hydroxycinnamic acids, chlorogenic acid (ChA), *p*-coumaric acid (*p*-CA), ferulic acid (FA), caffeic acid (CFA), sinapic acid (SNA); and 370 nm for the flavonoids; rutin (RU) and quercetin (QU). The results are expressed as µg/g extract.

Antioxidant Capacity

The antioxidant capacity of the extracts was determined using four methods; 2,2-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) reduction assay, the ferric reducing antioxidant power (FRAP) assay, and the oxygen radical absorbance capacity (ORAC) assay. For the DPPH assay, the extracts were mixed with 200 µM DPPH reagent in a 96-well plate and kept in dark conditions for 30 min.[10] The absorbance was measured at 517 nm using a microplate reader with Trolox as a standard. The inhibition was calculated as follows:

Inhibition (%) = [(Absorbance control – Absorbance sample)/Absorbance control)] × 100

The results are expressed as IC_{50} values determined from plotting inhibition versus extract concentration. For the ABTS assay, ABTS reagent consisting of 140 mM potassium persulfate ($K_2S_2O_8$) and 7 mM ABTS in a ratio of 1:1 (v/v) was prepared and kept in dark for 16-18 h to generate radical cations. The extracts were mixed with ABTS reagent and left to stand for 30 min. The absorbance was read at 415 nm. Trolox was used as the standard and ABTS capacity expressed as IC_{50} value.[11] FRAP assay was conducted as previously reported with slight modifications.[12] The FRAP reagent consisted of 300 mM acetic buffer (pH 3.6), 10 mM TPTZ and 20 mM $FeCl_3$ in 10:1:1 (v/v) ratio. The extracts and FRAP reagent were mixed and allowed to stand for 5 min in the dark. Afterward, the absorbance was measured at 595 nm using a microplate reader (Sunrise Tecan, Grödig, Austria). Ferrous sulfate was used as standard to generate a standard curve. The FRAP value is showed as mM Fe^{2+}/g extract. The ORAC assay was performed by mixing the extract with 25 mM of the fluorescent substance and 153 mM 2,2-azobis (2-amidinopropane) dihydrochloride (APPH) reagent. The mixture was incubated at 37°C for 10 min in a microplate reader (SpectroStar NANO, BMG Labtech, Germany) and the fluorescence intensity was read at 485 nm (Ex), 525 nm (Em) for 180 min.[13] The ORAC capacity was expressed as µmol Trolox Equivalent (TE)/ g DW.

Statistical Analysis

All experiments were performed in triplicate. The results are expressed as means ± standard deviation (SD). One-way ANOVA and Tukey's posthoc test ($p < 0.05$) were carried out using SPSS software version 26.0. Principal component analysis (PCA) and hierarchical cluster analysis (HCA) were performed to determine the relationship between extracts, phytochemical contents and antioxidant activity and to classify and cluster samples based on phytochemical contents and their antioxidant activities. The PCA and HCA were computed using XLSTAT software version 2022.4.1.1359.

Table 1: Herbal Ingredients of Phikud Bencha Thien (BT), Phikud Bencha Kot (BK) and Phikud Bencha Keson (BKS)

Common name (part used)	Scientific name
Phikud Bencha Thien (BT)	
Dill (seed)	*Anethum graveolens* L.
Cumin (seed)	*Cuminum cyminum* L.
Sweet fennel (seed)	*Foeniculum vulgare* Mill
Garden cress (seed)	*Lepidium sativum* L.
Black cumin (seed)	*Nigella sativa* L.
Phikud Bencha Kot (BK)	
Dong quai/Femail ginseng (root)	*Angelica sinensis* (Oliv.) Diels
Dahurian angelica (root)	*Angelica dahurica* (Hoffm.) Benth. & Hook.f. ex Franch. & Sav.
Wormwood (leaves)	*Artemisia annua* L
Atractylodes (root)	*Atractylodes lancea* (thunb.) DC
Szechuan lovage (root)	*Ligusticum striatum* DC.
Phikud Bencha Keson (BKS)	
Arabian jasmine (flower)	*Jasminum sambac* (L.) Aiton
Negkassar (flower)	*Mammea siamensis* T. Anderson
Iron wood (flower)	*Mesua ferrea* L.
Asian bulletwood (flower)	*Mimusops elengi* L.
Sacred lotus (Stamen)	*Nelumbo nucifera* Gaertn.

Results and discussion

Phytochemical Contents

Several studies have shown that phenolic constituents derived from plants are potential antioxidant agents.[14] Among extraction solvents, polar solvents such as water, methanol, and ethanol are more effective in extracting phenolic compounds than non-polar solvents[15], implying that the different solvent selected for an extraction can affect the phenolic constituents in an extract and its antioxidant properties. Therefore, the phenolic acid and flavonoid phytochemical components and the antioxidant activity were determined for BT, BK, and BKS extracts prepared by using different solvents in this study. The total phenolic contents (TPC) and total flavonoid contents (TFC) of the six extracts prepared from the three remedies varied according to the solvent used for the extraction and between remedies. The methanol extracts of all remedies were richer in phenolic constituents with 10 – 15fold higher TPC values compared to the aqueous extracts (Table 2). The highest TPC was found in the methanol extract of BKS (60.85 ± 0.16 mg GAE/g extract), followed by methanol extracts of BT and BK. The TFC values of methanol extracts were also higher than they were in aqueous extracts, but the TFC levels were the same across the different remedies. The highest level of TFC was found in methanol extract of BT - 5.81 ± 0.03 mg GAE/g extract.

A qualitative and quantitative analysis of the phenolic constituents present in aqueous and methanol extracts was performed by High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD). Several well characterized antioxidant phenolic acids (gallic acid; GA, protocatechuic acid; PCCA, *p*-hydroxy benzoic acid; *p*-HO, vanillic acid; VA, syringic acid; SyA, chlorogenic acid; ChA, caffeic acid; CFA, *p*-coumaric acid; *p*-CA, ferulic acid; FA, sinapic

acid; SNA) and flavonoids (rutin; RU and quercetin; QU) were selected to be standards. Among the three remedies it was the BKS extracts that showed the highest total amount of polyphenolic compounds with total values of 9285.83 ± 588.61 and 9045.11 ± 729.99 µg/g extract for the aqueous and methanolic extracts, respectively. BKS extract were also found to have higher GA (3746.31 ± 361.94 and 3494.94 ± 234.05 µg/g extract of aqueous and methanolic extracts) and PCCA (2259.35 ± 22.66 and 3028.80 ± 235.03 µg/g extract of aqueous and methanol extracts) content than the BT and BK extracts, as shown in Table 3.

Antioxidant Capacity

The antioxidant activity of the BKS remedy extracts had illustrated a significantly stronger antioxidant activity than the BT and BK remedy extracts in all antioxidant models, as shown in Table 4. However, the antioxidant activities of the extracts were lower than for the standard Trolox in the DPPH and ABTS assays for all remedies (p<0.05). In contrast, the BKS methanol extract had significantly more antioxidant activity (741.52 ± 1.76 mM Fe2+/g extract) than the standard Trolox (173.52 ± 12.47 mM Fe2+/g extract) in the FRAP assay. Interestingly, BKS extracts had very large amounts of GA and PCCA, with nearly 100-fold the amount found in the BT and BK extracts. GA is a well-characterized phenolic phytochemical found in many natural sources. Many studies have illustrated that GA is a strong antioxidant agent that is able to scavenge hydroxy and hydroperoxyl radicals.[16] PCCA is also reported to possess antioxidant activity.[17] Taken together, this indicates that GA and PCCA are the major phenolic constituents responsible for the strong antioxidant activity of BKS remedy extracts. The name "Keson" in Phikud Bencha Keson (BKS) can mean the whole flower (sepal, petal, stamen and stigma) or only the stamen. Flowers are rich in a great variety of natural antioxidants including flavonoids, anthocyanins and many other phenolic compounds.[18] The combination of five unique flowers in the BKS formulation corresponds with the high levels of phenolic and flavonoid contents in the extracts, as well as their antioxidant activities in all four assays.

Multivariate analysis

Principal component analysis (PCA) and hierarchical cluster analysis (HCA) were used to assess associations between the phytochemical contents (phenolic acids and flavonoids) and antioxidant activities (ABTS, DPPH, FRAP, and ORAC assays) of aqueous (W) and methanol (M) extracts of BT, BK, and BKS. HCA was performed to classify the extracts based on phytochemical compounds and antioxidant activities. The HCA generated three clusters (Figure 1A). Cluster I included BT-W, BK-W, and BK-M extracts. The extracts in cluster I had the lowest phytochemical contents and the lowest antioxidant activities. Cluster II consisted of BKS-W and BKS-M extracts, which had the highest antioxidant capacities and the highest phytochemical contents. Cluster III consisted of only BT-M extract. The extracts in clusters II and III were associated with the highest antioxidant capacities and phytochemical contents.

Table 2: Total Phenolic Acid Content (TPC) and Total Flavonoid Content (TFC) of BT, BK and BKS Remedies Extracts.

Sample	TPC (mg GAE/g extract)		TFC (mg QE/g extract)	
	Aqueous	MeOH	Aqueous	MeOH
BT	2.54 ± 0.01^{b,B}	29.64 ± 1.83^{b,A}	1.50 ± 0.11^{b,B}	5.81 ± 0.03^{a,A}
BK	1.38 ± 0.01^{c,B}	20.18 ± 0.84^{c,A}	0.63 ± 0.01^{c,B}	2.30 ± 0.01^{c,A}
BKS	5.72 ± 0.04^{a,B}	60.85 ± 0.16^{a,A}	1.97 ± 0.04^{a,B}	2.64 ± 0.04^{b,A}

Note: Letters within column were different from each other as determined by one-way ANOVA and Tukey test (p<0.05). Lowercase letters indicate significance within the column and uppercase letters indicate significance between aqueous and methanol extraction.

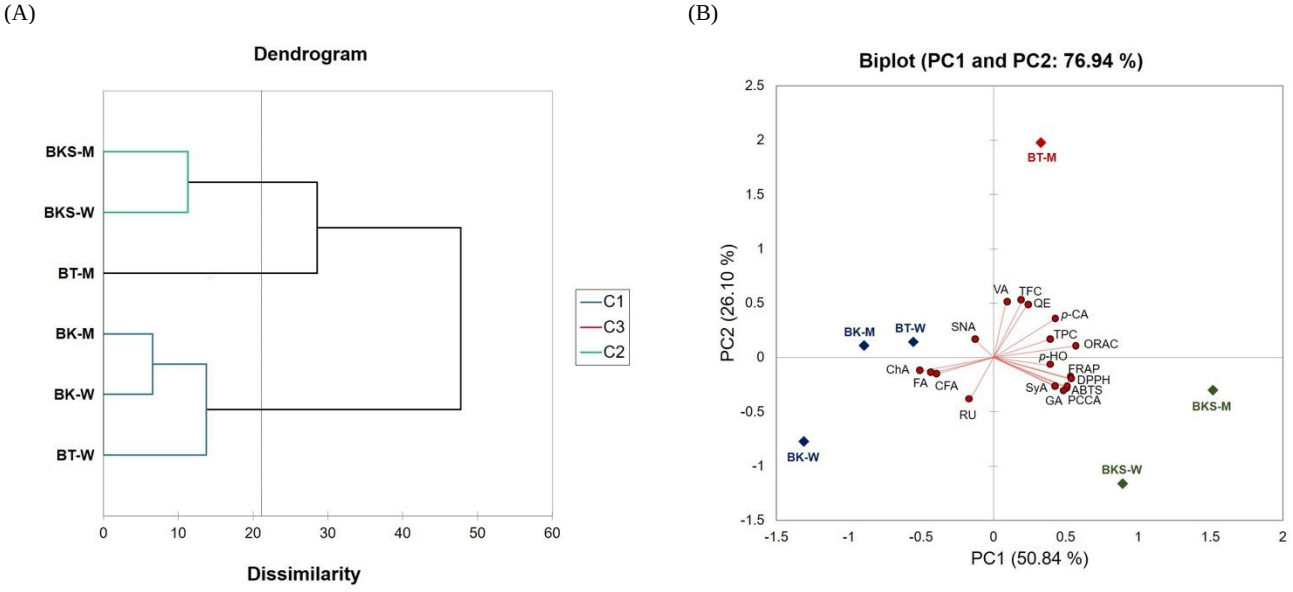

Figure 1: (A) Hierarchical Cluster Analysis (HCA) and (B) Principal Component Analysis (PCA) Biplot of Phikud Bencha Thien (BT), Phikud Bencha Kot (BK) and Phikud Bencha Keson (BKS) Remedies based on Phytochemical Contents and Antioxidant Activities.

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Table 3: Phytochemical Prefile by HPLC Analysis of BT, BK and BKS Remedies Extracts

Compounds	Phytochemical Contents (µg / g extract)					
	BT extracts		BK extracts		BKS extracts	
	Aqueous	MeOH	Aqueous	MeOH	Aqueous	MeOH
GA	46.56 ± 0.11 ^{d,A}	12.13 ± 0.38 ^{d,B}	13.58 ± 0.30 ^{h,i,B}	40.88 ± 1.11 ^{b,c,d,A}	3746.31 ± 361.94 ^{a,A}	3494.94 ± 234.05 ^{a,A}
PCA	40.64 ± 0.16 ^{d,A}	35.94 ± 1.98 ^{d,B}	30.75 ± 0.57 ^{h,B}	81.04 ± 1.18 ^{b,c,d,A}	2259.35 ± 22.66 ^{b,B}	3028.80 ± 235.03 ^{b,A}
p-HO	34.68 ± 0.75 ^{d,B}	46.50 ± 0.47 ^{d,A}	76.51 ± 0.56 ^{f,B}	165.93 ± 5.06 ^{b,c,d,A}	153.47 ± 0.54 ^{c,B}	211.97 ± 16.66 ^{d,A}
VA	203.66 ± 3.90 ^{c,A}	132.21 ± 4.48 ^{d,B}	61.78 ± 0.26 ^{f,g,B}	92.72 ± 1.77 ^{b,c,d,A}	147.67 ± 7.46 ^{c,A}	134.54 ± 11.25 ^{d,A}
SyA	44.14 ± 2.86 ^{d,A}	41.81 ± 0.22 ^{d,B}	49.75 ± 0.23 ^{g,B}	88.81 ± 1.90 ^{b,c,d,A}	146.75 ± 4.35 ^{c,B}	158.62 ± 12.25 ^{d,A}
ChA	891.29 ± 5.08 ^{b,B}	922.90 ± 36.35 ^{b,A}	460.84 ± 12.82 ^{c,A}	ND	181.44 ± 0.75 ^{c,A}	185.94 ± 0.73 ^{d,A}
CFA	6555.63 ± 506.49 ^{a,A}	3870.91 ± 172.39 ^{a,B}	917.92 ± 14.96 ^{a,B}	2225.64 ± 186.11 ^{a,A}	2014.88 ± 184.40 ^{b,A}	1165.38 ± 181.62 ^{c,B}
p-CA	49.06 ± 0.44 ^{d,A}	49.19 ± 0.25 ^{d,A}	76.30 ± 0.23 ^{f,B}	175.77 ± 5.46 ^{b,c,d,A}	92.73 ± 1.34 ^{c,B}	143.25 ± 10.23 ^{d,A}
FA	652.36 ± 10.69 ^{b,A}	491.70 ± 14.64 ^{c,B}	176.75 ± 0.17 ^{e,B}	203.63 ± 3.83 ^{b,c,A}	169.13 ± 0.81 ^{c,B}	211.19 ± 6.13 ^{d,A}
SNA	ND	ND	631.99 ± 29.66 ^{b,A}	153.41 ± 7.53 ^{b,c,d,B}	ND	ND
RU	541.38 ± 349.16 ^{b,c,A}	64.95 ± 0.76 ^{d,B}	245.85 ± 2.27 ^{d,A}	85.64 ± 1.06 ^{b,c,d,B}	374.10 ± 4.36 ^{c,A}	165.78 ± 11.14 ^{d,B}
QE	ND	76.08 ± 1.16 ^{d,A}	ND	260.24 ± 6.98 ^{b,A}	ND	144.70 ± 10.90 ^{d,A}
Total	9059.4 ± 879.53	5744.32 ± 233.08	2742.02 ± 62.03	3573.71 ± 221.99	9285.83 ± 588.61	9045.11 ± 729.99

Note: BT: Phikud Benja Thien remedy, BK: Phikud Benja Kot remedy, BKS: Phikud Benja Keson remedy. GA: gallic acid, PCCA: protocatechuic acid, *p*-HO: *p*-hydroxy benzoic acid, VA: vanillic acid, SyA: syringic acid, ChA: chlorogenic acid, CFA: caffeic acid, *p*-CA: *p*-coumaric acid, FA: ferulic acid, SNA: sinapic acid, RU: rutin, QE: quercetin. ND is no detection. Letters within column were different from each other as determined by one-way ANOVA and Tukey test ($p<0.05$). Lowercase letters indicate significance within the column and uppercase letters indicate significance between aqueous and methanol extraction.

Table 4: Antioxidant Capacities of BT, BK and BKS Remedies Extracts

Sample	IC_{50} value (µg /g extract)				FRAP value (mM Fe^{2+}/g extract)		ORAC assay (mgTE/g DW)	
	DPPH assay		ABTS assay					
	Aqueous	MeOH	Aqueous	MeOH	Aqueous	MeOH	Aqueous	MeOH
BT	202.59 ± 3.10 ^{c,B}	143.44 ± 0.63 ^{c,A}	154.90 ± 9.17 ^{c,A}	153.44 ± 1.79 ^{c,A}	124.43 ± 3.75 ^{c,B}	216.52 ± 10.68 ^{b,A}	273.42 ± 5.58 ^{b,B}	480.00 ± 30.18 ^{a,A}
BK	330.95 ± 8.14 ^{d,A}	557.35 ± 4.38 ^{d,B}	335.67 ± 0.92 ^{d,B}	238.99 ± 0.95 ^{d,A}	111.74 ± 2.38 ^{c,A}	115.76 ± 1.76 ^{c,A}	137.77 ± 5.60 ^{c,B}	226.84 ± 14.57 ^{b,A}
BKS	33.15 ± 0.56 ^{b,A}	39.69 ± 0.22 ^{b,A}	24.54 ± 0.06 ^{b,A}	21.36 ± 0.04 ^{b,A}	562.12 ± 19.75 ^{a,B}	741.52 ± 1.76 ^{a,A}	493.82 ± 17.39 ^{a,A}	547.56 ± 37.83 ^{a,A}
Trolox	4.73 ± 0.06 ^{a}		4.87 ± 0.03 ^{a}		173.52 ± 12.47 ^{b}		-	

Note: Letters within column were different from each other as determined by one-way ANOVA and Tukey test ($p<0.05$). Lowercase letters indicate significance within the column and uppercase letters indicate significance between aqueous and methanol extraction.

The PCA explained that components 1 (PC1) and 2 (PC2) were responsible for 76.94% of total variance, with associated eigenvalues greater than 1 according to the Kaiser criterion.[19] GA, PCCA, and SyA values were the variables in PC1 that explained about 51% of the total data variance based on antioxidant assays. Similarly, PC2 explained another 26.1% of the variance and separated the remedies extracts based on ChA, CFA, and FA content (Figure 1B). The PCA results agreed with the cluster analysis (HCA). The extracts in clusters II and II had the highest TPC, GA, PCCA, *p*-HO, SyA, *p*-CA, VA, RU, and QU contents and the highest antioxidant activities. Interestingly, BT remedy extracts were classified into 2 clusters based on the solvent used for extraction (methanol and water). The BT-M extract (cluster III) was revealed to have high TFC, QU, VA, and *p*-CA values, while the BT-W extract (cluster I) showed the lowest phytochemical content and antioxidant activity.

Conclusion

In conclusion, methanol proved to be a powerful solvent for extracting phenolic compounds from BT, BK, and BKS remedies. The highest CFA content was found in BT and BKS remedies, whereas the highest GA content was found in BT. BKS remedy had the strongest antioxidant activity, which correlated with high GA and PCCA. Multivariate analysis revealed that the methanol and water extracts of BKS displayed the highest antioxidant capacities and had the highest phytochemical contents.

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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