Original Article

Antioxidant and Anti-inflammatory Activities of Ethanol and Aqueous Extracts in the Amnouy Suksomjit's Thai Folk Remedy

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Abstract

Introduction: A Thai folk remedy is traditionally used to manage many diseases, such as diabetes mellitus, hyperlipidemia, and dementia, as they are related via oxidative stress and chronic inflammation pathways.

Objectives: This study investigates the antioxidant contents, and anti-oxidation and anti-inflammation properties of ethanol and aqueous extracts of Dr. Amnouy Suksomjit's Thai folk remedy (RM or the Remedy).

Methods: RM and its plant ingredients were extracted with ethanol maceration and water decoction. All extracts had their antioxidant compounds measured i.e. total phenolic contents (TPC) and total flavonoid contents (TFC), activity by DPPH, $ABTS^{\bullet+}$ and FRAP assays, and anti-inflammatory activity by determining the inhibition of nitric oxide (NO) production in LPS-induced RAW264.7 macrophage cells.

Results: The total phenolic contents, measured by TPC assay, for ethanol RM extract (RME) and water extract of the remedy (RMW) were 25.06 and 24.26 mg GAE/g Extract, respectively. Additionally, the flavonoid contents, determined by TFC, were 87.26 and 85.27 mg QE/g Extract for RME and RMW, respectively. RME and RMW exhibited antioxidant activity, with DPPH IC_{50} values of 56.69 and 46.35 µg/mL, and FRAP values of 20.28 and 13.07 µM TE/g Extract, respectively. RME and RMW showed no anti-$ABTS^{\bullet+}$ effect. Furthermore, RME demonstrated a significant anti-inflammatory effect by reducing NO production in lipopolysaccharide (LPS) induced cells, with an IC_{50} value of 37.68 µg/mL, compared to prednisolone's IC_{50} value of 51.45 µg/mL. Conversely, RMW did not exhibit any anti-inflammatory effect.

Conclusions: The ethanol and the water extracts of the Remedy showed antioxidant effects as found in the phenolics and flavonoids. However, only the ethanolic extract exhibited anti-inflammatory activity. These results support the use of the Remedy for treating diseases related to oxidative stress and inflammation pathways.

Keywords: Antioxidant Activity, Anti-inflammation, Thai Folk Remedy, Antioxidant Compound, Nitric Oxide

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Introduction

Chronic inflammation and oxidative stress play an important role in the development of chronic diseases and are contributed to by cytokines and free radicals produced during metabolic processes in the human body.[1-3] Furthermore, nitric oxide (NO) production is activated by the inducible nitric oxide synthase (iNOS) signaling pathway from excessive inflammation.[4] It is well known that lipopolysaccharide (LPS)-stimulated inflammation in the RAW264.7 macrophage cells can increase NO production and release. Many phenolic chemicals, such as flavonoids, tannins, and curcumins, are believed to exert their effects by scavenging free radicals or inhibiting pro-inflammatory enzymes in the inflammatory response.[5,6] Nowadays, herbal remedies are widely used in Thai traditional medicine for treating the diseases that are related via oxidation and inflammation pathways.

A herbal remedy created by Dr. Amnouy Suksomjit, a Thai folk healer in Ayutthaya province, Thailand, has been traditionally used to manage many diseases such as diabetes mellitus, hyperlipidemia, dementia and inflammation for 30 years. Dr. Amnouy Suksomjit's Thai folk remedy consists of five herbs: the aerial parts of *Acanthus ebracteatus* (*A. ebracteatus*; AE), the shell nuts and the leaves of *Plukenetia volubilis* (*P. volubilis*; PV), the *Piper nigrum* (*P. nigrum*; PN) fruits, the *Phlogacanthus pulcherrimus* (*P. pulcherrimus*; PP) leaves and the leaves of *Vernonia amygdalina* (*V. amygdalina*; VA). Previous studies showed that the plant ingredients of the recipe possessed antioxidant activity and included compounds such as phenolics and flavonoids. From several literature sources, the total flavonoid contents (TFC) and/or total phenolic contents (TPC), and radical scavenging activity by DPPH, FRAP or ABTS assays were, in both absolute and 70% ethanol extracts, from AE leaves,[7,8] PV seeds,[9,10] PN fruits,[11-13] methanol PP leaf extract,[14,15] and the VA leaves made from various solvents, including aqueous,[16-18] methanol,[16,18,19] ethanol,[16] chloroform,[18] petroleum ether[18] and acetone.[20] Furthermore, *P. nigrum fruits* and *V. amygdalina leaves* exhibited anti-inflammatory effects by inhibiting nitric oxide (NO) production.[21-23] These literature sources indicate that the plant ingredients of the Remedy can inhibit oxidation and inflammation, supporting its use for the prevention and treatment of inflammatory-induced diseases, but RM itself has not been extensively studied.

Because of this lack of scientific evidence supporting the use of this folk remedy, RM, in treating diseases associated with oxidative stress and inflammation, we aimed to investigate the phytochemical contents, including total phenolic and flavonoid compounds, as well as the antioxidant activity and anti-inflammatory properties of both ethanol and aqueous extracts obtained from the Remedy.

Methods

Chemicals and Reagents

Aluminum chloride, potassium acetate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), butylated hydroxytoluene (BHT), Iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), sodium acetate, sodium nitrite ($NaNO_2$), quercetin, gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and lipopolysaccharides (LPS) obtained from *Escherichia coli* (serotype 0111:B4) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Folin-Ciocalteu reagent, dimethyl sulfoxide (DMSO), sodium carbonate (Na_2CO_3), and potassium persulfate were obtained from Merck (Darmstadt, Germany). Ethanol (EtOH), acetic acid, and hydrochloric acid (HCl) were obtained from RCI Labscan Limited (Bangkok, Thailand). Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin (P/S), and 2-mercaptoethanol were obtained from Gibco BRL Life Technologies (Gaithersburg, MD, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was obtained from Invitrogen (Merelbeke, Belgium). Mouse leukemic macrophages (RAW 264.7 cells) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA).

Plant Extraction

Aerial part of *A. ebracteatus* leaves (AE), *P. nigrum* fruits (PN), *P. pulcherrimus* leaves (PP), *P. volubilis* shell nuts (PVS), *P. volubilis* leaves (PVL) and *V. amygdalina* leaves (VA) were purchased from a traditional drug store, in Nakhon Pathom, Thailand. All plants were identified and collected

by Dr. Amnouy Suksomjit, a Thai folk doctor, and a botanist of the Thai Traditional Medicine Herbarium, Department of Thai Traditional and Alternative Medicine, Thailand. All herbs were washed and crushed before they were extracted. The folk remedy (RM) powder was made to combine all herbs in a ratio of 2:1:1:1:1:1 (AE, PN, PP, PVS, PVL, and VA). 200 gm of AE, PN, PP, PVS, PVL, VA and RM powders were macerated with 1 L of 95% ethanol at room temperature for 72 h by shaking. The residues were extracted under the same conditions, twice again. Each ethanolic extract was filtered through a Whatman's No. 1 filter paper and dried using a rotary evaporator (Buchi, Swizerland). 200 gm of the powders were decocted by boiling in 1 L distilled water for 15 min. The residues were boiled in 1 L of DI for 15 min, twice again. Each aqueous extract was dried using a freeze dryer (Telster, Spain). All extracts were calculated for percentage of yield and stored in a sealed container at -20 ◦C until use.

Table 1 Thai medicinal plants in Dr. Amnouy Suksomjit's remedy, RM.

Scientific name (Family)	Thai name	Part of use	Extraction	Code
Acanthus ebracteatus (AE) (Acanthaceae)	Nguak-Pla-Mor	Aerials	Ethanol Maceration	AEE
			Water Decoction	AEW
Phlogacanthus pulcherrimus (PP) (Acanthaceae)	Dee-Pla-Kung	Leaves	Ethanol Maceration	PPE
			Water Decoction	PPW
Piper nigrum (PN) (Piperaceae)	Prik-Thai-Dum	Fruits	Ethanol Maceration	PNE
			Water Decoction	PNW
Plukenetia volubilis (PV) (Euphorbiaceae)	Daw-In-Ca	Leaves (PVL)	Ethanol Maceration	PVLE
			Water Decoction	PVLW
		Shell nuts (PVS)	Ethanol Maceration	PVSE
			Water Decoction	PVSW
Vernonia amygdalina (VA) (Asteraceae)	Nhan-Choa-Wei	Leaves	Ethanol Maceration	VAE
			Water Decoction	VAW
Remedy (RM)	-	-	Ethanol Maceration	RME
			Water Decoction	RMW

Determination of Total Phenolic Contents (TPC)

TPC was determined with Folin-Ciocalteu reagent assay.[24,25] 20 µL of the extracts were mixed with 100 µL Folin-Ciocalteu (10%) reagent in 96-well plates. After incubation at room temperature in dark for 5 min, 80 µL of Na_2CO_3 solution was added and then incubated for 30 min in dark with room temperature. The absorbance was measured using a microplate reader (Bio-Rad Laboratories Inc., CA, USA) at 765 nm. Gallic acid was used to establish the standard curve (R^2 = 0.9993-0.9996). The TPC was expressed as milligrams of gallic acid equivalent per gram of the extract (mg GAE/g Extract).

Determination of Total Flavonoid Contents (TFC)

TFC was determined using the aluminum chloride and potassium acetate method.[24,25] 500 µL of each sample was mixed with 75 µL of 5% $NaNO_2$ solution and 150 µL of 10% aluminum chloride and potassium acetate solution in 2 mL centrifuged tube. After incubation for 5 min at room temperature, 500 µL of 1 M NaOH and 275 µL distilled water were added and then incubated for 30 min. 100 µL of each sample mixture was added to a new well-plate. The absorption at a wavelength of 510 nm was measured with a microplate reader (Bio-Rad Laboratories Inc., CA, USA). Quercetin concentrations ranging from 20 to 1000 mg/mL were prepared and used to

establish the standard calibration curve by using a linear fit (R^2 = 0.9958-0.9974). TFC was expressed as milligrams of quercetin equivalent per gram of extract (mg QE/g Extract).

Determination of Antioxidant Activities

DPPH radical scavenging assay

The method of Nutmakul and Chewchinda[24] was followed to determine DPPH radical scavenging activity. 100 µL of various concentrations (1-100 µg/mL) extracts were added 100 µL freshly prepared DPPH solution (6×10^{-5} M concentration) in 96-well plates. After dark incubation at room temperature for 30 min, the optical density was determined at 520 nm using a microplate reader (Bio-Rad Laboratories, Inc., CA, USA). BHT was used as a positive control. The percentage of DPPH radical scavenging activity was calculated using the equation below:

$$\% \text{ DPPH reduction} = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$$

Assay was carried out in triplicates and a plot of percentage DPPH reduction and sample concentration was drawn. Antioxidant activity was expressed as 50% of inhibitory concentration (IC_{50}) value and was calculated from regression analysis conducted by Prism software (GraphPad Software, Inc., CA, USA).

$ABTS^{\bullet+}$ radical scavenging assay

This assay was performed according to the method described by Nutmakul and Chewchinda[24] with slight modification. The $ABTS^{\bullet+}$ radical was prepared by mixing equal proportions of 7.4 mM $ABTS^{\bullet+}$ solution and 2.6 mM potassium persulfate solution at room temperature for 16 hours in the dark. 10 µL of the different concentrations (1, 10, 50 and 100 µg/mL) of each sample was mixed with 1 mL the $ABTS^{\bullet+}$ working solution in 96-well plates. After incubation for 6 minutes at room temperature in the dark, 100 µL sample were collected in a new plate and the absorbance was measured at 734 nm. Trolox was used to establish the standard curve. Percentage of $ABTS^{\bullet+}$ scavenging inhibition was calculated by using the equation below and then the IC_{50} value was calculated.

$$\% \text{ ABTS inhibition} = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$$

Ferric ion reducing antioxidant power assay

FRAP assay was carried out according to the method described by Olatunji et al.[26] with slight modification. FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl and 20 mM $FeCl_3 \bullet 6H_2O$ solution in the ratio of 10:1:1. The 20 µL of the sample solution at 1, 10, 50 or 100 µg/mL concentration was mixed with 180 µL of the FRAP working reagent and incubated at 37 ºC for 4 minutes. After dark incubated at room temperature for 8 minutes, the absorbance of the reaction mixture was read at 593 nm. The standard curve was prepared using Trolox ranging from 5 to 300 µM (R^2 = 0.9986). The FRAP values of extracts were calculated from the calibration curve of Trolox and expressed as µM Trolox equivalent (TE)/g Extract.

Determination of Anti-inflammatory Activity

Measurement of NO production by Griess reagent assay

The inhibitory activity of extracts on NO production of the LPS-induced RAW 264.7 cells was investigated according to the protocol of Makchuchit and coworkers.[27] This protocol was approved by Thammasat University institutional biosafety committee in the certificate letter No. 084/2566.

The cells were cultured in DMEM, supplemented with 10% FBS and 1% P/S, to confluence at 37ºC in a humidified incubator of 5% CO_2. Briefly, the cells (1×10^6 cells/well) were seeded in 96 well-plate and then incubated for 24 hours. Subsequently, the medium was replaced with a 100 µL/well fresh medium with 5 ng/mL LPS final concentration and without LPS. The cells were treated with 100 µL/well of 12.5, 25, 50 and 100 µg/mL concentrations of the extracts, and then incubated for 24 hours. An aliquot of the supernatant (100 µL/well) was transferred into a new 96 well-plate and added 100 µL/well of Griess reagent. The absorbance was measured by a microplate reader (Bio-Rad Laboratories Inc., CA, USA) at 570 nm. The percent of inhibition on LPS-induced NO production was calculated using the equation below:

$$\text{Inhibition (\%)} = [(Abs_{negative\ control} - Abs_{sample})/Abs_{negative\ control}] \times 100$$

where, $Abs_{negative\ control}$ = mean of control solvent (0.2% DMSO in final concentration) containing LPS Abs - mean of control solvent (0.2% DMSO in final concentration) without LPS Abs; Abs_{sample} = mean of sample containing LPS Abs - mean of sample without LPS Abs. Finally, IC_{50} value (µg/mL) was calculated by using the GraphPad Prism program (CA, USA). Prednisolone was used as a positive control.

Cell viability by MTT assay

The RAW264.7 cells were used to determine the percentage of cell survival after being treated under the same conditions for 48 hours. After 100 µL of the supernatant was removed, the cells were incubated with 10 µL of 5 mg/mL MTT solution for 2 hours. The MTT solution was removed and then added 100 µL of DMSO to dissolve the formazan. Absorbance at 570 nm was measured using a microplate reader (Bio-Rad Laboratories Inc., CA, USA).

Statistical Analysis

The data were presented as mean ± SEM from triplicate analyses. Statistical analysis was determined using one-way ANOVA with a post-hoc analysis by GraphPad Prism (GraphPad Software, Inc., CA, USA). The differences were considered significant at $p < 0.05$.

Results

Extraction Yield, Total Phenolics and Total Flavonoid Contents of the Extracts

The yield, TPC and TFC of the ethanolic and aqueous extracts of the remedy and the plants are shown in **Table 1**. The highest yield (28.67%) was observed in the water extract of the remedy (RMW), while the ethanol RM extract (RME) had the extraction yield of 11.23%. The lowest yield (2.22%) was shown in PVSE. Among the ethanolic extracts, percentages of the yield extraction were aligned as PP> VA> PP> RM> PN> AE> PVS in descending order. The yields of the aqueous extracts ranged from 8.46% to 28.67% and aligned as RM> PVL> PP> PN> VA> PVS in descending order.

The TPC values of the extracts ranged between 8.79 ± 1.26 and 84.90 ± 0.99 mg GAE/g Extract. The highest TPC was observed in the water PVS extract (TPC = 84.90 ± 0.99 mg GAE/g Extract), whereas the lowest TPC was found in the aqueous PP extract (mg GAE/g Extract). However, RME and RWM did not differ significantly in total phenolics, with TPC values of 25.06 ± 4.02 and 24.26 ± 3.98 mg GAE/g Extract, respectively (**Figure 1A**). The different solvent used for extraction resulted in varying TPC values, with water extraction showing significantly higher TPC than the ethanol extraction in PP, PVL and PN. Conversely, the TPC of PVS and VA extracted with water was significantly higher than those extracted using ethanol solvent (**Figure 1A**).

The highest TFC (189.14 ± 4.08 mg QE/g Extract) was observed in the water extract from the VAW, whereas the lowest TFC (12.63 ± 7.34 mg QE/g Extract) was found in PVLE. In addition, total flavonoids were found in RME and RMW, with TFC values of 87.26 ± 4.49 and 85.27 ± 2.15 mg QE/g Extract, respectively. In the different extraction, the water AE and PN extracts exhibited significantly higher TFC than those extracted with ethanol, whereas the PVL, PVS and VA aqueous extracts had TFC higher than those extracted with ethanol (**Figure 1B**).

Table 2 Extraction yield, TPC, TFC and antioxidant activities of the extracts.

Extracts	Extraction yield (%)	TPC [a] (mg GAE/g Extract)	TFC [a] (mg QE/g Extract)	DPPH IC_{50} value [a] (μg/mL)	$ABTS^{\cdot+}$ IC_{50} value [a] (μg/mL)	FRAP value [a] (μM TE/g Extract)
AEE	8.37	15.61 ± 2.84	122.74 ± 4.98	54.07 ± 6.51	> 100	18.66 ± 1.56
AEW	8.46	12.12 ± 1.04	89.32 ± 4.44	62.67 ± 4.26	> 100	9.76 ± 0.24
PPE	13.74	16.17 ± 0.63	54.67 ± 1.73	83.44 ± 6.38	> 100	15.88 ± 0.56
PPW	24.82	8.79 ± 1.26	59.50 ± 1.46	> 100	> 100	7.69 ± 1.06
PNE	8.44	55.53 ± 1.73	152.17 ± 4.62	59.72 ± 2.59	> 100	21.91 ± 0.75
PNW	22.77	11.01 ± 0.60	47.53 ± 2.42	> 100	> 100	6.35 ± 0.39
PVLE	23.18	31.16 ± 0.44	12.63 ± 7.34	> 100	> 100	19.16 ± 1.33
PVLW	28.25	12.36 ± 1.45	53.85 ± 5.18	> 100	> 100	8.57 ± 0.11
PVSE	2.22	21.17 ± 1.04	99.74 ± 3.05	41.70 ± 0.52	> 100	26.12 ± 1.02
PVSW	12.13	84.90 ± 0.99	120.04 ± 8.13	20.18 ± 7.11	49.62 ± 6.96	39.78 ± 0.58
VAE	19.68	28.47 ± 4.29	122.26 ± 7.90	25.34 ± 1.40	> 100	27.87 ± 5.11
VAW	21.71	39.82 ± 3.85	189.14 ± 4.08	27.65 ± 5.88	> 100	20.44 ± 1.64
RME	11.23	25.06 ± 4.02	87.26 ± 4.49	56.69 ± 5.76	> 100	20.28 ± 2.26
RMW	28.67	24.26 ± 3.98	85.27 ± 2.15	46.35 ± 6.80	> 100	13.07 ± 0.80
BHT	-	-	-	13.59 ± 1.04	-	-
Trolox	-	-	-	-	13.11 ± 1.31	-

[a] Values represent the mean ± SEM of three measures.

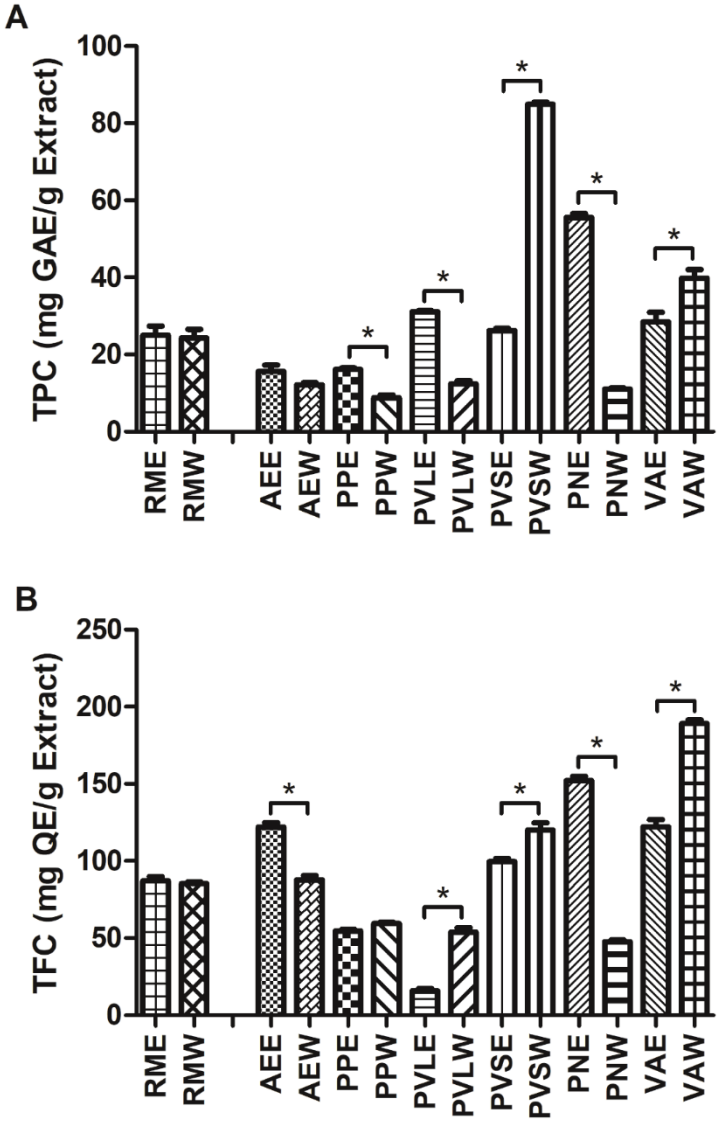

Figure 1 Comparative total phenolic content (TPC; A) and total flavonoid content (TFC; B) of the ethanol and the water extracts of the Thai folk remedy and its components, * $p < 0.05$ vs. water extraction.

Effects of the Extracts on the Antioxidant Activities

The antioxidant activity measured by using DPPH tests are presented in **Figure 2A**, **Figure 2B**, **Table 2** and **Figure 3A**. RME and RMW exhibited DPPH scavenging activity with IC_{50} values of 56.59 and 46.35 µg/mL, respectively. Within the ethanolic extract group, the ethanol VA extracts had the highest DPPH radical scavenging activity with an IC_{50} value of 25.34 ± 1.40 µg/mL. The PVSW exhibited the highest DPPH IC_{50} value (20.18 ± 7.11 µg/mL) in the aqueous extraction group. However, all extracts had the lower DPPH inhibitory activities compared to the positive control BHT (IC_{50} value = 13.59 µg/mL) except PVSW and VAE.

From the results of the $ABTS^{\bullet+}$ activity (**Figure 2C**, **Figure 2D**, **Table 2** and **Figure 3B**), RME and RMW had no $ABTS^{\bullet+}$ scavenging effects (the IC_{50} value of both extracts > 100 µg/mL). The highest $ABTS^{\bullet+}$ scavenging ability were the water PVS extract (IC_{50} value = 49.62 ± 6.96 µg/mL) and the other extracts had no obvious antioxidant activity in $ABTS^{\bullet+}$ scavenging system. Moreover, the PVSW had significant lower the $ABTS^{\bullet+}$ scavenging activity than Trolox as the positive control by the IC_{50} value was 13.11 ± 1.31 µg/mL.

The FRAP capacity for each extract was showed in **Figure 3C** and **Table 2**. The FRAP standard curve had R^2 = 0.9986 (**Figure 3D**). The FRAP values of RME and RMW were 20.28 ± 2.26 and 13.07 ± 0.80 μM TE/g Extract, respectively. The PVSW had the highest ferric radical antioxidant potential by the FRAP value was 39.78 ± 0.58 μM TE/g Extract.

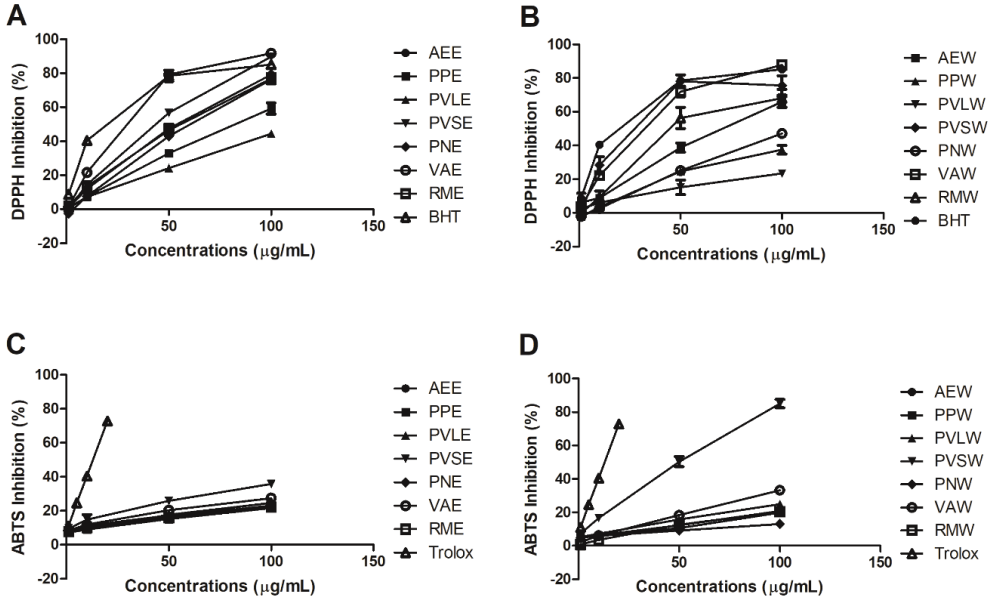

Figure 2 Percent inhibition of the DPPH radicals by the ethanol (A) and water (B) extracts, and the $ABTS^{\bullet+}$ inhibitory activity (%) of the ethanol (C) and water (D) extracts.

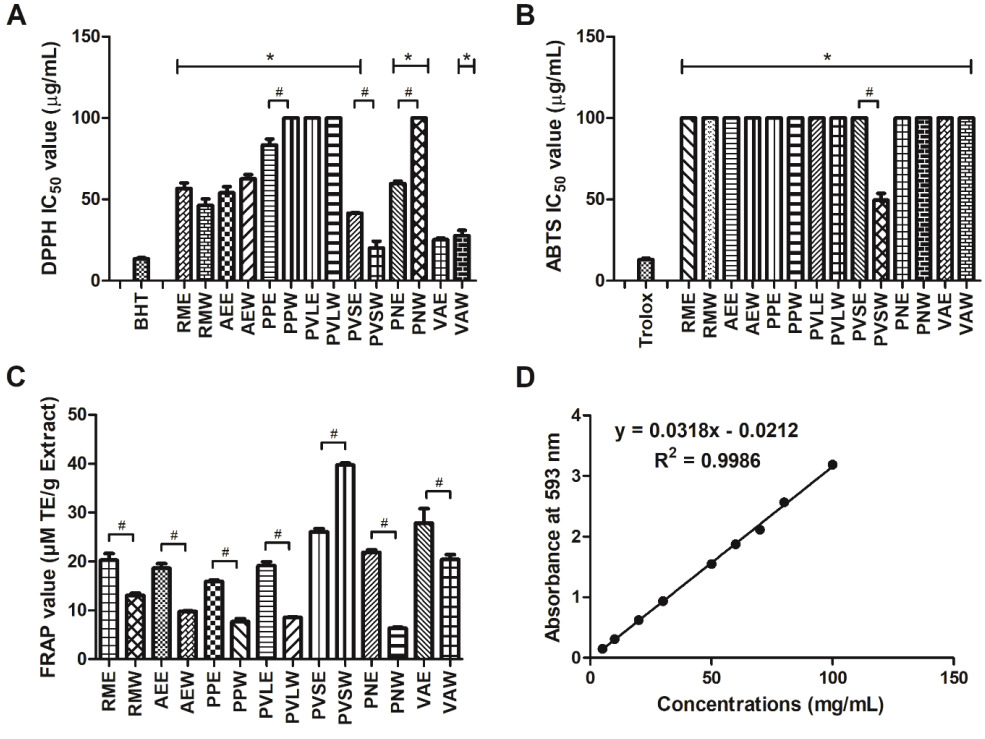

Figure 3 The antioxidant activity of the remedy and the plant ingredient extracting with ethanol and aqueous solvents on DPPH (A), ABTS (B) and FRAP (C) methods, and the standard curve for calculating FRAP value (D). * $p < 0.05$ vs. BHT or Trolox, # $p < 0.05$ vs. water extraction.

Inhibitory Effect of the Extracts on the Inflammatory Activity

The anti-inflammatory activity of the extracts was determined by the inhibition of NO production in the LPS-induced RAW264.7 cells. The inhibition percentages of the NO releases in the cells treated with the various concentrations of the extracts and the IC_{50} values are presented in **Figure 4A**, **Figure 4B** and **Table 3**. Our results presented that RME exhibited an inhibitory effect on NO production with an IC_{50} value of 37.68 ± 2.28 µg/mL, but RMW had no effect (IC_{50} value > 100 µg/mL). Among the ethanol extracts of the plant ingredients, four plant extracts showed the inhibitory potential on NO production, with IC_{50} values aligned as follows: PNE> VAE> PVSE> PPE in descending order (14.01 ± 0.48, 37.68 ± 2.28, 49.53 ± 2.53 and 97.45 ± 1.91 µg/mL, respectively). AEE and PVLE had no anti-inflammatory effect on NO production with each IC_{50} value of more than 100 µg/mL. Furthermore, it is important to note that the three ethanol extracts, PNE, RME and VAE, significantly down-regulated NO production when compared to Prednisolone group as the positive control (IC_{50} value = 51.45 ± 0.98 µg/mL) as shown in **Figure 4A**. On the other hand, all aqueous extracts at 100 µg/mL cannot inhibit NO production in the LPS-induced the RAW264.7 cells.

The cytotoxicity of the extracts at various concentrations on RAW264.7 macrophage cells was measured by MTT assay. All extracts at the different concentrations had no cytotoxicity on the RAW264.7 cells by the percentages of the cell survival more than 80% (**Figure 4C** and **Table 3**).

Table 3 Anti-inflammatory activity and cell viability of the extracts on the NO production in LPS-stimulated the RAW264.7 cells.

Extract	NO Inhibition of the different concentrations; % (Cell viability; %)				IC_{50} value (µg/mL)
	12.5 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL	
AEE	-	-	21.78 ± 4.20	39.28 ± 3.76	> 100
	-	-	(106.19 ± 12.86)	(101.98 ± 2.56)	
AEW	-	-	8.65 ± 1.03	11.14 ± 1.65	> 100
	-	-	(93.62 ± 2.73)	(89.10 ± 2.30)	
PNE	32.70 ± 1.56	47.17 ± 0.41	64.73 ± 4.54	74.44 ± 2.94	14.01 ± 0.48
	(106.48 ± 1.17)	(103.46 ± 7.65)	(101.12 ± 6.21)	(97.70 ± 10.98)	
PNW	-	-	7.04 ± 2.06	11.14 ± 2.05	> 100
	-	-	(95.88 ± 10.96)	(90.80 ± 2.52)	
PPE	0.37 ± 2.08	12.31 ± 0.68	21.31 ± 2.59	52.08 ± 1.24	97.45 ± 1.91
	(97.49 ± 6.15)	(95.14 ± 0.98)	(92.91 ± 6.06)	(92.97 ± 2.51)	
PPW	-	-	12.24 ± 2.69	21.83 ± 2.38	> 100
	-	-	(111.19 ± 10.21)	(117.03 ± 12.02)	
PVLE	-	-	15.06 ± 4.08	36.83 ± 1.55	> 100
	-	-	(96.33 ± 3.30)	(98.55 ± 2.56)	
PVLW	-	-	5.09 ± 1.24	8.53 ± 0.42	> 100
	-	-	(107.65 ± 8.48)	(109.06 ± 9.28)	
PVSE	17.50 ± 4.23	23.38 ± 7.93	49.49 ± 2.91	69.31 ± 4.91	49.53 ± 2.53
	(104.66 ± 1.99)	(107.73 ± 3.01)	(110.85 ± 3.25)	(114.43 ± 4.60)	
PVSW	-	-	14.83 ± 1.26	30.59 ± 3.05	> 100
	-	-	(113.67 ± 4.69)	(111.82 ± 6.38)	

Table 3 Anti-inflammatory activity and cell viability of the extracts on the NO production in LPS-stimulated the RAW264.7 cells. (cont.)

Extract	NO Inhibition of the different concentrations; % (Cell viability; %)				IC_{50} value (µg/mL)
	12.5 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL	
VAE	23.64 ± 4.02	36.65 ± 1.87	57.92 ± 1.98	79.32 ± 4.65	39.76 ± 0.89
	(98.17 ± 1.66)	(104.64 ± 0.62)	(110.70 ± 5.19)	(109.72 ± 8.24)	
VAW	-	-	26.69 ± 3.56	37.60 ± 2.50	> 100
	-	-	(112.61 ± 12.12)	(110.86 ± 10.25)	
RME	26.30 ± 2.69	36.67 ± 3.17	63.15 ± 1.38	87.23 ± 1.06	37.68 ± 2.28
	(104.34 ± 6.76)	(110.45 ± 2.76)	(109.06 ± 5.02)	(112.80 ± 2.06)	
RMW	-	-	9.57 ± 2.31	13.68 ± 1.82	> 100
	-	-	(111.11 ± 6.09)	(109.78 ± 12.54)	
Pred.	33.48 ± 12.84	37.81 ± 6.20	49.38 ± 0.32	67.42 ± 6.43	51.45 ± 0.98
	(88.72 ± 12.43)	(89.14 ± 9.43)	(87.76 ± 6.86)	(82.43 ± 1.83)	

All data expressed Mean ± SEM., Pred. is Prednisolone as the positive control.

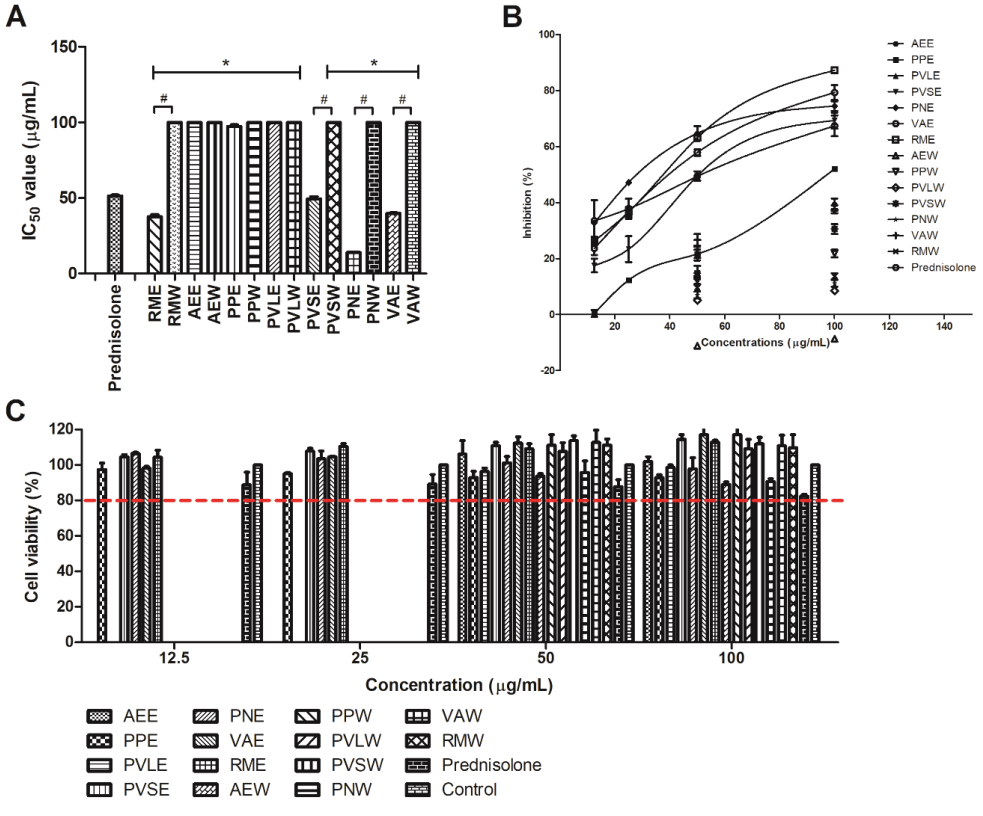

Figure 4 The anti-inflammatory activity (IC_{50}) (A), %inhibition at various concentration (B) and the percent of RAW264.7 survival cells (C) of the ethanol and the aqueous extracts of the recipe and the ingredients. Red dot line is 80% survival level. * $p < 0.05$ vs. Prednisolone, # $p < 0.05$ vs. water extraction.

Discussion

This study is a first scientific exploration into the antioxidant capacity and anti-inflammatory potential of the Thai folk formulation created by Dr. Amnouy Suksomjit (RM or the Remedy). He is a folk doctor in Ayutthaya province of Thailand and has been using the Remedy for treating patients with several diseases such as diabetes, dyslipidemia, dementia for 30 years. It is known that these diseases are stimulated from oxidative stress and inflammation. Our study investigated the Remedy extracts antioxidant and anti-inflammatory activities. The Remedy consists of five plants with six herbal parts i.e. the aerial part of *A. ebracteatus* leaves, the *P. nigrum* fruits, the *P. pulcherrimus* leaves, the *P. volubilis* shell nuts, the *P. volubilis* leaves and the *V. amygdalina* leaves. These ingredients are mixed according to the ratio described in the methodology. RM and the plant ingredients were extracted using two methods, by maceration with 95% ethanol, a universal solvent, and water decoction as a Thai traditional method. The ethanolic and water RM extracts had their total phenolic and total flavonoid contents determined. Our results show that both the ethanol (25.06 mg GAE/g Extract TPC and 87.26 mg QE/g Extract TFC, respectively) and the water RM extracts (24.26 mg GAE/g Extract TPC and 85.27 mg QE/g Extract TFC, respectively) contained significant levels of TPC and TFC. Interestingly, the different solvents used in the extraction process did not affect the phenolic and flavonoid contents in the herbal recipe. The phenolics in the dry plant extract are classified into three categories based on TPC levels: low, medium and high (<10, 10-50 and > 50 mg GAE/g, respectively).[28] The results indicated that the ethanol and the water extracts of the remedy were classified as medium in total phenolics. Therefore, our results related to previous literature indicating that phenolics and flavonoids are generally known to exhibit antioxidant activity.[3]

Next, RM extracts were measured for their antioxidant activity using DPPH, $ABTS^{\bullet+}$ and FRAP methods. We found that the ethanolic and the aqueous RM extracts exhibited significant inhibition of the oxidant stress pathways, as measured in DPPH scavenging (IC_{50} values = 56.69 and 46.35 μg/mL, respectively) and FRAP (FRAP values = 20.28 and 13.07 μM TE/g Extract, respectively). However, the two extracts cannot reduce the free radicals in the $ABTS^{\bullet+}$ assay. Interestingly, there was no significant difference observed in the antioxidant activity between ethanolic and water RM extracts. Furthermore, the results aligned with previous studies that indicated the extraction with different solvents affects different antioxidant DPPH, ABTS and FRAP activities.[18]

Anti-inflammatory activity was determined by inhibitory assay of NO production in the LPS-induced macrophage cells. Our results showed that the ethanolic RM extract had an anti-inflammatory effect in inhibition of NO production in macrophage cells stimulating LPS (IC_{50} = 37.68 ± 2.28 μg/mL), but the water extract of the remedy had no effect (IC_{50} = > 100 μg/mL). In addition, the individual plant components of the remedy had anti-inflammatory effects by inhibiting the production of NO.[15,21-23,29-31] Therefore, it is plausible that the water RM extract may exhibit anti-inflammatory activity on other mediators of inflammation, such as TNF-α, INF-γ, IL-1β, IL-6, and IL-10.

The results presented show that the Thai folk remedy, RM, may be effective in treating diseases associated with free radicals and inflammation. We suggest this plant remedy could be investigated with other antioxidant assays, for example enzymatic antioxidant status, or bioactive compounds with high performance liquid chromatography or gas chromatography-mass spectrometry. In addition, the inhibitory pathways of NO production as measured by the expression of iNOS or other intracellular signaling proteins, or the reduction of the other cytokines such as TNF-α, IL-1β could be used for confirming the anti-inflammatory effects in a future study.

In conclusion, this study evaluated the ethanol and water extracts of Dr. Amnouy Suksomjit's Thai folk remedy, which exhibited antioxidant effect in DPPH and FRAP assays, with significant levels of total phenolic and flavonoid contents. Moreover, the ethanolic extract of the formulation showed anti-inflammatory activity by inhibiting NO secretion in LPS-induced murine macrophage cells.These results could support using this Thai folk remedy for managing diseases relating to the oxidative stress and inflammation pathways.

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Compliance with Ethics Requirements

No.

Conflict of interest

Authors declare no conflict of interest.

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Author Contributions

Conceptualization, methodology, software, data curation and original draft preparation, Wichida Larsomsri; conceptualization, methodology, validation, formal analysis, writing review and editing, visualization and supervision, Pannawat Chaiyawatthananthn; methodology and data curation, Thanaporn Intarawan; data curation and formal analysis, Yupa Ngankogsoong; writing review and editing, Katanchalee Houngiam. All authors have read and agreed to the published version of the manuscript.

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