Original Article

Quality Control and Evaluation of Anti-inflammatory and Antioxidant Activities of Ethanolic Extracts from Ha-Rak remedy, *Piper betle* Linn., *Garcinia mangostana* Linn., and Their Combined Remedies

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Abstract

Introduction: A Thai herbal remedy, Ha-Rak (HR), *Piper betle* Linn. (PB) leaves, and *Garcinia mangostana* Linn. (GM) pericarps tend to relieve inflammatory-related atopic dermatitis (AD). Additionally, antioxidants have important role in the AD prevention.

Objectives: To develop combined formulas of HR, PB and GM extracts and investigate them on anti-inflammatory and antioxidant activities, the total flavonoid content (TFC) and the total phenolic content (TPC).

Methods: The quality of each dried plant material was evaluated according to Thai Herbal Pharmacopoeia (THP) criteria. Each was extracted by maceration with 95% ethanol, and then combined with a remedy. The inflammatory properties of each extract were assessed using the production inhibition of TNF- α from RAW 264.7 cells. The antioxidant properties were assessed using DPPH and ABTS radical scavenging. The TFC and TPC were also analyzed.

Results: The quality of all plant materials passed Thai herbal pharmacopeia standards. HR showed the highest anti-inflammatory activity. The combination HMB-321, which is composed of HR:GM:PB in a ratio of 3:2:1, showed the highest anti-inflammatory activity among other combined formulas. Moreover, HMB-321 showed moderate antioxidant activities and capacities. PB showed the highest antioxidant activity but less anti-inflammatory activity. HMB-123, whose proportion of HR:GM:PB was 1:2:3, showed the highest antioxidant activity among the combination of extracts but showed less anti-inflammatory activity.

Conclusions: The combination HMB-321 could be a candidate remedy for the prevention and treatment of AD. Further study should be directed to *in vivo* testing and clinical trials for anti-inflammatory and antioxidant activities.

Keywords: Ha-Rak remedy, *Piper betle*, *Garcinia mangostana*, Antioxidant activity, Anti-inflammatory activity

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Introduction

Atopic dermatitis (AD) is a chronic skin disease that produces inflammation, redness, itchiness, and irritation. It is a prevalent disorder that typically begins in childhood.[1,2] Increasing amounts of the inflammatory mediator, plasma tumor necrosis factor-alpha (TNF-α), contributes to skin inflammation in atopic dermatitis and correlates with the severity of the condition.[3,4] AD pathogenesis includes being more susceptible to reactive oxygen species (ROS) or oxidants, as malondialdehyde increased, and enzymatic and non-enzymatic antioxidants decreased. Several foods and natural products containing antioxidants such as vitamins E and C, flavonoids, carotenoids, and phenolics can reduce free radicals, and play a significant role in the prevention of AD.[5,6]

Thai traditional medicine (TTM) has three well-known herbal treatments for skin disorders such as AD. For burning skin, Ha-Rak remedy (HR) is formulated from five plant roots in equal proportions.[7] *Piper betle* Linn. (PB) leaves are used to relieve skin irritation and itching. *Garcinia mangostana* Linn. (GM) pericarps are used to treat acute and chronic wounds.[8] These herbal medicines are used topically to treat skin diseases such as AD. Biological activities relevant to AD pathogenesis showed that HR ethanolic extract had anti-inflammatory and anti-allergic properties. Ethanolic extract of PB decreased histamine level in IgE-mediated hypersensitivity reactions and suppressed IL-8 release in TNF-α and IL-4-induced allergy reactions.[9] The extract also inhibited *S. aureus* and *S. epiderdimis* growth. It was found that GM pericarp extract inhibited *S. epidermidis* and *P. acnes* and lessened A23187-induced PGE_2 synthesis in C6 rat glioma cells and histamine release in RBL-2H3 cells.[10,11] In this study, we developed three combined formulas comprising HR, GM, and PB to enhance the effectiveness of the formulations and reduce the potential irritation or side effects associated with single-use extracts. Consequently, the combination of these extracts will be a potential product for AD patients. Therefore, the objectives of this study involved the investigation of the *in vitro* anti-inflammatory activity, focusing on inhibiting TNF-α production, as well as assessing antioxidant activity through DPPH and ABTS scavenging assays of HR, GM, PB, and their combined extracts. Additionally, we evaluated the total phenolic content (TPC) and total flavonoid content (TFC) to ascertain their chemical composition. Furthermore, all plant ingredients were also evaluated for quality control parameters in accordance with the Thai Herbal Pharmacopoeia to ensure the quality of plants utilized in this investigation.

Methods

Preparation of Plant Extracts and Combined Extracts

Preparation of Plant Extracts

Five plant roots of HR were collected in Chachoengsao province, Thailand. GM pericarps and PB leaves were purchased from the Pathum Thani province market as shown in **Table 1**. They were cleaned, sliced, and oven-dried at 50 °C before grinding. To obtain HR, GM, and PB ethanolic extracts, HR, GM, and PB were macerated in 95% ethanol for 72 hours. The extracts were filtered with Whatman paper No. 1 and evaporated to dryness by a rotary evaporator. The extracts were combined after three macerations. The percentage yield was calculated by the following equation.

$$\text{The percentage of yield} = \frac{\text{Weight of the extract (g)}}{\text{Weight of dried powder of plant (g)}} \times 100$$

The combined extracts were combined from GM extract, PB extract, and HR extract (which combines the same amounts of botanical roots). The three extracts were combined in different proportions as shown in **Table 1** according to Thai traditional medicine techniques for improving the therapeutic efficacy and reducing side effects and toxicity. In TTM, novel drug remedies require medicinal plant components including primary, secondary, and supplementary components. For a treatment, the main plant ingredients were chosen by their taste, which relate to its disease treatments. A pungent main component balances the circulatory system, relieving allergy symptoms, including itching. Sweet-astringent plants are used to cures wounds, whereas anti-inflammatory plants having a bitter taste, minimize burning.[7]

Table 1 Plant ingredients, voucher specimen number and the proportion of combination extracts

Plants/ Remedy	Thai name	Scientific name	Voucher specimen number	Part used	The taste of plant	Proportion in formula		
						HMB -321	HM B-231	HMB -123
Ha-Rak remedy (HR)	Maduae-Chumporn	*Ficus racemosa* Linn.	SKP 391 03 13 01	Root	Bitter-cool	3	2	1
	Ching-Chee	*Capparis micracantha* DC.	SKP 202 03 09 01	Root	Bitter-cool			
	Tao-Yai-Mom	*Clerodendrum petasites* S. Moore	SKP 117 06 18 01	Root	Bitter-cool			
	Khon-Tha	*Harrisonia perforata* Merr.	SKP 178 08 16 01	Root	Bitter-cool			
	Ya-Nang	*Tiliacora triandra* (Colebr.) Diels.	SKP 114 20 20 01	Root	Bitter-cool			
Mangosteen (GM)	Mung-Kud	*Garcinia mangostana* Linn.	SKP 214 09 13 01	Pericarp	Sweet-astringent	2	3	2
Betel (PB)	Plu	*Piper betle* Linn.	SKP 146 16 02 01	leaf	Spicy	1	1	3

The Quality Control of Plant Materials

Quality control is regulated according to Thai Herbal Pharmacopoeia (THP 2022). This study uses physical criteria such as moisture content, extractive value total ash, and acid insoluble ash.[12]

Moisture Content

Loss on Drying

The moisture analyzer (105 °C) measured two grams of dry powder from each plant and HR except PB. The moisture content was calculated using this equation:

$$\% \text{ Moisture content} = \frac{\text{Weight of beginning sample - Weight of drying sample (g)}}{\text{Weight of beginning sample (g)}} \times 100$$

Azeotropic Distillation Method

Approximately 2 mL of water and 200 mL of toluene were distilled in a dry flask for 2 hours. The flask was slowly heated for 15 minutes. When the toluene boiled, 2 drops per second were distilled until the all the water was distilled, then the distillation rate was raised to 4 drops per second. The condenser tube was rinsed with toluene after the water was distilled. After 5 minutes of distillation, the heat was stopped, and the receiving tube was allowed to cool to room temperature. After separating the water and toluene, the water volume was measured and calculated as a percentage using the equation:

$$\% \text{ Moisture content} = \frac{\text{The substance to be examined (g) - water obtained in first distillation (ml)}}{\text{The total volume of water obtained in the two distillations (ml)}} \times 100$$

Total Ash

The crucibles were heated at 105 °C until stable. 2 g of ground-dried material were added and burned in a muffle furnace at 450 °C for 9 hours. Then the ash was weighed and placed in a 450 °C muffle furnace for 5 hours. The Ash in the crucibles was weighed repeatedly until stable. Total ash was calculated using this equation:

$$\% \text{ Total ash} = \frac{\text{Stable weight after burning (g)}}{\text{Weight before burning (g)}} \times 100$$

Acid Insoluble Ash

The total ash was heated for 5 minutes with 10% HCl. The sample was filtered through an ashless paper filter and rinsed with distilled water to pH 7. The filter paper was put into the crucible and burned at 550 °C for 9 hours in a muffle furnace. Acid-insoluble

product was weighed until the weight was stable. The following equation calculated acid insoluble ash from the following equation:

$$\% \text{ Acid insoluble ash} = \frac{\text{Stable weight after burning (g)}}{\text{Weight before burning (g)}} \times 100$$

Extractive Value

Ethanol Soluble Extractive Value

The dried powder of each plant was macerated in a closed flask with 5 g of 100 mL ethanol. After shaking repeatedly for 6 hours, the flasks were let to stand for 18 hours before being rapidly filtered. In a tare dish at 105 °C, 20 mL of filtrate was evaporated to a constant weight. Estimated ethanol-soluble extractive percentage by the equation below.

$$\% \text{ Extractive value} = \frac{\text{Weight of the extract (g)}}{\text{Weight of dried powder of plant (g)}} \times 100 \times 5$$

Water Soluble Extractive Value

Following the ethanol-soluble extraction process, chloroform water (5% chloroform in Distilled water) was used instead of ethanol.

Determination of LPS-induced TNF-alpha Release from RAW 264.7 Cells

The extract was tested for its efficacy to inhibit TNF-α release from RAW 264.7 cells using a mouse TNF-α Quantikine ELISA kit as instructed by the manufacturer. RPMI 1640 medium with 10% heat inactivated FBS (Fetal bovine serum), 10,000 units/mL penicillin, and 10,000 mg/mL streptomycin was used to culture the RAW 264.7 cells. The cells were incubated at 37 °C in humidified 5% CO_2/95% air. In 96-well plates, 1 x 10^5 cells/well were seeded and left attached for 24 h at 37 °C in 5% CO_2. The medium was replaced with RPMI 1640 containing 10 ng/mL LPS (100 µL/well) and treated with different test sample concentrations (100 µL/well) for 24 hours at 37 °C in 5% CO_2. The culture supernatant was transferred to a 96-well ELISA plate after 24 h to measure TNF-α. The absorbance was measured with a microplate reader at 405 nm. The following equation determined the TNF-α release inhibition (%) of the test samples, and the IC_{50} values were calculated by Prism (GraphPad, USA).

$$\text{Inhibition (\%)} = \left[\frac{OD_{control} - OD_{sample}}{OD_{control}}\right] \times 100$$

To assess the cytotoxicity, the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric technique was used. Each well was incubated with 10 µL MTT solution for 2 hours after transferring the supernatant to another plate. After removing the medium, 100 µL of 0.04 M HCl was applied to dissolve the formazan crystal in the well. Formazan solution optical density was 570 nm.

Determination of DPPH Radical Scavenging Activity

The DPPH radical scavenging effect was measured using Yamasaki et al.'s technique.[13] The extract was dissolved in 100% ethanol at doses of 1–100 µg/mL. After transferring 100 µL of extracts, a 96-well microplate was prepared by adding 100 µL of 6×10^{-5} M DPPH in 100% ethanol. For 30 minutes, the microplate was incubated at ambient temperature in the dark. The absorbance was measured at 520 nm. Butylated hydroxytoluene (BHT) was used as positive control. The experiment was repeated three times. Prism (GraphPad, USA) was used to calculate the 50% effective concentration of DPPH scavenging activity to determine the EC_{50} value.

Determination of $ABTS^{\bullet+}$ Radical Scavenging Activity

ABTS was modified from the previous method.[14] ABTS reagent was prepared with 7.7 mg/mL $ABTS^{\bullet+}$ in MQ water and 1.2 mg/mL potassium persulphate ($K_2O_8S_2$) in MQ water in a 1:1 ratio. 20 µL sample solution was dissolved (1-100 µg/mL) with 180 µL $ABTS^{\bullet+}$ reagent and incubated for 6 minutes at the ambient temperature. Positive control was Trolox. A microplate reader measured absorbances at 734 nm. EC_{50} of ABTS activity indicated antioxidant activity.

Measurement of Total Phenolic Contents (TPC)

TPC was measured using the modified Folin-Ciocalteu method.[15] A 20 µL extract (1000 µg/mL) was mixed with 100 µL of Folin-Ciocalteu's reagent and 80 µL of Na_2CO_3. A microplate reader

evaluated sample absorbance at 765 nm after 30 minutes at ambient temperature incubation. All measurements were performed in triplicate. The sample absorbance was compared to the gallic acid calibration curve to calculate TPC in mg gallic acid equivalent per gram (mg GAE/g).

Measurement of Total Flavonoid Contents (TFC)

TFC was determined using Zou, et al.'s technique with minor modifications.[16] A 500 µL of extract (1000 µg/mL) was mixed with 75 µL of 5% $NaNO_2$ and 150 µL of 10% $AlCl_3$. After 5 min of incubation at the ambient temperature, 500 µL of 1 M NaOH was added to the reaction solution. The mixture was added to 275 µL water and incubated at ambient temperature for 30 minutes. The absorbance was measured at 510 nm by a microplate reader. TFC was estimated by comparing the sample with Catechin's calibration curve and represented as mg catechin equivalent per gram (mg CE/g).

Statistical Analysis

All data were means of triplicates. Results were shown as mean ± standard error of the mean (SEM) and mean ± standard deviation (SD) for all extracts. The IC_{50} and EC_{50} were determined using Prism program (GraphPad, USA). One-way analysis of variance (ANOVA) and Dunnett's test determined statistical significance.

Results

Quality Control of Plant Materials

The quality control of each plant ingredient was conducted following the Thai Herbal Pharmacopoeia (THP, 2022). **Table 2** displays the values of moisture content, extractive value, total ash and acid insoluble ash, and toxic heavy metals (As, Cd, and Pb).

Regarding the moisture content of plant materials, all plant materials except PB were tested for loss on drying by a moisture analyzer. PB was tested by the azeotropic distillation method. The moisture content of all plant materials was in the range of 5.80 - 8.03%. The plant material with the highest moisture content was *Clerodendrum petasites*'s root (8.03 ± 0.30%). All plant materials passed the standard requirement of moisture content stated in the THP. For the extractive values, the water-soluble extractive values of all plant materials were in the range of 3.97 - 19.34%. PB contained the highest water-soluble extractive value (19.34 ± 0.03%). The ethanol-soluble extractive values of all plant materials were in the range of 0.64 - 4.73%. PB contained the highest ethanol-soluble extractive value (4.73 ± 0.16%). All plant materials passed the standard requirement of extractive values stated in the THP. For the total ash and acid-insoluble ash, the total ash contents of all plant materials were in the range of 3.14 - 17.66%, whereas the acid-insoluble ash contents of all plant materials were in the range of 0.02 - 5.21%. PB contained the highest acid-insoluble ash contents (5.21 ± 0.07%). The total ash and acid-insoluble ash of all plant materials passed the standard requirement stated in the THP. These quality control results indicated the quality of plants used throughout this entire investigation.

Table 2 Moisture content, ash content, extractive values, total ash acid, insoluble ash of Ha-Rak remedy and its ingredients, *Piper betle* Linn. and *Garcinia mangostana* Linn.

Scientific name	% Moisture content	% Water- soluble Extractive value	% Ethanol- soluble Extractive value	% Ash content	
				Total ash	Acid insoluble ash
Capparis micracantha standard [12]	≤ 9	≥ 1	≥ 4	≤ 5	≤ 2
Capparis micracantha	6.52 ± 0.03	9.10 ± 0.30	0.64 ± 0.04	4.83 ± 0.09	1.90 ± 0.20
Clerodendrum petasites standard [12]	≤ 9	≥ 4	≥ 1.5	≤ 5	≤ 1
Clerodendrum petasites	8.03 ± 0.30	6.65 ± 0.92	1.62 ± 0.03	4.92 ± 0.59	0.37 ± 0.14
Ficus racemosa standard [12]	≤ 10	≥ 2	≥1	≤ 7	-
Ficus racemose	7.20 ± 0.13	3.97 ± 0.16	1.19 ± 0.04	4.04 ± 0.29	0.37 ± 0.10
Harrisonia perforate standard [12]	≤ 9	≥ 3	≥ 2	≤ 4	-
Harrisonia perforate	6.88 ± 0.27	4.49 ± 0.91	4.08 ± 0.11	3.14 ± 0.15	0.02 ± 0.00
Tiliacora triandra standard [12]	≤ 9	≥ 6	≥ 4	≤ 8	-
Tiliacora triandra	7.41 ± 0.18	6.18 ± 0.41	4.37 ± 0.38	7.77 ± 0.24	0.17 ± 0.04
GM standard [17]	4.52–9.27%	29.58	37.78	14.488	0.684
GM	7.48 ± 0.34	12.10 ± 0.88	1.05 ± 0.03	3.16 ± 0.03	0.02 ± 0.02
HR standard [12]	-	-	-	-	-
HR	7.53 ± 0.14	4.89 ± 0.52	2.04 ± 0.25	5.23 ± 0.20	0.90 ± 0.15
PB standard [12]	≤ 14	≥ 8	≥ 4	≤ 18	≤ 7
PB	5.80 ± 0.21	19.34 ± 0.03	4.73 ± 0.16	17.66 ± 0.36	5.21 ± 0.07

Note: Data were expressed as the means ± standard deviation (SD) of three determinations (n = 3)

Determination of TNF-α from LPS-induced RAW 264.7 Cells

The results on the reduction of TNF-α release by the tested extracts and the positive control anti-inflammatory drug, Prednisolone, are shown in **Table 3**. To confirm that the inhibitory effect of TNF-production was not a result of cell death, the MTT assay was used for cell viability testing (cell viability > 70%). The ethanolic extract from HR showed the most potent anti-inflammatory activity by inhibiting TNF-α releasing with IC_{50} of 49.06 ± 0.38 µg/mL. GM and PB showed much lower activity with IC_{50} > 50 µg/mL, respectively. The combined formula, HMB-321 showed the strongest TNF-α releasing inhibition with IC_{50} of 47.41 ± 1.74 µg/mL, while HMB-231 and HMB-321 showed much lower activity with IC_{50} > 50 µg/mL. Prednisolone, positive control, inhibited TNF-α production with an IC_{50} of 0.11 ± 0.01 µg/mL.

Table 3 Anti-inflammatory and antioxidant activities and capacities of the ethanolic extract of combined remedies and plant ingredients

Sample	IC_{50} (µg/mL)			TPC	TFC
	TNF-α	DPPH	ABTS		
HR	49.06 ± 0.3[a]	72.17 ± 4.38[b]	78.53 ± 6.10[c]	30.25 ± 0.51	29.32 ± 1.15
GM	> 50[a]	8.25 ± 0.39	8.01 ± 0.07	156.33 ± 3.39	102.39 ± 6.37
PB	> 50[a]	4.58 ± 0.03[b]	7.63 ± 0.39	266.07 ± 3.57	711.56 ± 3.85
HMB-321	47.41 ± 1.74[a]	11.15 ± 0.27	13.91 ± 0.79	142.00 ± 3.41	201.03 ± 2.13
HMB-231	> 50[a]	7.38 ± 0.37	9.19 ± 0.31	172.22 ± 3.37	196.38 ± 1.21
HMB-123	> 50[a]	5.17 ± 0.08[b]	8.25± 0.27	246.47 ± 2.46	466.91 ± 7.22
Prednisolone	0.11 ± 0.01	-	-	-	-
BHT	-	13.40 ± 0.77	-	-	-
Trolox	-	-	4.85 ± 0.21	-	-

Note: Data were expressed as the means ± standard error of the mean (SEM) of three determinations (n = 3).
[a] Significant differences ($p < 0.05$) compared with positive control (Prednisolone)
[b] Significant differences ($p < 0.05$) compared with positive control (BHT)
[c] Significant differences ($p < 0.05$) compared with positive control (Trolox)

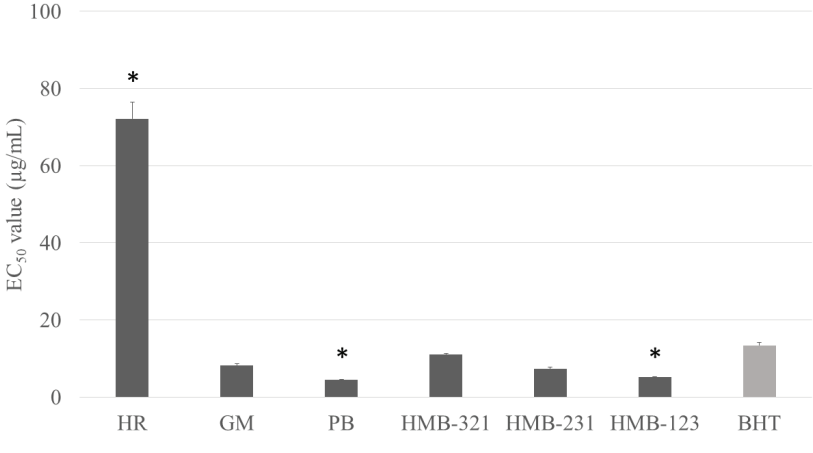

Figure 1 DPPH activity of the ethanolic extract of combined remedies and plant ingredients when compared with BHT

*P-value < 0.05 vs BHT

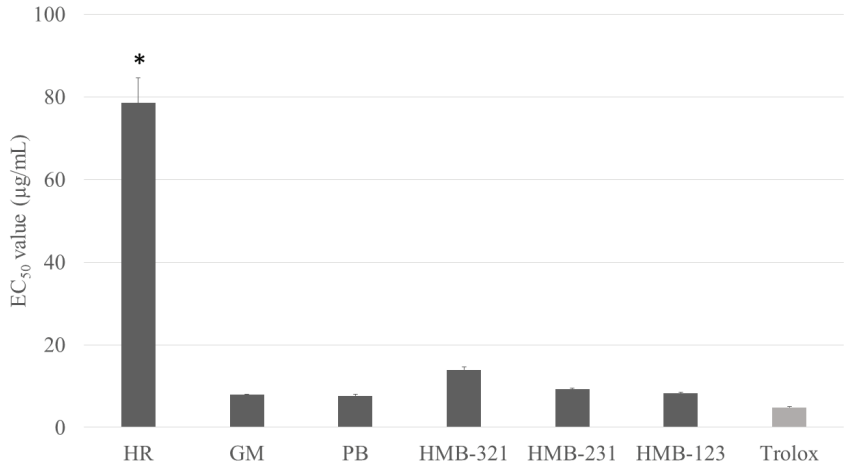

Figure 2 ABTS activity of the ethanolic extract of combined remedies and plant ingredients when compared with Trolox and BHT

* P-value < 0.05 vs Trolox

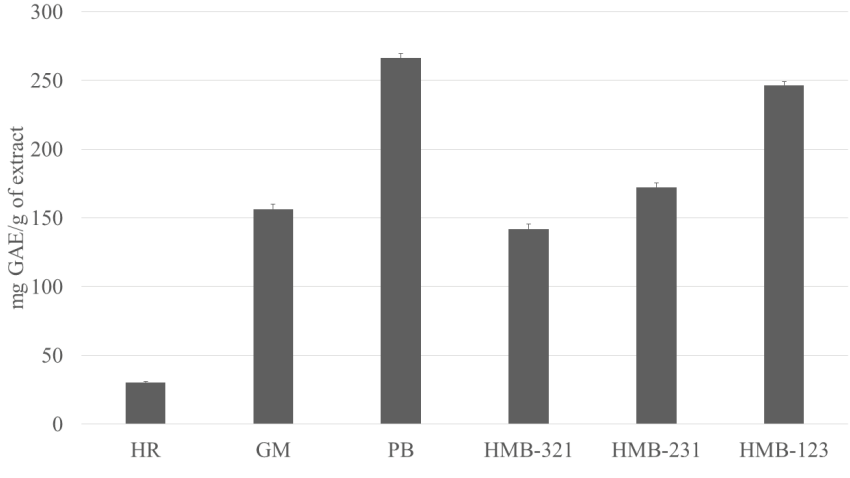

Figure 3 TPC of the ethanolic extract of combined remedies and plant ingredients

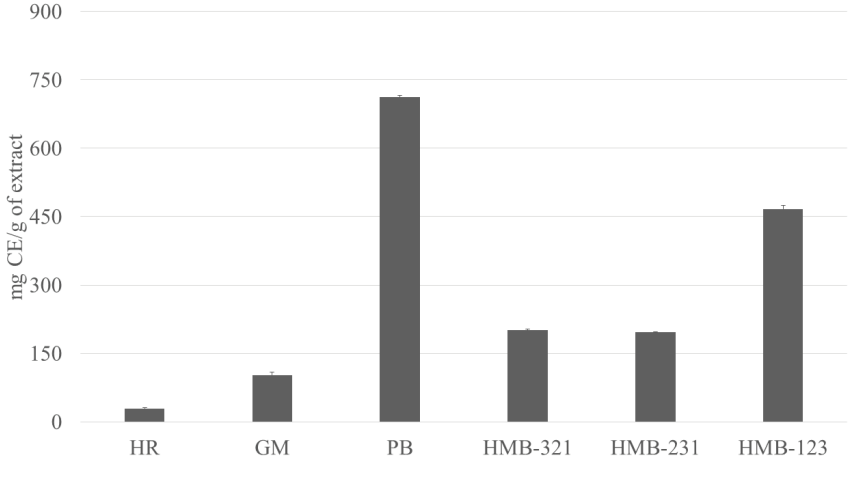

Figure 4 TFC of the ethanolic extract of combined remedies and plant ingredients

Determination of DPPH Radical Scavenging Activity

The results of DPPH radical scavenging activity of the extracts and the positive control, butylated hydroxytoluene (BHT), are shown in **Table 3** and **Figure 1**. The ethanolic extract from PB showed the most potent antioxidant activity with an EC_{50} value of 4.58 ± 0.03 µg/mL, which higher than BHT (EC_{50}= 13.40 ± 0.77 µg/mL) following by GM ethanol extract (EC_{50}= 8.25 ± 0.39 µg/mL). However, ethanolic extract of HR showed lower activity with EC_{50} of 72.17 ± 4.38 µg/mL. The combined formula, HMB-123 showed the strongest antioxidant activity, higher than BHT with EC_{50} of 5.17 ± 0.08 µg/mL. while HMB-231 and HMB-321 showed much lower activity with EC_{50} of 7.38 ± 0.37 and 11.15 ± 0.27 µg/mL.

Determination of ABTS Radical Scavenging Activity

The results of ABTS radical scavenging activity of the tested extracts and the standard, trolox, are shown in **Table 3** and **Figure 2**. The results were consistent with the results of DPPH radical scavenging activity. The ethanolic extract from PB showed the most potent antioxidant activity with EC_{50} of 7.63 ± 0.39 µg/mL which did not significantly differ from trolox (EC_{50} = 4.85 ± 0.21 µg/mL). This was followed by GM and HR, respectively. The combined formula, HMB-123, showed the strongest antioxidant activity with EC_{50} of 8.25 ± 0.27 µg/mL, while HMB-231 and HMB-321 showed lower activity.

Measurement of Total Phenolic Contents (TPC) and Total Flavonoid Contents (TFC)

The content of antioxidants in plant material were measured by the TPC and the TFC values. TPC of all extracts were between 30.25 ± 0.51 to 266.07 ± 3.57 mg GAE/g of extract. In addition, PB showed the highest TPC value as shown in **Table 3** and **Figure 3**. TFC of all extracts ranged between 29.32 ± 1.15 to 711.56 ± 3.85 mg CE/g of extract. Moreover, PB showed the highest TFC (**Table 3** and **Figure 4**).

Discussion

Quality control testing on plant materials is needed to ensure their reliable results throughout the entire study. High humidity could stimulate microbial growth and hydrolysis, which degrades plant compounds. Total ash includes physiological ash like calcium oxalate and calcium carbonate and non-physiological ash such as soil and sand contamination. Higher acid-insoluble ash can be indicated when silica is present.[18] Lower extractive value implies that the plant material is substandard, contaminated, or encounters problems during multiple processes. All plant materials, according to the quality control results of this study, satisfied the standards specified in the Thai Herbal Pharmacopoeia guideline. However, plant materials should be stored in zip lock bags and sealed in moisture-proof bags before use.

According to the results of the anti-inflammatory effect on TNF-α releasing inhibition HR and HMB-321, in which HR as the main ingredient, gave better inflammatory effect than GM and PB, or the combinations in which PB or GM was the main ingredient. Previous reports revealed that the 95% ethanolic extract of the HR remedy has potent anti-inflammatory activity by using inhibition of nitric oxide production from LPS-induced RAW264.7 cells with an IC_{50} of 40.4 µg/mL.[15] Simultaneously, previous research on PB and GM have demonstrated anti-inflammatory effects through their inhibition of many cytokines, including NO, IL-, IL-6 which are also associated with the AD pathway.[20-22]

Phytochemicals including phenolics compounds and flavonoids are found in plants, and act as a natural antioxidant that plays an important role in limiting the damaging effect of free radicals and retard the process of many chronic diseases including AD.[23] PB and HMB-123, of which PB was a main ingredient, showed the strongest antioxidant effect by DPPH and ABTS radical scavenging activities. The results of antioxidant activities correlated with the TPC and TFC contents. Higher TPC and TFC demonstrated better antioxidant activities. Moreover, a previous report found that methanolic extract of PB possessed higher DPPH radical scavenging activity than vitamin E and BHT.[24] The highest TPC was possibly due to hydroxychavicol, the main phenolic compounds in the PB which consist of a monocyclic aromatic ring with an alcoholic, aldehydic or carboxylic group. Furthermore, PB leaves contained 12 phenolic

compounds, including 6 flavonoid derivatives, 5 cinnamoyl, and phenyl propanoid.[25] Another study reported that hydroxychavicol has been reported to possess anti-inflammatory properties against Interleukin-2, Interferon- γ, and TNF-α.[26]

According to Chaovanalikit et al. (2012),[27] phenolic compounds including procyanidin, prodelphinidin, stereoisomers of afzekechin/epiafzelechin, catechin/epichatechin, and gallocatechin/epigallocatechin were also present in the pericarps of GM, suggesting their presence in the GM extract and the combined formulas. Moreover, the pericarp samples possessed the highest flavonoid content, specifically anthocyanins, cyanidin 3-glucoside and cyanidin 3-sophoroside, which are two widely distributed natural pigments in the pericarp of plants and fruits.[27-28] These results are also related to the amount of TPC and TFC.

Regarding the combined formula, HMB-321, of which HR is the main ingredient, should have the most promising medicinal activity because it exhibited anti-inflammatory effects by suppressing TNF-α release and demonstrated higher antioxidant effects than HR alone. Using a combination of medicinal plants in traditional Thai medicine may reduce the adverse effects associated with the use of a single herb and maintains long-term safety. Moreover, combination therapy has also been reported to help patients with significant drug-resistant bacterial infections. Reduced dose and toxicity reduction are the two primary objectives of synergistic treatment which minimize drug resistance as well.[29] Therefore, proportionate amounts of each ingredient should provide biological action based on medicinal taste. The findings indicated that the HMB-321 combination extract may offer potential benefits in the prevention and treatment of AD. *In vivo* testing models and clinical trials may also be needed to further assess the anti-inflammatory and antioxidant properties of the combination.

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Conflict of Interest

The authors have no conflict of interest to declare.

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Author Contributions

Ubonwan Saesiw: Reviewing, Data curation, Methodology, Writing- Original draft preparation. **Arunporn Itharat:** Supervision. **Srisopa Ruangnoo:** Methodology, Editing, Supervision. **Pranporn Kuropakornpong:** Data curation, Methodology, **Sunita Makchuchit:** Methodology, **Saovapak Poomirat:** Data curation. **Pattama Sriumpai:** Data curation, **Jagavet Tontan:** Data curation.

References

1. *Atopic dermatitis* (2023) *National Institute of Arthritis and Musculoskeletal and Skin Diseases*. Available at: https://www.niams.nih.gov/health-topics/atopic-dermatitis (Accessed: 07 November 2023).
2. *Reports* (no date) *Global Atopic Dermatitis Atlas*. Available at: https://www.atopicdermatitisatlas.org/en/explore-data/reports (Accessed: 07 November 2023).
3. Hadi HA, Tarmizi AI, Khalid KA, Gajdács M, Aslam A, Jamshed S. The Epidemiology and Global Burden of Atopic Dermatitis: A Narrative Review. *Life (Basel)*. 2021;11(9):936. doi: 10.3390/life11090936
4. Sumimoto S, Kawai M, Kasajima Y, Hamamoto T. Increased plasma tumour necrosis factor-alpha concentration in atopic dermatitis. *Arch Dis Child*. 1992;67(3):277-279. doi: 10.1136/adc.67.3.277
5. Sivaranjani N, Rao SV, Rajeev G. Role of reactive oxygen species and antioxidants in atopic dermatitis. *J Clin Diagn Res*. 2013;7(12):2683-2685. doi: 10.7860/JCDR/2013/6635.3732

6. Boo YC. Can Plant Phenolic Compounds Protect the Skin from Airborne Particulate Matter? *Antioxidants*. 2019;8(9):379. https://doi.org/10.3390/antiox8090379
7. Foundation of Thai Traditional Medicine and Ayurvedathamrong school. Tumrakarnphatthaideim (Phaetthayasat songkhro conservation issue). Bangkok: Supawanichkarnpimm; 2007.
8. National List of Essential Medicines. Notification of Ministry of Public Health 162 No. 2 2016. Bangkok; 2016.
9. Wirotesangthong M, Inagaki N, Tanaka H, Thanakijcharoenpath W, Nagai H. Inhibitory effects of *Piper betle* on production of allergic mediators by bone marrow-derived mast cells and lung epithelial cells. *Int. Immunopharmacol*, 2008;8:453-457. doi: 10.1016/j.intimp.2007.11.005
10. Dilokkunanant U, Mongkolsook N, Komgkathip U, Sukatta W, Rakthaworn P. Antimicrobial activity of betel vine (*Piper betle* Linn.) extracts in Thailand. The Proceedings of the 42nd Kasetsart University Annual Conference, 2004. Bangkok, Thailand.
11. Chomnawang MT, Surassmo S, Nukoolkarn V, Gritsanapan W. Effect of *Garcinia mangostana* on inflammation caused by *Propionibacterium acnes*. *Fitoterapia*. 2007;78(6):401-408. doi: 10.1016/j.fitote.2007.02.019
12. Department of Medical Sciences, Ministry of Public Health. Thai Herbal Pharmacopoeia 2021 Supplement 2022. (Available from: https://bdn.go.th/thp/ebook/qQIcZatkpR9gC-3q0GT5gMJq0qT5co3uw). (Accessed: 07 November 2023).
13. Yamasaki K, Hashimoto A, Kokusenya Y, Miyamoto T, Sato T. Electrochemical method for estimating the antioxidative effects of methanol extracts of crude drugs. *Chem Pharm Bull (Tokyo)*. 1994;42(8):1663-1665.
14. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med.* 1999;26(9-10):1231-1237.
15. Folin O, Ciocalteu V. On Tyrosine and Tryptophane Determinations in Proteins. *Journal of Biological Chemistry.* 1927;73(2):627-650.
16. Zou Y, Lu Y, Wei D. Antioxidant activity of a flavonoid-rich extract of *Hypericum perforatum* L. in vitro. *J Agri Food Chem*. 2004;52(16):5032-5039.
17. Pothitirat W, Pithayanukul P, Chomnawang MT, Gritsanapan W. Standardization of Garcinia mangostana fruit rind extract. *Planta Med*. 2007;73:892-893. doi: 10.1055/s-0029-1234655
18. Quality Control Methods for - World Health Organization (WHO). Available at: https://www.who.int/docs/default-source/medicines/norms-andstandards/guidelines/quality-control/quality-control-methods-for-medicinal-plantmaterials.pdf?sfvrsn=b451e7c6_0 (Accessed: 14 November 2023).
19. Juckmeta T, Itharat A. Anti-Inflammatory and Antioxidant Activities of Thai Traditional Remedy Called "Ya-Ha-Rak". *Journal of Health Research*. 2011;26(4):205-210.
20. Chen LG, Yang LL, Wang CC. Anti-inflammatory activity of mangostins from *Garcinia mangostana*. *Food Chem. Toxicol*. 2008;46:688-693.
21. Sharma S, Ali Khan I, Ali I, Ali F, Kumar M, Kumar A. Evaluation of the Antimicrobial, Antioxidant, and Anti-Inflammatory activities of Hydroxychavicol for Its Potential Use as an Oral Care Agent. *Antimicrob Agents Chemother*. 2009;53(1):216-222.
22. Tewtrakul S, Wattanapiromsakul C, Mahabusarakam W. Effects of compounds from *Garcinia mangostana* on inflammatory mediators in RAW264.7 macrophage cells. *J Ethnopharmacol*. 2009;121(3):379-82.
23. Sivaranjani N, Rao SV, Rajeev G. Role of reactive oxygen species and antioxidants in atopic dermatitis. *J Clin Diagn Res*. 2013;7(12):2683-5. doi: 10.7860/JCDR/2013/6635.3732
24. Sundang M, Nasir SNS, Sipaut CS, Othman H. Antioxidant Activity, Phenolic, Flavonoid and Tannin Content of *Piper Betle* and *Leucosyke Capitella*. *Malaysian Journal of Fundamental and Applied Sciences*. 2014;8(1):1-6. doi: 10.11113/mjfas.v8n1.115

25. Ferreres F, Oliveira AP, Gil-Izquierdo A, Valentão P, Andrade PB. *Piper betle* leaves: profiling phenolic compounds by HPLC/DAD-ESI/MS(n) and anti-cholinesterase activity. *Phytochem Anal*. 2014;25(5):453-60. doi: 10.1002/pca.2515
26. Pin K, Chuah AL, Rashih AA, Mazura M, Fadzureena J, Vimala S, Rasadah M. Antioxidant, and anti-inflammatory activities of Extracts of betel leaves (*Piper betle*) from solvents with different polarities. *Journal of Tropical Forest Science*. 2010;22(4): 448-455.
27. Chaovanalikit A, Mingmuang A, Kitbunluewit T, Choldumrongkool N, Sondee J, Chupratum S. Anthocyanin and total phenolics content of mangosteen and effect of processing on the quality of mangosteen products. *International Food Research Journal*. 2012;19(3):1047-1053.
28. Mosquera M, Oscar M, Obando C, María A, Ortega C. Chemistry characterization and antioxidant activity of mangosteen (*Garcinia mangostana* L, Clusiaceae) cultivated in Colombia. *Bol Latinoam Caribe Plant Med Aromat.* 2020;19(2):167-178. doi: 10.37360/blacpma.20.19.2.10
29. Pezzani R, Salehi B, Vitalini S, Iriti M, Zuñiga FA, Sharifi-Rad J, Martorell M, Martins N. Synergistic effects of plant derivatives and conventional chemotherapeutic agents: an update on the cancer perspective. *Medicina*. 2019;55(4):110. doi: 10.3390/medicina 55040110