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Asian Medical Journal and Alternative Medicine (AMJAM) aims to publish high quality, peer-review articles. This journal publishes Original article, Review article, Special article, Brief research, Case report, and Letter to the Editor in all health sciences, medical specialties and alternative medicine. Manuscripts submitted to AMJAM should consist of new material that focuses on broad topics in health sciences published in English language. AMJAM published 3-4 issues annually (January-April, May-August, September-December, and 1 supplemental issue per year). All submitted articles will be evaluated using double-blinded review process by 3-4 reviewers.

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Editor's Note

Amidst conflicts and changes in politics, climate and environment of our world, health sciences and alternative medicine have still been driven by the results from high-quality research papers conducted in varied settings around the world. The editorial team of AMJAM which includes Thai and international editors commit to select and publish great research articles in AMJAM to disseminate advanced and evolving knowledge in the fields of medicine and alternative medicine to its readers. We are now in the process of quality improvement to be well-recognized as an international journal and eventually be accepted in world-renowned scientific citation databases. This process may take some time and our readers will notice these changes soon. For this current issue, AMJAM publishes original articles addressing anti-inflammatory property of different Thai traditional medicine remedies and impact of pterygium, one of the most common eye diseases on tear and dry eye symptoms. Brief research paper should be of interest for those taking care of preschool children with attention deficit hyperactivity disorder, while the case report section illustrates the interesting cases of warthin-like papillary thyroid carcinoma and hypertrophic pachymeningitis from neuro-behçet's disease.

Thana Khawcharoenporn, M.D., M.Sc.
Professor of Medicine
Editor-in-Chief
Asian Medical Journal and Alternative Medicine (AMJAM)

Editorial

Thai Medicinal Treatment Theory in Hypertension

Pannawat Chaiyawatthananthn*

In Thai traditional medicine hypertension or high blood pressure is understood through the lens of imbalance in the body's elements and energies. The treatment approach typically involves a combination of herbal remedies, dietary adjustments, lifestyle modifications, and sometimes therapeutic practices like Thai massage or meditation. Here are some key aspects for the treatment of hypertension in Thai traditional theory:

1. Herbal Remedies - Thai traditional medicine utilizes various herbs and herbal formulations that are believed to help balance the body's energies and support cardiovascular health, such as Centella asiatica (Gotu kola), Andrographis paniculata (King of Bitters), and Orthosiphon stamineus (Java tea).

2. Dietary Modifications - A key component of treatment involves dietary adjustments aimed at reducing sodium intake and promoting a diet rich in fresh fruits, vegetables, lean proteins, and whole grains. Certain herbs and spices are also recommended to be integrated into cooking for their health-promoting properties.

3. Lifestyle Changes - Thai traditional medicine emphasizes lifestyle modifications including regular physical activity, stress reduction techniques (such as meditation or mindfulness practices), and adequate sleep to support overall health, as well as manage hypertension.

4. Therapeutic Practices - Traditional Thai massage (Nuad-Thai) and therapeutic Thai herbal compress (Luk-Pra-Kob) are sometimes used to promote circulation, reduce muscle tension, and support relaxation, which may indirectly help in managing hypertension.

5. Energy Balancing - Practitioners of Thai traditional medicine often focus on balancing the body's energy channels and imbalance of four elements (e.g., fire, wind, water and earth) believed to contribute to hypertension.

6. Individualized Approach - Treatment plans in Thai traditional medicine are often tailored to the individual's specific constitution, symptoms, and risk factors of hypertension. This personalized approach may involve a combination of different herbs and therapies based on the practitioner's assessment.

Several herbs are known for their benefits in managing hypertension. It's important to note that while some herbs may show promise in clinical studies, their effectiveness and safety can vary, and they should be used cautiously and preferably under medical supervision. Here are some herbs commonly used or studied for their potential effects on hypertension:

1. Garlic (*Allium sativum*) - Known for its cardiovascular benefits, garlic may help lower blood pressure due to its ability to widen blood vessels and improving circulation,[1]

2. Hawthorn (*Crataegus pinnatifida*)- Often used in traditional medicine for heart health, hawthorn may help dilate blood vessels and improve blood flow, potentially lowering blood pressure,[2]

3. Hibiscus (*Hibiscus sabdariffa*)- Hibiscus tea has been studied for its antihypertensive effects by increasing urination, possibly due to its antioxidant properties and ability to relax blood vessels,[3]

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4. Cinnamon (*Cinnamomum verum*)- While more commonly associated with blood sugar control, cinnamon may also have a modest effect on blood pressure reduction,[4]

5. Ginger (*Zingiber officinale*) - Known for its anti-inflammatory properties, ginger may help lower blood pressure by improving blood circulation and relaxing muscles surrounding blood vessels,[5]

6. Turmeric (*Curcuma longa*) – Contains curcumin, which has antioxidant and anti-inflammatory properties that may contribute to cardiovascular health,[6]

7. Basil (*Ocimum basilicum*) - Contains eugenol, which may help lower blood pressure by dilating blood vessels,[7]

8. Cardamom (*Elettaria cardamomum*)- Often used in traditional medicine, cardamom may help lower blood pressure due to its antioxidant properties and potential diuretic effects,[8]

9. French lavender (*Lavandula stoechas*) - Known for its calming effects, lavender may help reduce stress and anxiety, which can contribute to lowering blood pressure,[9]

10. Celery seed (*Apium graveolens*)-Contains compounds that may help lower blood pressure by relaxing the muscles in and around arterial walls,[10]

It's crucial to consult with a healthcare provider before using herbs for hypertension. This precaution is especially important if you are already taking medication or have other health conditions. Herbal remedies should not replace prescribed medications without medical guidance, as they may interact with medications or have unintended effects. The collaboration between traditional and modern medicine can offer a combined strategy for managing hypertension effectively and safely.

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Original Article

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

Wichida Larsomsri[1], Pannawat Chaiyawatthananthn[2,3*], Thanaporn Intarawan[1], Yupa Ngankogsoong[2], Katanchalee Houngiam[2]

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.

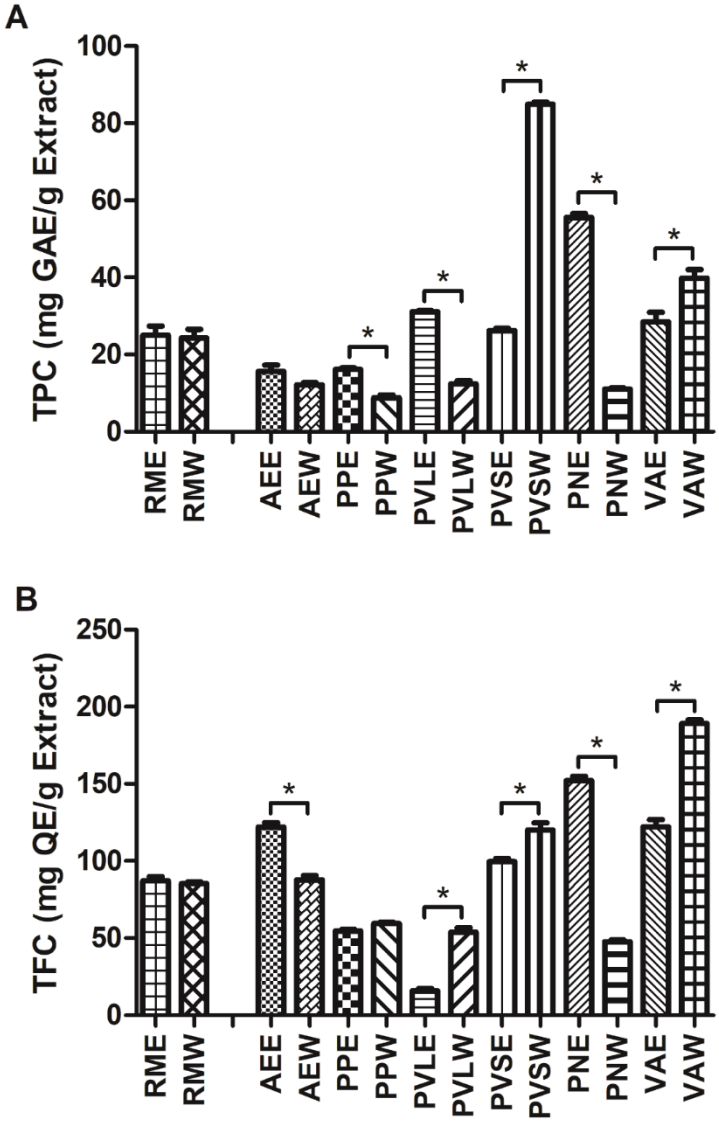

Figure 1 Comparative total phenolic content (TPC; A) and total flavonoid content (TFC; B) of the ethanol and the water extracts of the Thai folk remedy and its components, * $p < 0.05$ vs. water extraction.

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.

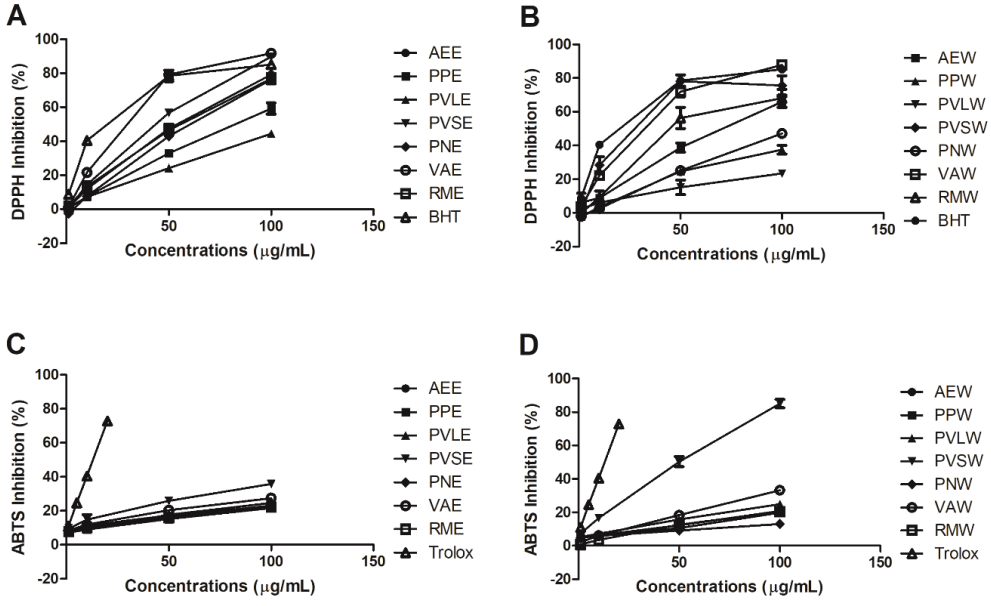

Figure 2 Percent inhibition of the DPPH radicals by the ethanol (A) and water (B) extracts, and the $ABTS^{\bullet+}$ inhibitory activity (%) of the ethanol (C) and water (D) extracts.

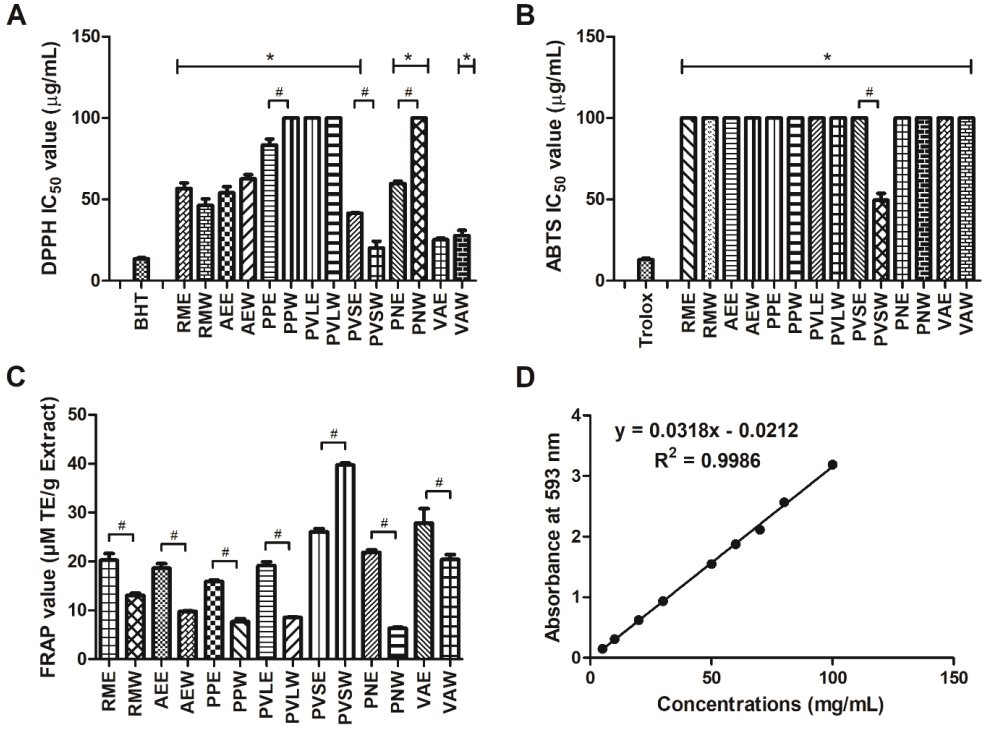

Figure 3 The antioxidant activity of the remedy and the plant ingredient extracting with ethanol and aqueous solvents on DPPH (A), ABTS (B) and FRAP (C) methods, and the standard curve for calculating FRAP value (D). * $p < 0.05$ vs. BHT or Trolox, # $p < 0.05$ vs. water extraction.

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.

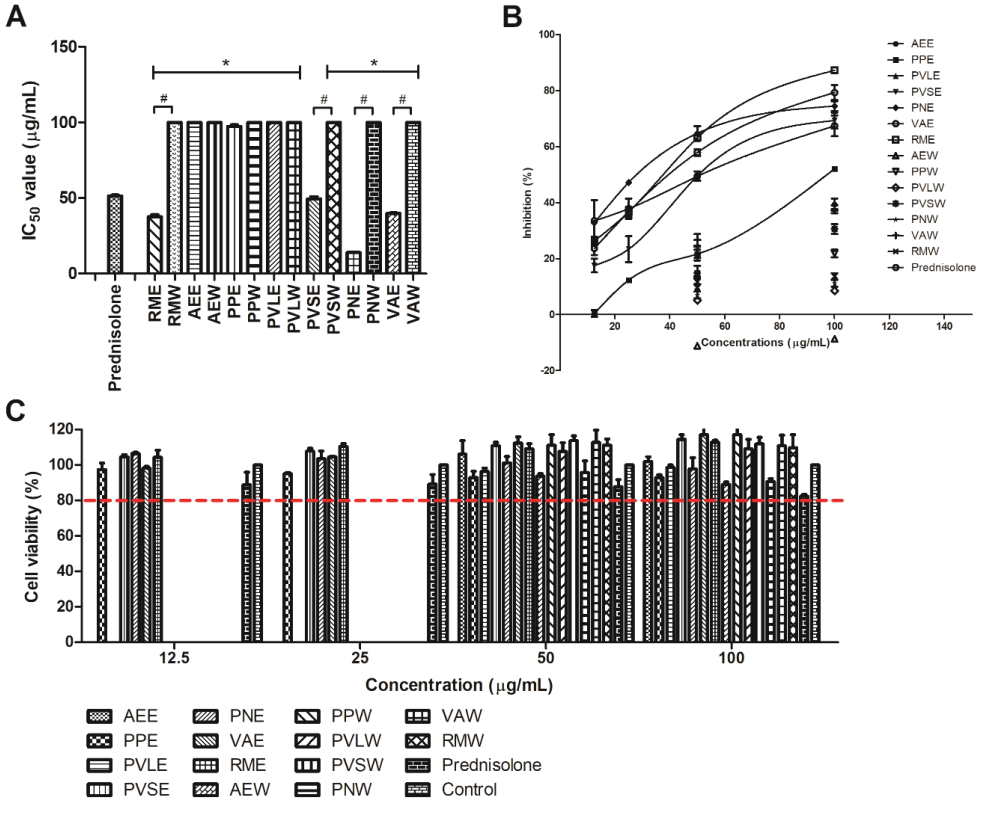

Figure 4 The anti-inflammatory activity (IC_{50}) (A), %inhibition at various concentration (B) and the percent of RAW264.7 survival cells (C) of the ethanol and the aqueous extracts of the recipe and the ingredients. Red dot line is 80% survival level. * $p < 0.05$ vs. Prednisolone, # $p < 0.05$ vs.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

Correlation of Size and Redness of Pterygium on Tear Film and Dry Eye Symptoms

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Abstract

Purpose: This study aimed to investigate the correlation of size and redness of pterygium to tear film and dry eye symptoms.

Setting/Venue: Thammasat University Hospital, Thailand

Materials and Methods: 976 patients joined a hospital-based, retrospective cross-sectional study in Thammasat University Hospital. Severity of pterygium was measured and collected by size and redness; Tear film was measured and collected by Tear meniscus height (TMH) and Tear break up time (TBUT); Dry eye syndrome was measured and collected by Ocular surface visual analogue scale (VAS), Ocular Surface Disease Index (OSDI), Oxford corneal staining scale and Meibomian gland dysfunction grading. The study was analyzed for correlation, using statistical tools of simple linear regression, Pearson correlation and Anova.

Results: 328 pterygium patients were identified, and information collected. The average horizontal and vertical size of pterygium was 2.74 mm and 2.45 clock hours. The most common redness grading was intermediate (176/328:53.66%). The relationship between horizontal size, vertical size and redness of pterygium with dry eye symptoms and tear film was (R = 0.32, 0.29 and 0.64) with a significance of <0.001, 0.005 and <0.001. The strongest correlation found was in redness of pterygium with OSDI scores, TBUT and Oxford corneal staining scale (R = 0.58, -0.46 and 0.38) with a significance of <0.001.

Conclusions: Pterygium patients were found to be 33.6 percent in this hospital-based, retrospective cross-sectional study. Horizontal size, Vertical size and redness of pterygium were related to tear film and dry eye symptoms. Redness of pterygium was the most important clinical feature affecting tear film and dry eye symptoms.

Keywords: Size, Redness, Pterygium, Tear film, Dry eye symptoms

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Introduction

Pterygium, one of the most common ocular surface diseases in ophthalmology practice, is a wing-shaped conjunctival tissue growth from the conjunctiva to the cornea.[1] Blurred vision due to astigmatism, appearance, and an irritated eye are important chief complaints with pterygium patients. Prevalence is roughly around 10% depending on the geographical location and is more common in the equator area or pterygium belt.[2,3] Many studies have researched the cause of pterygium, but it is still unclear. However, hereditary factors have been noted as a possible cause, and pterygium progression has a higher prevalence in areas exposed to greater ultraviolet radiation.[4-9] Since pterygium is an abnormal conjunctival tissue growth, there is an impact on symptoms from the ocular surface and tear film. Many studies have evidence of a correlation between pterygium and dry eye disease.[10-11]

Based on the report from the Dry Eye Workshop (DEWS II), dry eye disease is the most common problem of eye health. Moderate to severe dry eye disease was shown to be similar to moderate to severe angina, in quality-of-life studies. Dry eye disease has been defined as a disease of the ocular surface which has multifactorial causes and many various pathogenesis. The disease disrupts homeostasis of the tear film which appears in conjunction with ocular symptoms associated with hyperosmolar tears, tear film instability, inflammation of the eye surface, and loss of sensory perception of the eyes.[12-13] As population surveys show, electronic tools are a factor that cause eye disorders among office workers, particularly dry eye symptoms. The prevalence of dry eye was found to be more than half in a population of digital usage users.[14]

In a study conducted by Lekhanont and colleagues, dry eye disease had a prevalence rate of 34% among 550 participants who visited the ophthalmology department for their annual eye exam, with more than 50% of these patients also having pterygium.[15,16] Another study in Pathum Thani showed more than 70-85% of pterygium patients have dry eye symptoms.[17]

Erkut Kucuk and colleagues performed a study which investigated tear film function and dry eye syndrome, and it showed young patients with pterygium had lower Schirmer II test results, lower TBUT values, and higher OSDI scores compared to the control group.[18] Another study, by Jeremy Tan and colleagues, showed pterygium recurrence was associated with a greater severity of dry eye, possibly by perpetuating ocular surface inflammation in the postoperative period.[19] Also, Huping Wu and colleagues showed a correlation between Meibomian Gland Dysfunction (MGD) parameters and ocular discomfort, as well as dry eye indexes, and these findings suggest that MGD correlates with tear film instability and ocular discomfort as seen in patients with pterygium.[20]

As there are many ways for grading severity of pterygium, size and redness were chosen as two common characteristics. This study intended to assess the correlation of size and redness of pterygium to tear film and dry eye symptoms. These results could be useful in pterygium care.

Materials and Methods

This study was approved by the Research Ethics committee 1, the Faculty of Medicine, Thammasat University. All methods were performed in accordance with the guidelines and regulations under Thammasat University Hospital.

The study was performed by retrospective reviews of data from the Pterygium Screening Project at Thammasat Hospital, which collected data in a hospital-based, cross-sectional study at Thammasat University Hospital. The criteria for enrollment included patients with pterygium, aged between 15-80 years. The criteria for exclusion included those who were not mentally capable and could not provide the data required for the study.

Data collection included the following information: age, gender, education, occupation and type of pterygium. Moreover, severity levels of symptoms and signs on the ocular surface such as eye pain, eye irritation, eye tearing, blurred vision, red eye, and level of disturbances in daily life were collected as a visual analog scale (VAS) (1-10 points). Evaluation of dry eye symptoms were recorded by ocular surface disease index (ODSI).[21] All patients were examined by standard slit lamp for evaluating severity of pterygium, including size and redness. The horizontal size of pterygium

was calculated by slit lamp measurements, and the vertical size by using clock hours. Redness severity was observed as Tan grading,[22] and was evaluated by morphology and fleshiness: 1 for atrophic, 2 for intermediate and 3 for fleshy. Tear film was collected by tear meniscus height (TMH), tear break up time (TBUT) with fluorescein staining, Oxford corneal staining scale and Meibomian gland dysfunction grading.

The quantitative data was displayed as numbers, which were subsequently analyzed to obtain percentages and mean by using ANOVA, Regression analysis and correlation analysis by Pearson correlation coefficient. The results were considered statistically significant at $p \leq 0.05$. Statistical analysis was performed using the SPSS software version 22.0 (SPSS Inc, Chicago, IL).

The data was reviewed from the questionnaire collected at Thammasat University Hospital. The questionnaire obtained patient's information and was then tested with the simple content validity method by two ophthalmologists, specializing in cornea and glaucoma. Administrative staff also helped to verify the questionnaire's linguistic accuracy.[23-24] The Pterygium Screening Project at Thammasat Hospital was performed by the department of ophthalmology. All included patients were voluntary, and all the data was collected by 5 general ophthalmologists who were studying in a fellowship program (1 cornea and refractive surgery, 2 glaucoma and 2 retina fellowship).

Results

A total of 976 individuals participated in The Pterygium Screening Project at Thammasat University Hospital and answered the questionnaire. The study was collected as inclusion and exclusion criteria. According to the collected data, 328 patients of pterygium were collected, 314 patients as primary type and 14 patients as recurrent type. The range of age was 15-80 years, and the majority age group was 51-60 years of age which was represented by 105 patients (32%). The average age was 51.2 years. Two hundred seventeen male participants (66.2%) were more populous than the 111 female participants (33.8%). 102 patients (31.1%) graduated from primary education, and General labor was the most represented group with 97 patients (29.6%) (Table 1).

By horizontal size of pterygium: the average size was 2.74 millimeters, and grouped by size, less than 1.5 millimeters was 53 patients (16.2%), 1.5-4.0 millimeters were was 235 patients (71.6%) and more than 4.0 millimeters was 40 patients (12.2%). The three groupings of horizontal size, from small to large, showed a correlation with increasing dry eye severity levels of symptoms and signs as visual analog scale (VAS) (1-10 points) included eye pain, eye irritation, eye tearing, blurred vision, red eye, level of disturbances in daily life, OSDI scores. Size correlated with severity level of symptoms and signs. Tear film as tear meniscus height (TMH), tear break up time (TBUT) with fluorescein staining, Oxford corneal staining scale and meibomian gland dysfunction grading are shown in Table 2. Average vertical size was 2.45 clock hours. Vertical size of pterygium was grouped into 5 categories following 1, 2, 3, 4 and 5 clock hours, and a correlation with increasing dry eye severity level of symptoms and signs as visual analog scale (VAS), and OSDI scores, was noted too. correlated with severity of levels of. Tear film as tear meniscus height (TMH), tear break up time (TBUT) with fluorescein staining, Oxford corneal staining scale and meibomian gland dysfunction grading are shown in Table 3. The most common redness grading was intermediate in 176 patients (53.7%). Redness level, separated into 3 groups, showed increasing dry eye severity levels of symptoms and signs as visual analog scale (VAS), and OSDI scores, and these groups correlated with severity of levels of symptoms and signs as well.

Table 1 Demographic and clinical data of patients (n = 328)

Characteristics	Type of pterygium		
Age (years)	Primary N (%) n = 314	Recurrent N (%) n = 14	Total N (%)
< 30	16 (5.1)	0 (0.0)	16 (4.9)
30-40	53 (16.9)	4 (28.6)	57 (14.7)
41-50	71 (22.6)	2 (14.3)	73 (22.3)
51-60	99 (35.1)	6 (42.9)	105 (32.0)
61-70	61 (19.4)	1 (7.1)	62 (18.9)
> 70	14 (4.5)	1 (7.1)	15 (4.6)
Mean age	51.21	50.9	51.2
Gender			
Female	105 (33.4)	6 (42.9)	111 (33.8)
Male	209 (66.6)	8 (57.1)	217 (66.2)
Education			
Uneducated	36 (11.5)	4 (28.6)	36 (11.0)
Primary education graduates	98 (31.2)	1 (7.1)	102 (31.1)
High school graduates	72 (22.9)	7 (50.0)	73 (22.3)
Bachelor's degree graduates	75 (23.9)	2 (14.3)	82 (25.0)
Vocational education graduates	33 (10.5)	0 (0%)	35 (10.7)
Occupation			
Unemployed	76 (24.2)	1 (7.1)	77 (23.5)
General labourers	93 (29.6)	4 (28.6)	97 (29.6)
Officer	28 (8.9)	3 (21.4)	31 (9.5)
Farmer	41 (13.1)	2 (14.3)	43 (13.1)
Officialdom	27 (8.6)	1 (7.1)	28 (8.5)
Private business owners	13 (4.1)	2 (14.3)	15 (4.6)
Merchant	36 (11.5)	1 (7.1)	37 (11.3)

The relationship between horizontal size of pterygium, dry eye symptoms and tear film was assessed by using Pearson correlation coefficient and the simple linear regression analysis at the statistical significance level of 0.05. The results revealed a significant relationship between horizontal size of pterygium with eye tearing, blurred vision, red eye, VAS total, and OSDI scores. The overall Pearson correlation coefficient was 0.32 as shown in Table 2 and Figure scatterplot 1.

Furthermore, the relationship between vertical size of pterygium, dry eye symptoms and tear film were assessed by using Pearson correlation coefficient and the simple linear regression analysis at the statistical significance level of 0.05. The results revealed a significant relationship between vertical size of pterygium and blurred vision, red eye, VAS total, OSDI scores and Tear meniscus height. The overall relationship was 0.29 as shown in Table 3 and Figure scatterplot 2.

In addition, the relationship between redness of pterygium, dry eye symptoms and tear film were assessed by using Pearson correlation coefficient and the simple linear regression analysis at the statistical significance level of 0.05. The results revealed a significant relationship between vertical size of pterygium and every dry eye symptom and tear film parameter except tear meniscus height. The overall relationship was 0.64 as shown in Table 4 and Figure scatterplot 3.

Table 2 Relationship between Horizontal size of Pterygium and tear film and dry eye symptoms

Horizontal size of Pterygium	**Mean**	**SD**	**Mode**	**R (Pearson correlation)**	**P-value**	**Summary R**	**Summary R square**	**Summary p-value**
VAS Eye pain	3.83	2.88		0.21	0.35	0.32	0.10	<0.001
VAS Eye irritation	5.61	2.89		0.78	0.08			
VAS Eye tearing	4.50	3.14		0.17	<0.001*			
VAS Blur vision	5.83	3.09		0.16	0.02*			
VAS Red eye	5.71	3.25		0.16	0.02*			
VAS Disturb daily life	6.36	3.13		0.08	0.07			
VAS total	31.87	14.38		0.15	0.004*			
OSDI scores	21.57	10.06		0.17	<0.001*			
Tear meniscus height: TMH (mm)	0.285	0.13		0.05	0.17			
Tear break up time: TBU (s)	8.87	4.11		-0.03	0.30			
Oxford corneal staining scale			Grade 2 (118/328) (35.98%)	0.04	0.26			
Meibomian gland dysfunction grading			Grade 1 (138/328) (42.07%)	-0.04	0.25			

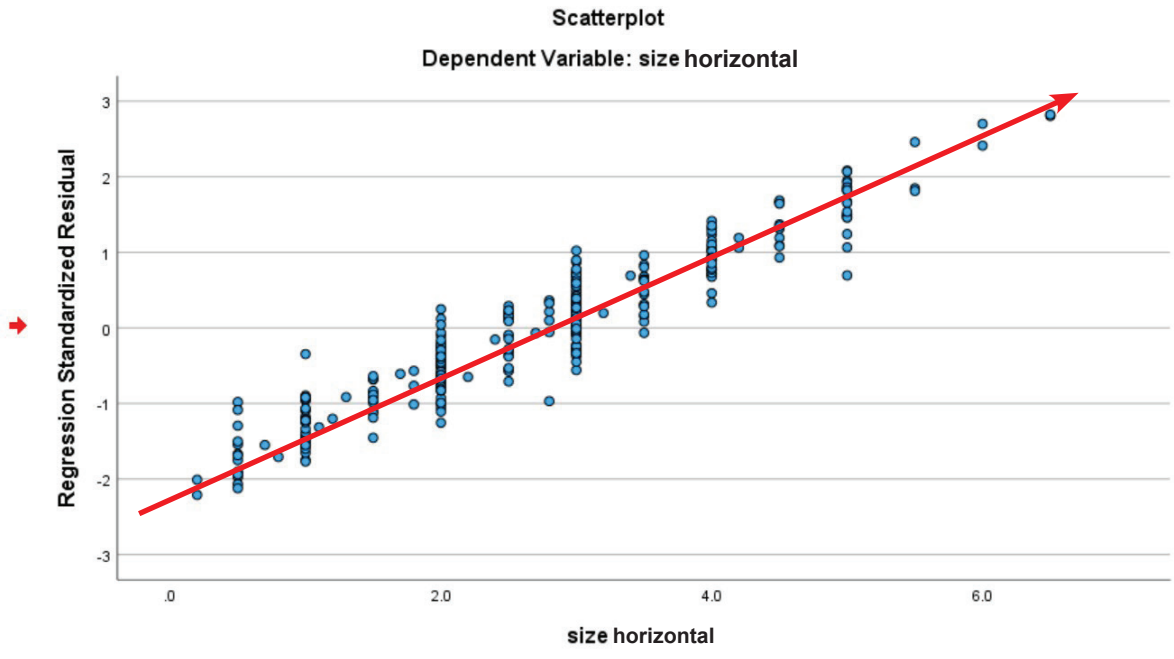

Figure 1 Relationship between horizontal size of pterygium and tear film and dry eye symptoms scatterplot

Table 3 Relationship between vertical size of Pterygium and tear film and dry eye symptoms

Vertical size of Pterygium	Mean	SD	Mode	R (Pearson correlation)	P-value	Summary R	Summary R square	Summary p-value
VAS Eye pain	3.83	2.88		0.18	0.38	0.29	0.08	0.005
VAS Eye irritation	5.61	2.89		0.07	0.10			
VAS Eye tearing	4.50	3.14		0.07	0.20			
VAS Blur vision	5.83	3.09		0.17	<0.001*			
VAS Red eye	5.71	3.25		0.13	0.01*			
VAS Disturb daily life	6.36	3.13		0.07	0.12			
VAS total	31.87	14.38		0.11	0.02*			
OSDI scores	21.57	10.06		0.11	0.02*			
Tear meniscus height: TMH (mm)	0.285	0.13		0.11	0.02*			
Tear break up time: TBU (s)	8.87	4.11		-0.10	0.30			
Oxford corneal staining scale			Grade 2 (118/328) (35.98%)	0.31	0.29			
Meibomian gland dysfunction grading			Grade 1 (138/328) (42.07%)	0.29	0.30			

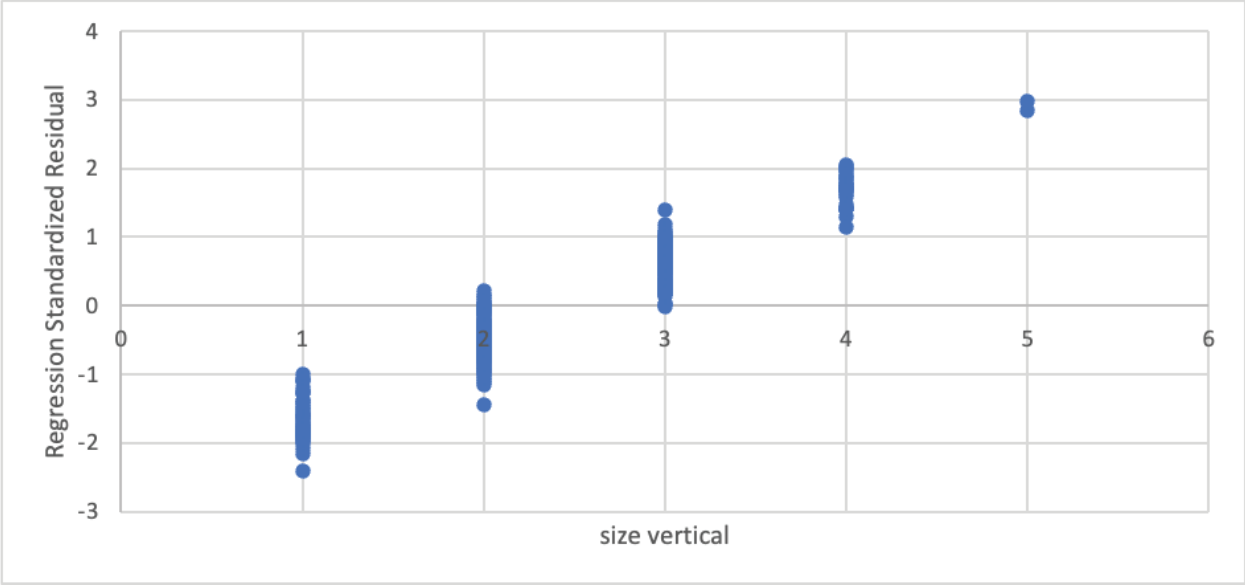

Figure 2 Relationship between vertical size of pterygium and tear film and dry eye symptoms

Table 4 Relationship between redness of pterygium and tear film and dry eye symptoms

Pterygium Redness	Mean	SD	Mode	R (Pearson correlation)	P-value	Summary R	Summary R square	Summary p-value
VAS Eye pain	3.83	2.88		0.25	<0.001*	0.643	0.414	<0.001
VAS Eye irritation	5.61	2.89		0.17	<0.001*			
VAS Eye tearing	4.50	3.14		0.18	<0.001*			
VAS Blur vision	5.83	3.09		0.20	<0.001*			
VAS Red eye	5.71	3.25		0.20	<0.001*			
VAS Disturb daily life	6.36	3.13		0.21	<0.001*			
VAS total	31.87	14.38		0.26	<0.001*			
OSDI scores	21.57	10.06		0.58	<0.001*			
Tear meniscus height: TMH (mm)	0.285	0.13		-0.05	0.17			
Tear break up time: TBU (s)	8.87	4.11		-0.46	<0.001*			
Oxford corneal staining scale			Grade 2 (118/328) (35.98%)	0.38	<0.001*			
Meibomian gland dysfunction grading			Grade 1 (138/328) (42.07%)	0.26	<0.001*			

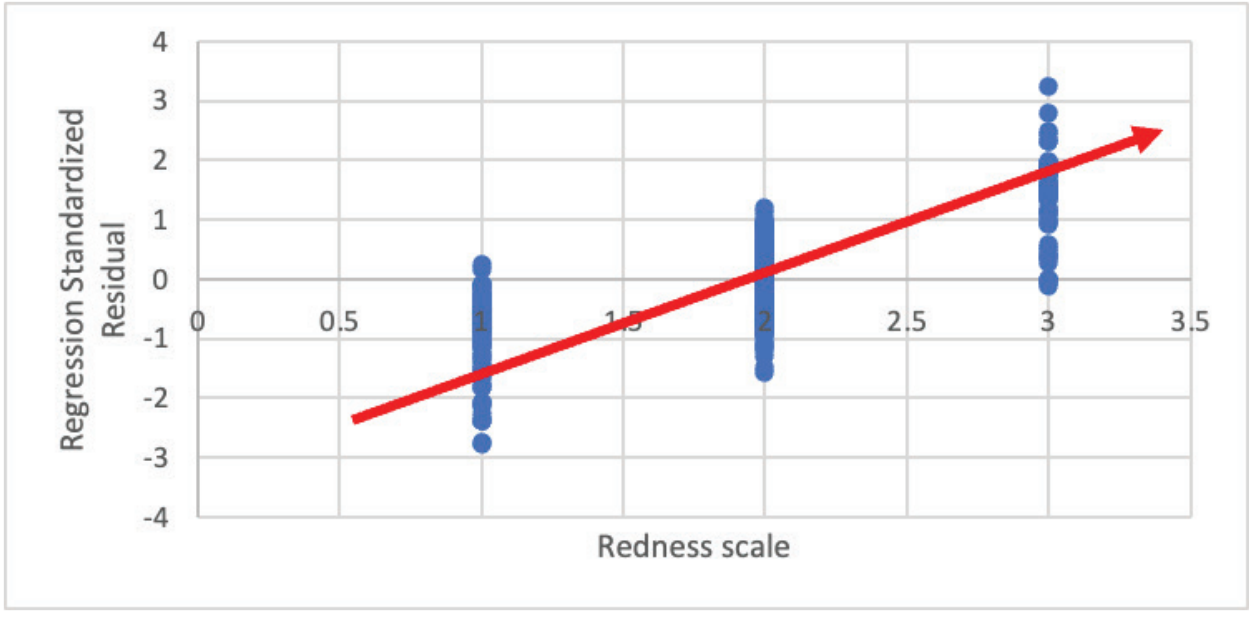

Figure 3 Relationship between redness of pterygium and tear film and dry eye symptoms

Discussion

Among 976 patients who joined in this project, we found 328 pterygium patients, of which the incidence was 33.6%. This Pterygium incidence was more than a prior incidence report.[25] It was assumed this project included patients who were aware of their own pterygium problems, and this could have influenced the incidence of pterygium in our study, since it gave patients an opportunity to be in a pterygium study.

In terms of severity of pterygium, there are many characteristics. This study observed size and redness of pterygium because these characteristics are two of the most common in clinical practice and could be applied in a real clinical setting. However, in terms of clinical symptoms which causes suffering to patients, these involved ocular surface symptoms. Qian L and colleagues,[26] in a meta-analysis study investigating risk factors for dry eye syndrome, found pterygium as one of risk factors influencing clinical dry eye. Regarding the pathogenesis of pterygium,[27] many theories exist with explanations such as tear film changes, inflammatory stimulation by cytokines and growth factor imbalance. Therefore, tear film expression and dry eye symptoms affect severity of pterygium.

For the size of pterygium, this study observed and measured horizontal and vertical dimensions. Horizontal size of pterygium, organized into three groups following small to large, showed that dry eye severity levels as OSDI scores and tear break up time (TBUT) correlated with severity. These results were similar to results reported in Erkut Kucuk and colleagues' study, investigating tear film function and dry eye syndrome, which showed that young patients with pterygium had lower TBUT values, and higher OSDI scores compared to the control group.[19] However, our study investigated more dry eye severity levels of symptoms, which included a visual analog scale (VAS) (1-10 points), eye pain, eye irritation, eye tearing, blurred vision, red eye, level of disturbances in daily life, Tear film as tear meniscus (TMH), Oxford corneal staining scale and meibomian gland dysfunction grading, and these correlated with larger horizontal size. Similarly, vertical size of pterygium, separated into five groups following 1, 2, 3, 4 and 5 clock hours, showed correlation with increasing dry eye severity levels of symptoms and signs too, including visual analog scale (VAS), and OSDI scores. Tear film as tear meniscus height (TMH), tear break up time (TBUT) with fluorescein staining, Oxford corneal staining scale and meibomian gland dysfunction grading correlated with the larger vertical size.

Redness of pterygium, separated into 3 groups, showed increasing dry eye severity levels of symptoms and signs as visual analog scale (VAS),

and OSDI scores, and these groups correlated with severity of levels of symptoms and signs. Tear film as tear meniscus height (TMH), tear break up time (TBUT) with fluorescein staining and Oxford corneal staining scale were shown to have a correlation with the severity of redness. Ozsutcu M and colleagues investigated tear break up time (TBUT), fluorescein corneal staining, and conjunctival redness in pterygium patients compared with a control group, and they found a correlation between these characteristics and pterygium.[28] These outcomes were the same in our study.

In addition, meibomian gland dysfunction grading was a parameter our study evaluated. Huping Wu and colleagues showed a correlation between MGD parameters and ocular discomfort, as well as dry eye indexes, and these findings suggest that MGD correlates with tear film instability and ocular discomfort as seen in patients with pterygium.[20] Our study found a correlation between severity of redness and meibomian gland dysfunction grading, but regarding size, we did not find a correlation. We suggest further studies should investigate in this issue.

The relationships between horizontal size, vertical size, and redness with dry eye symptoms and tear film, were also assessed by using Pearson correlation coefficient and simple linear regression analysis. We found that redness of pterygium had the strongest relationship, 0.64, the next was horizontal size, 0.32 and then vertical size, 0.29.

There are limitations associated with this research which retrospectively collected data with a subjective questionnaire from patients who may have data collection bias. Even this study, which was a hospital-based, retrospective cross-sectional study, collected from a diverse population, may have selection bias and limited generalizability. Although there are some limitations of this study, the obtained information may be beneficial for future research.

Conclusion

The incidence of pterygium was 33.6 percent in this hospital-based study. Horizontal size, Vertical size and redness of pterygium were related to tear film and dry eye symptoms. Redness of pterygium was the most important clinical feature associated with tear film and dry eye symptoms.

What is already known on this topic?

According to previous studies, the results suggested that pterygium affected with dry eye symptoms and tear film. However it still has no data about relationship severity of pterygium and dry eye symptoms and tear film. Moreover, Thai population particularly the patients with pterygium, remained limitation of data.

What is this study add?

Report affecting size and redness of pterygium to tear film and dry eye symptoms.

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No

Potential conflicts of interest

The authors declare no conflict of interest.

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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)

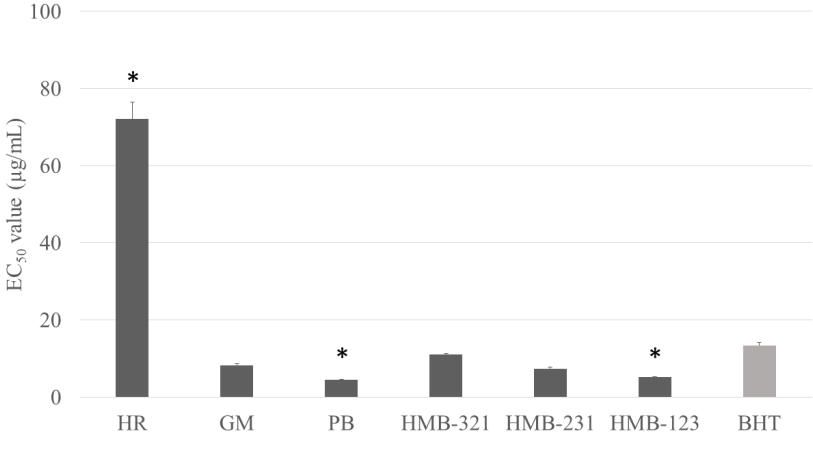

Figure 1 DPPH activity of the ethanolic extract of combined remedies and plant ingredients when compared with BHT

*P-value < 0.05 vs BHT

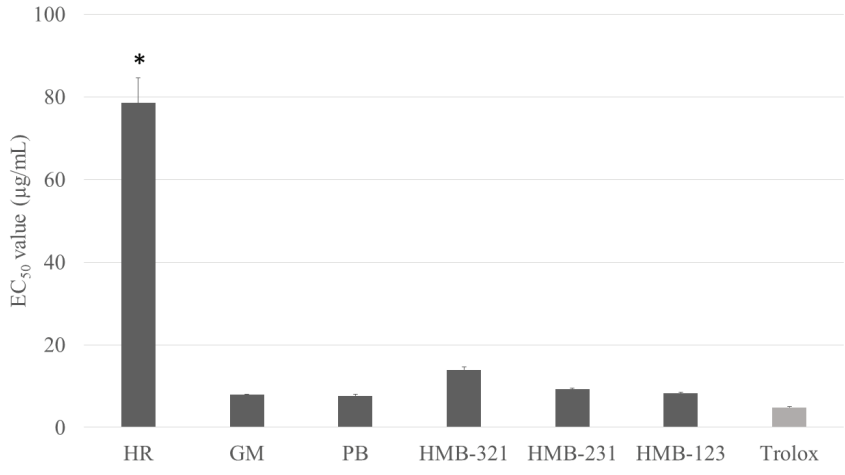

Figure 2 ABTS activity of the ethanolic extract of combined remedies and plant ingredients when compared with Trolox and BHT

* P-value < 0.05 vs Trolox

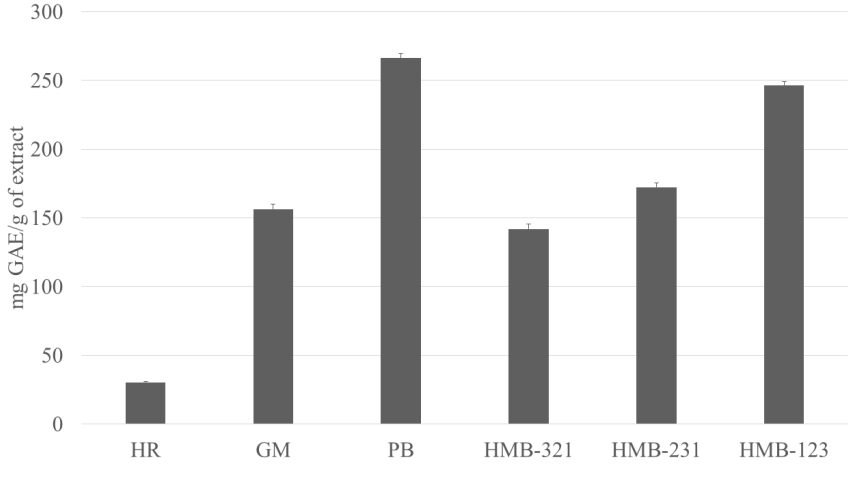

Figure 3 TPC of the ethanolic extract of combined remedies and plant ingredients

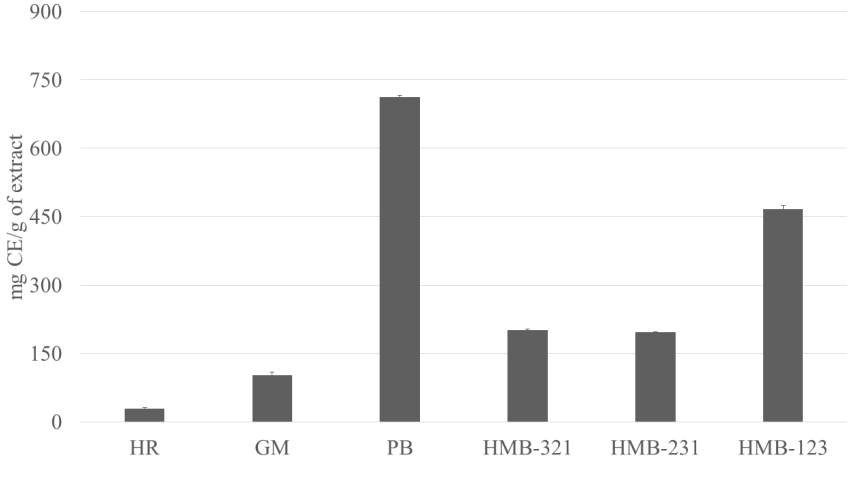

Figure 4 TFC of the ethanolic extract of combined remedies and plant ingredients

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.

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Original Article

Unveiling the Botanical Riches: Enhancing Quality Control and Stability Assessment of Sa-Tri-Lhung-Klod Remedy Extract Through HPLC Profiling and Anti-inflammatory Potency Evaluation

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Abstract

Introduction: Sa-Tri-Lhung-Klod (ST) remedy is a Thai traditional medicine preparation, used for post-partum care including excreting amniotic fluid, reducing inflammation, and improving blood circulation. The standard quality values and the stability of ST have not been reported. Quality control and stability testing are necessary to determine and insure the standard quality of ST remedy.

Objectives: To evaluate the quality control of herbal components according to Thai Herbal Pharmacopoeia (THP). The stability of ST extracts was examined using high performance liquid chromatography (HPLC) and anti-inflammatory activity was measured.

Methods: The quality assessment of the ST and plant components was performed by moisture content, total ash, acid-insoluble ash, extractive value, and tannin content, following the THP method. The stability testing of ST extracts stored under accelerated conditions for six months was performed, and the percentage of the remaining compounds were evaluated using HPLC and tested for anti-inflammatory activity on nitric oxide (NO) inhibitory assay in RAW264.7 cells.

Results: Quality control of ST and plant components met THP requirements, and the tannin content of ST increased upon extraction. ST ethanolic extract (STE) and the remaining compounds exhibited stability after 6 months and anti-inflammatory activity on nitric oxide inhibition assay was retained from day 0 to day 180. However, ST aqueous extract (STW) was unstable from day 30.

Conclusions: The first report of quality control and stability testing of ST is outlined. ST and plant components comply with THP standard requirements. STE can be stored at room temperature for up to two years, whereas STW requires refrigeration in order to extend its shelf life.

Keywords: Sa-Tri-Lhung-Klod remedy, Quality control, Stability testing, HPLC, Anti-inflammatory activity

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Introduction

The Sa-Tri-Lhung-Klod (ST) remedy is a Thai traditional medicine remedy, which is described in the list of The National List of Essential Herbal Medicines (NLEM). It consists of seventeen herbs, including *Angelica sinensis* (Oliv.) Diels, *Artocarpus heterophyllus* Lam, *Caesalpinia sappan* Linn, *Carthamus tinctorius* Linn, *Curcuma comosa* Roxb, *Jasminum sambac* Ait, *Maclura cochinchinensis* (Lour.) Corner, *Mammea siamensis* Kosterm, *Mesua ferrea* Linn, *Mimusops elengi* L, *Nelumbo nucifera* Gaertn, *Piper longum* Linn, *Piper nigrum* Linn, *Piper ribesioides* Wall, *Piper sarmentosum* Roxb, *Plumbago indica* L, and *Salacia chinensis* L. The main medicinal taste of the ST is spicy, complemented with an astringent, sweet, and fragrant taste. Currently, ST remedy is prepared by decoction as an aqueous extract and maceration as an ethanolic extract in order to use for post-partum care and promote maternal health. Previously, clinical trials reported that ST is effective for uterus contraction, amniotic fluid excretion, and maintenance of overall health after childbirth.[1] In addition, ST extract has been reported to demonstrate biological activities, e.g., anti-inflammatory activity on cyclooxygenase-2 enzyme (Cox-2) inhibition and cytotoxic activity on cervical and ovarian cancer cells.[2,3]

However, standardization and quality control of the ST remedy and its constituent plant components are important to assess and determine their quality and stability for use. Quality control and stability testing are performed to guide the development of herbal pharmacopeia, which includes the correct selection of herbs, the development of quality standards, and determination of the shelf-life of herbal extracts, which varies with time under the influence of a variety of environmental factors such as temperature, light, and humidity.[4] Stability studies ensure the quality of active ingredients and predict the shelf life of herbal formulations. Storage conditions can also be recommended.[5] To date, there have been no monograph reports on quality control and stability studies of the ST remedy in Thai Herbal Preparation Pharmacopoeia (THPP).[6] However, the standardized monographs in the THP appear for 11 plant components listed in ST remedy, such as *Angelica sinensis*, *Carthamus tinctorius*, *Curcuma comosa*, *Jasminum sambac*, *Mesua ferrea*, *Mimusops elengi*, *Nelumbo nucifera*, *Piper longum*, *Piper nigrum*, *Piper ribesioides*, and *Piper sarmentosum*.[7] The quality assessment of ST remedy is important to ensure further acceptance in the modern medical system. Therefore, it is necessary to evaluate the quality control and stability analysis of the ST remedy accordingly. This study aims to determine the quality control of the ST remedy and plant components, including moisture content, total ash, acid-insoluble ash, extractive value, and tannin content, following the Thai Herbal Pharmacopoeia (THP) method. Additionally, the stability of the ST extracts under accelerated storage conditions was evaluated by determining the percentage of remaining compounds using HPLC and tested for anti-inflammatory activity on nitric oxide (NO) inhibitory assay in RAW264.7 cells.

Methods

Chemicals and Reagents

Dimethyl sulfoxide (DMSO) and isopropanol were purchased from RCl Labscan, Thailand. Fetal bovine serum (FBS), penicillin-streptomycin (P/S), RPMI medium 1640 (BIOCHROM^{AG}), Trypan blue stain 0.4%, and Trypsin-EDTA were purchased from Gibco, USA. Lipopolysaccharide from *E. coli* 055:B5 (LPS), *N*-(1-Naphthyl) ethylenediamine dihydrochloride, phosphoric acid (H_3PO_4), sulfanilamide, and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich, USA. Phosphate buffered saline (PBS) was purchased from Biochrom, Germany. Hide powder was purchased from Eurofins BLC Leather Technology Centre Limited, UK.

Plant Material

Sa-Tri-Lhung-Klod remedy (ST) consists of 17 different herbs. **Table 1** shows the botanical name, family, Thai name, collected from, voucher specimens, part used, and proportion used. The voucher specimens were obtained at the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Science, Prince of Songkla University, Songkhla, Thailand.

Extraction

Each plant was washed in water, sliced into small pieces, dried in an oven at a temperature of 50 °C, and pulverized into a coarse powder. Then they were weighed and mixed to make the ST according to NLEM. The proportion of maceration and decoction was 1 g of the coarse powder with 20 mL of 95% ethanol and distilled water, respectively. ST was macerated with 95% ethanol for 3 days/3 times and concentrated to dryness by an evaporator (Rotavapor R-205, Germany), obtaining an 8.51% weight per weight (w/w) yield of ethanolic extract of ST (STE). For the aqueous extract, ST was boiled for 30 min in distilled water at the boiling point and concentrated to dryness by lyophilizer (Lyophilization System Inc, USA). An aqueous extract of ST (STW) was obtained at 12.34% w/w yield.

Assays for Quality Control

Loss on Drying[7]

Two grams of sample powder were put into an electronic moisture analyzer (Scaltec instrument, Germany) at 105 °C until a constant weight was obtained. Each sample was examined in triplicate and the mean weight loss on drying was reported.

$$\% \text{ Loss on drying} = \frac{\textit{Start weight (g)-End weight (g)}}{\textit{Start weight (g)}} \times 100$$

Total Ash[7]

A crucible was prepared by putting it in a hot air oven at 105 °C until the weight was stable. Then, two grams of sample powder are put in the crucible and burned using a muffle furnace (Nabertherm, Germany) at 450 °C for 9 hours. Next, the crucible was cooled in a desiccator and then weighed. This process was repeated except it was heated for 5 hours instead of nine until the weight of the crucible and ash was constant.

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

Acid-insoluble Ash[7]

After the weight of total ash was determined to be constant the total ash was boiled in 25 mL of 10% HCl for 5 minutes. After cooling, it was filtered through Whatman paper no.42. The ash in the paper was washed with hot water until the filtrate was neutral, then burned in a muffle furnace at 500 °C for 9 hours. This process is repeated until the crucible's weight is stable.

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

Ethanol-soluble Extractive Value[7]

Sample powder (5 grams) was macerated in 100 mL of 95% ethanol for 24 hours, shaking frequently during the first 6 hours, and then allowed to stand for 18 hours. The plant extract was filtered and 20 mL filtrate was put in a shallow dish. Then, the extract was dried at 105 °C until constant weight.

$$\% \text{ Ethanol-soluble extractive} = \frac{\textit{Weight of the extract (g)}}{\textit{Weight of dried powder (g)}} \times 100$$

Water-soluble Extractive Value[7]

The procedure is similar to the ethanol-soluble extractive value method but uses 0.25% chloroform in water instead of ethanol.

$$\% \text{ Water-soluble extractive} = \frac{\textit{Weight of the extract (g)}}{\textit{Weight of dried powder (g)}} \times 100$$

Determination of Tannins[7]

Tannins testing used the gravimetric technique. Coarse powder (STP), aqueous extract (STW), and 95% ethanolic extract (STE) of ST were the substances employed to determine tannins. Four grams of each sample were accurately weighed, 150 mL of distilled water was added, and the samples were heated in a water bath at 80 °C for 30 minutes. The mixture was transferred quantitatively to a 250 mL volumetric flask and diluted to volume with distilled water.

Determination of total water-extractives: 50 mL of the sample was evaporated. The residue was weighed after 4 hours of drying at 105 °C (*T1*).

Determination of water-extractives not bound with hide powder: hide powder (6 g) was added to 80 mL of sample and shaken for 1 hour. After filtering and evaporating 50 mL of the filtrate was completely dried. The residue was weighed after 4 hours of drying at 105 °C (*T2*).

Determination of water-soluble hind powder: hide powder (6 g) was added to 80 mL of distilled water and shaken for 1 hour. Subsequently, the same process was followed with T_2 (*T0*).

Calculate the percentage of tannins from the expression:

$$\% \text{ Tannins} = \frac{[(T1-T2+T0)\ x\ 5]}{W} \times 100$$

Where, W is the weight in grams of the substance taken, calculated on a dried basis.

Stability Testing [8]

The purpose of drug stability testing is to control the quality and storage methods to maintain therapeutic efficacy. STE and STW were exposed for six months, under 40 ± 2 ºC with 75 ± 5% relative humidity (RH) as the accelerated condition. The samples were sampled on days 0, 15, 30, 60, 90, 120, 150, and 180. All samples were determined for the percentage of remaining compounds using HPLC and tested for anti-inflammatory activity on NO inhibitory effect in RAW264.7 cells.

Determination of the Remaining Compounds of ST Remedy Extracts Using HPLC

Brazilin, (3*S*)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6*E*)-6-hepten-3-ol, Piperine, Pipernonaline, (3*S*)-1,7-diphenyl-(6*E*)-6-hepten-3-ol, (3*R*)-1,7-diphenyl-(4*E*,6*E*)-4,6-heptadiene-3-ol, and Artocarpin are the main chemical compounds determined in ST remedy. These compounds showed peaks which can be integrated at RT 5.433, RT 23.519, RT 25.136, RT 31.585, RT 32.160, RT 33.063, and RT 35.687, respectively.[9] The stability analysis of these compounds was conducted using an Agilent 1200 HPLC system (Agilent Technologies, USA), with a diode array detector and automatic injector. A Phenomenex Luna 5u C18(2) 100A column (250 × 4.6 mm) (Phenomenex, USA) was used for chromatographic separation. Agilent ChemStation software was used to analyze the data. The mobile phase was composed of acetonitrile: water (v/v) with the following gradient elution: 0 min-20 : 80 v/v, 30 min-5 : 95 v/v, and 40 min 10 seconds-20 : 80 v/v. The flow rate was set at 1.0 mL/min with an injection volume of 10 µg/mL and the total run time of analysis was 45 min. A diode array detector was set at 210 nm.

STE and STW were prepared by dissolving them with methanol and deionized water at a concentration of 5 mg/mL, respectively. The solution was filtered through 0.45 µM nylon membrane filters. 10 µL of sample solutions were injected into the HPLC column and separated under the above chromatographic condition. The mean peak areas (mAU) of each chemical compound were calculated as the percentage of remaining compounds, with Day 0 set to 100%.

Anti-inflammatory Activity on Nitric Oxide (NO) Inhibitory Assay[10]

STE was dissolved in sterile DMSO to a concentration of 50 mg/mL. STW was dissolved to a concentration of 10 mg/mL in sterile deionized water and filtered through a Millipore 0.22 µM filter. STE and STW were diluted in the cultured medium to obtain a final concentration of 1-100 µg/mL.

RAW 264.7 cells (ATCC® TIB-71™) were obtained from the Center of Excellence in Applied Thai Traditional Medicine Research (CEATMR), Thammasat University, Pathum Thani, Thailand. The cells were grown in RPMI 1640 medium containing 10% FBS, and 1% P/S in an incubator with 5% CO_2 at 37 °C. Cells were seeded in 96-well plates at $1x10^5$ cells/well and incubated for 24 hours. Then, cells were stimulated with lipopolysaccharide (LPS) (10 ng/mL)and treated with extracts at various concentrations for 24 hours. Later, the supernatant was collected to detect NO production using the Griess reagent [1% sulfanilamide/0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride, each in 2.5% H_3PO_4]. The optical density (OD) was measured using a microplate reader (Bio Tex, USA) at 570 nm.

MTT assay was used to measure cell viability. MTT solution (10 µL, 5 mg/mL in PBS) was added to the wells without LPS and incubated for 2 hours. Subsequently, isopropanol containing 0.04 M HCl was added to dissolve the formazan production. The OD was measured at 570 nm. No cytotoxicity was observed when the survival rate was greater than 70% compared with the control.

Statistical Analysis

The data were performed in triplicate and the results were presented as mean ± standard Deviation (SD). Statistical analysis was determined by one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests using the SPSS

program (IBM SPSS Statistics 26). Statistical significance was indicated when the *p*-value < 0.05.

Results

Quality Control

Physical standardization of ST remedy and plant components were investigated as follows: loss on drying (moisture content), extractive value, total ash, and acid-insoluble ash for inorganic contamination. The quality of the plants complied with standard values of Thai Herbal Pharmacopoeia loss on drying < 10%, total ash < 10%, and acid-insoluble ash < 2%. The results of the quality control of the ST and its ingredients are shown in **Table 2**. The percentage of loss on drying of ST remedy was 9.17 ± 0.04% w/w. *Angelica sinensis* showed the highest percentage of loss on drying with 9.84 ± 0.14% w/w, while *Piper longum* had the lowest percentage with 6.45 ± 0.68% w/w.

The percentage of total ash and acid-insoluble ash of ST remedy were 6.37 ± 0.18 and 1.03 ± 0.09% w/w, respectively. Among all the samples tested, *Mammea siamensis* showed the highest percentage of total ash (8.16 ± 0.14% w/w) and *Plumbago indica* exhibited the highest percentage of acid-insoluble ash (1.75 ± 0.03% w/w). On the other hand, *Caesalpinia sappan* demonstrated the lowest in total and acid-insoluble ash (1.03 ± 0.06 and 0.07 ± 0.01% w/w).

The percentage of ethanol- and water-soluble extractive values of ST remedy were 1.69 ± 0.09 and 2.99 ± 0.40%, respectively. *Mesua ferrea* had the highest ethanol-soluble extractive value of 3.35 ± 0.28%, while *Angelica sinensis* had the highest percentage of water-soluble extractive value of 9.87 ± 0.01%. However, *Artocarpus heterophyllus* showed the lowest percentage in both ethanol- and water-soluble extractive values (0.21 ± 0.04 and 0.45 ± 0.03%, respectively).

The results revealed that the quality control of ST and plant components met THP requirements. The result of tannin content in the ST remedy is presented in **Figure 1**. A coarse powder of ST (STP) had the lowest tannin content of 2.48 ± 2.14%, whereas the tannin content obtained in ST extracts (STW and STE) gave higher values than that of STP. The highest values of tannins are found in STW. It provided a tannin content of 5.38 ± 2.58%. STE had a tannin content of 4.14 ± 0.71%. These results showed that the tannin content was increased upon extraction, especially the aqueous extraction.

Stability Testing of STE and STW Storage under Accelerated Conditions

The stability testing results of STE and STW storage under accelerated conditions at day 0, 15, 30, 60, 90, 120, 150, and 180 on the percentage of remaining compounds and inhibitory effects of LPS-induced NO production in RAW-264.7 cells are shown in **Table 3**.

Percentage of Remaining Compounds of STE and STW

STE and STW used the same chromatographic conditions to determine the chemical characteristics of their crude extracts as HPLC fingerprints (see **Figure 2**). STE demonstrated 7 main chemical compounds. Brazilin was obtained from *Caesalpinia sappan*. Piperine was obtained from *Piper nigrum* and *Piper longum*. In addition, *Piper longum* also provided Pipernonaline. Subsequently, (3*S*)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6*E*)-6-hepten-3-ol, (3*S*)-1,7-diphenyl-(6*E*)-6-hepten-3-ol, and (3*R*)-1,7-diphenyl-(4*E*,6*E*)-4,6-heptadiene-3-ol were derived from *Curcuma comosa*. Finally, Artocarpin was obtained from *Artocarpus heterophyllus*. For STW, the chemical compound was determined to be Brazilin.

The stability under accelerated conditions of ST remedy extracts was examined in the percentage of the various remaining compounds using HPLC. The results are shown in **Figure 2** and **Table 3**. The results showed that the seven main compounds of STE were stable from day 0 to day 180. Nevertheless, Brazilin, a compound of STW was not stable and was not detectable from day 30. Therefore, HPLC fingerprints of ST extracts can be used to verify drug stability and quality control.

Anti-inflammatory Activity on NO

Inhibition

The stability of ST extracts was tested with the anti-inflammatory activity against LPS-induced NO production and survival of RAW264.7 cells. As the results are shown in **Figure 3** and **Table 3**. Day 0 of STE showed anti-inflammatory values with IC_{50} of 20.59 ± 0.03 μg/mL and also showed similar values all under accelerated conditions from day 0 to day 180 (IC_{50} range of 20.59 to 25.61 μg/mL). However, from day 15 to day 180 STE showed a statistically significant difference compared to day 0 ($p < 0.05$). STW, Day 0 displayed an IC_{50} of 52.93 ± 0.90 μg/mL, and Day 15 to Day 180 showed an IC_{50} of more than 100 μg/mL. In addition, the cytotoxicity of all extracts was examined using the MTT assay to ensure that the inhibitory effect of NO production was not the result of cell death. The results showed that none of the extracts were toxic to RAW264.7 cells.

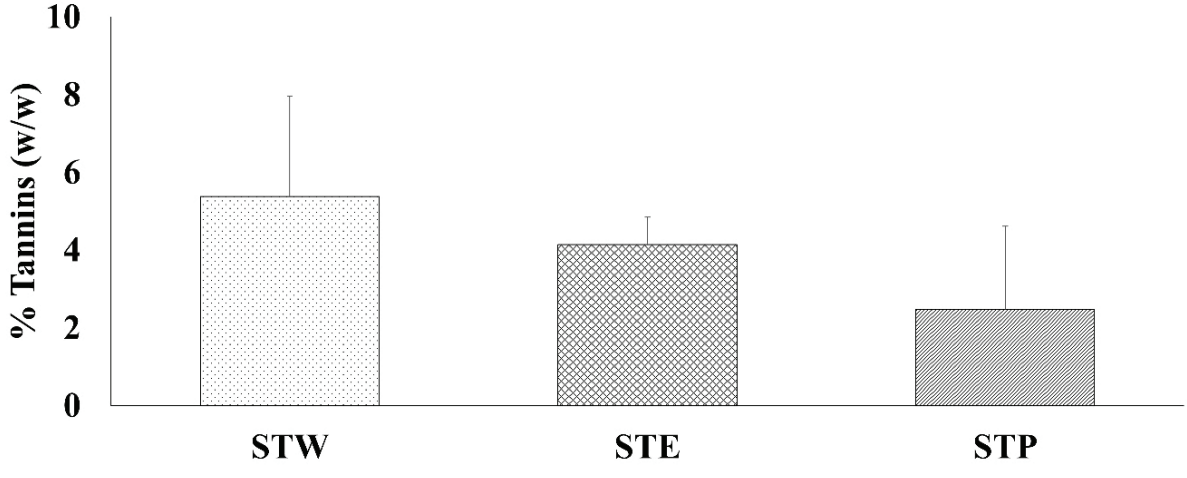
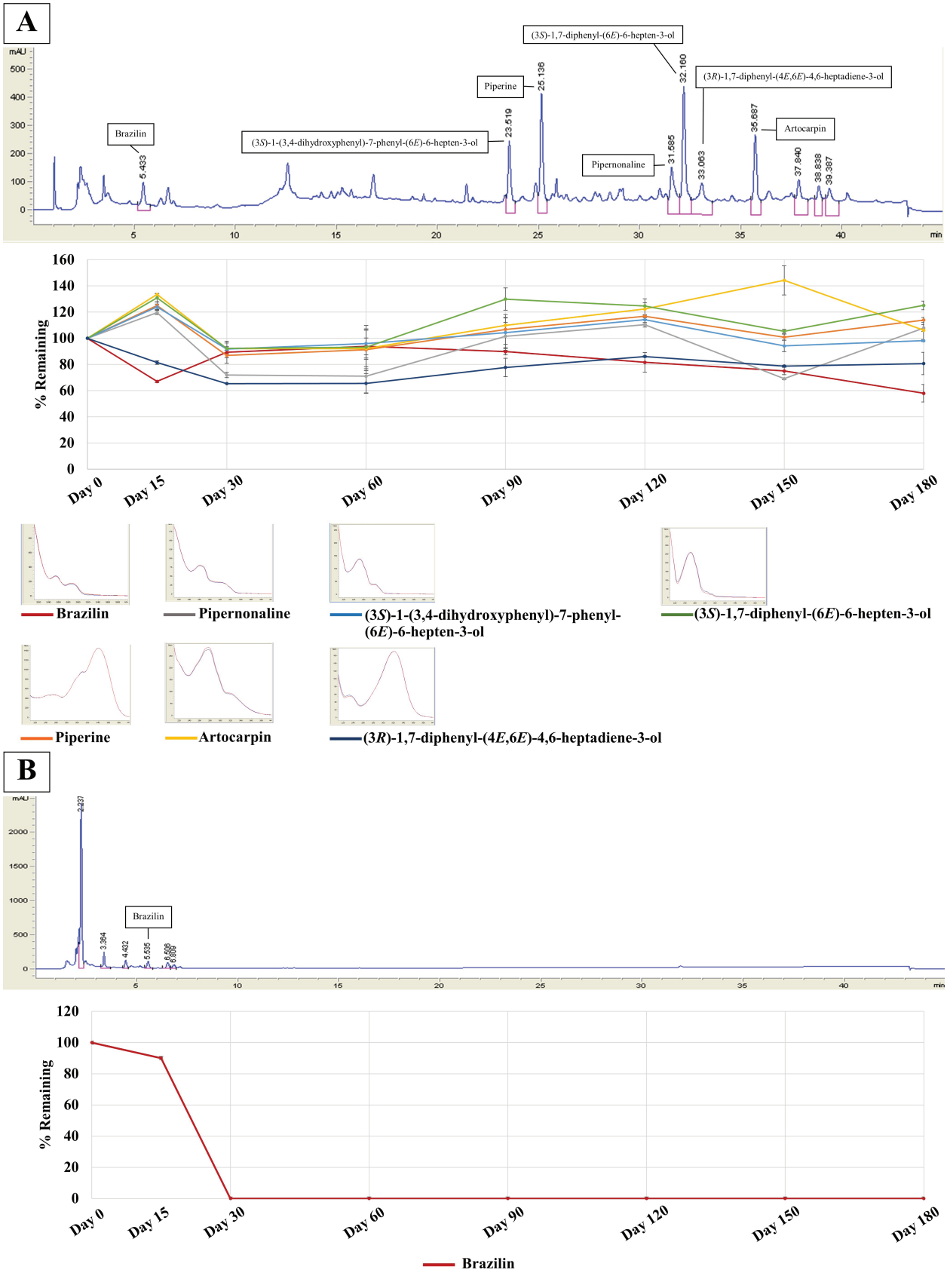


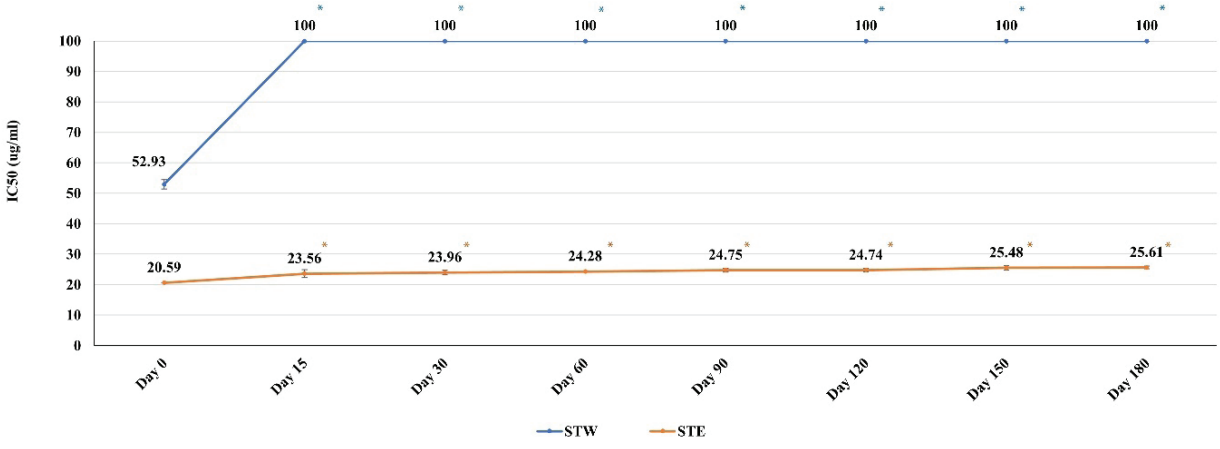
Figure 1 The percentage of tannins obtained from the Sa-Tri-Lhung-Klod remedy. Each value represents the mean ± SD., n = 3 for each group. STW: aqueous extract of Sa-Tri-Lhung-Klod remedy, STE: 95% ethanolic extract of Sa-Tri-Lhung-Klod remedy, and STP: coarse powder of Sa-Tri-Lhung-Klod remedy.

Figure 2 HPLC chromatogram and the percentage of the various remaining compounds of (A) STE and (B)



STW (n = 3).

Figure 3 The stability of anti-inflammatory activity (IC_{50}) of the STE and STW under accelerated condition



at 40 ± 2 ºC, 75 ± 5% RH for 6 months at different times (n = 3). * $p < 0.05$ compared with Day 0 in the same sample. The positive control was indomethacin (IC_{50} = 28.37 ± 0.28 µg/mL).

Table 1 Component plants and their parts in Sa-Tri-Lhung-Klod remedy

Botanical name	Family	Thai name	Collected from	Voucher specimen number	Part used	Proportion (% w/w)
Angelica sinensis (Oliv.) Diels	UMBELLIFERAE	Kot-Chiang	China	SKP199010901	Root	7.4
Artocarpus heterophyllus Lam.	MORACEAE	Kha-Nun	Nakhon Ratchasima	SKP117010801	Stem	7.4
Caesalpinia sappan Linn.	LEGUMINOSAE	Fang-Sen	Bangkok	SKP098031901	Stem	7.4
Carthamus tinctorius Linn.	COMPOSITAE	Kham-Foi	Chiang Mai	SKP051032001	Flower	3.7
Curcuma comosa Roxb.	ZINGIBERACEAE	Wan-Chak-Mod-Look	Phetchabun	SKP201030301	Rhizome	7.4
Jasminum sambac Ait.	OLEACEAE	Ma-Li	Nakhon Pathom	SKP129101901	Flower	3.7
Maclura cochinchinensis (Lour.) Corner	MORACEAE	Kae-Lae	Prachuap Khiri Khan	SKP117130301	Stem	7.4
Mammea siamensis Kosterm.	GUTTIFERAE	Sa-Ra-Pee	Ratchaburi	SKP083131901	Flower	3.7
Mesua ferrea Linn.	GUTTIFERAE	Boon-Nak	Ratchaburi	SKP083130601	Flower	3.7
Mimusops elengi L.	SAPOTACEAE	Phi-Kul	Ratchaburi	SKP171130501	Flower	3.7
Nelumbo nucifera Gaertn.	NELUMBONACEAE	Bua-Luang	Ratchaburi	SKP125141401	Pollen	3.7
Piper longum Linn.	PIPERACEAE	Di-Plee	Chanthaburi	SKP146160301	Fruit	7.4
Piper nigrum Linn.	PIPERACEAE	Prik-Thai	Chanthaburi	SKP146161401	Fruit	7.4
Piper ribesioides Wall.	PIPERACEAE	Sa-Kan	Sakon Nakhon	SKP146161801	Stem	7.4
Piper sarmentosum Roxb.	PIPERACEAE	Cha-Phlu	Ratchaburi	SKP146161901	Root	3.7
Plumbago indica L.	PLUMBAGINACEAE	Chet-Ta-Mun-Phloeng-Daeng	Bangkok	SKP148160901	Root	7.4
Salacia chinensis L.	CELASTRACEAE	Kam-Phaeng-Chet-Chan	Ratchaburi	SKP044190301	Stem	7.4

Table 2 Quality control of Sa-Tri-Lhung-Klod remedy and its plant ingredients (n = 3)

Botanical name	% Loss on drying (w/w)		% Ash content (w/w)				% Extractive value (w/w)	
			Total ash		Acid-insoluble ash		95% EtOH soluble	Water soluble
	Mean ± SD	Limit	Mean ± SD	Limit	Mean ± SD	Limit	Mean ± SD	Mean ± SD
Angelica sinensis	9.84 ± 0.14	≤ 10	7.07 ± 0.28	≤ 7 [a]	1.71 ± 0.04	≤ 2 [a]	2.55 ± 0.03	9.87 ± 0.01
Artocarpus heterophyllus	8.51 ± 0.86	≤ 10	1.64 ± 0.09	≤ 10	0.63 ± 0.04	≤ 2	0.21 ± 0.04	0.45 ± 0.03
Caesalpinia sappan	8.34 ± 0.15	≤ 10	1.03 ± 0.10	≤ 10	0.07 ± 0.01	≤ 2	0.26 ± 0.02	0.47 ± 0.09
Carthamus tinctorius	9.68 ± 0.24	≤ 14 [a]	7.68 ± 0.22	≤ 15 [a]	1.69 ± 0.02	≤ 5 [a]	2.76 ± 0.08	5.90 ± 0.10
Curcuma comosa	9.66 ± 0.21	≤ 11 [a]	5.02 ± 0.47	≤ 10 [a]	1.50 ± 0.12	≤ 3 [a]	1.33 ± 0.12	2.63 ± 0.10
Jasminum sambac	9.74 ± 0.20	≤ 11 [a]	8.00 ± 0.46	≤ 8 [a]	0.36 ± 0.06	≤ 1.5 [a]	1.42 ± 0.15	5.16 ± 0.26
Maclura cochinchinensis	9.55 ± 0.31	≤ 10	4.79 ± 0.10	≤ 10	0.21 ± 0.05	≤ 2	0.28 ± 0.03	0.63 ± 0.05
Mammea siamensis	8.41 ± 0.14	≤ 10	8.16 ± 0.24	≤ 10	0.56 ± 0.03	≤ 2	2.25 ± 0.07	3.73 ± 0.02
Mesua ferrea	9.41 ± 0.15	≤ 11 [a]	3.41 ± 0.36	≤ 5 [a]	0.59 ± 0.17	≤ 1.5 [a]	3.35 ± 0.28	3.03 ± 0.32
Mimusops elengi	9.48 ± 0.08	≤ 16 [a]	5.43 ± 0.24	≤ 7 [a]	1.15 ± 0.30	≤ 3 [a]	0.28 ± 0.02	1.88 ± 0.14
Nelumbo nucifera	8.04 ± 0.04	≤ 12 [a]	4.36 ± 0.05	≤ 6 [a]	0.30 ± 0.02	≤ 1 [a]	1.67 ± 0.06	2.92 ± 0.03
Piper longum	6.45 ± 0.68	≤ 10	5.48 ± 0.54	≤ 7.5 [a]	0.28 ± 0.04	≤ 0.4 [a]	1.76 ± 0.09	2.60 ± 0.11
Piper nigrum	8.20 ± 0.02	≤ 10	4.44 ± 0.19	≤ 4 [a]	0.39 ± 0.04	≤ 0.5 [a]	1.03 ± 0.01	0.52 ± 0.07
Piper ribesioides	7.85 ± 0.46	≤ 8 [a]	3.73 ± 0.13	≤ 9 [a]	0.27 ± 0.04	≤ 2	0.76 ± 0.04	1.54 ± 0.04
Piper sarmentosum	7.91 ± 1.09	≤ 10 [a]	6.82 ± 0.27	≤ 14 [a]	0.23 ± 0.01	≤ 4 [a]	0.46 ± 0.06	2.60 ± 0.14
Plumbago indica	9.39 ± 0.36	≤ 10	6.75 ± 0.28	≤ 10	1.75 ± 0.04	≤ 2	0.34 ± 0.06	5.44 ± 0.16
Salacia chinensis	9.25 ± 0.25	≤ 10	2.24 ± 0.03	≤ 10	0.15 ± 0.03	≤ 2	1.09 ± 0.08	0.89 ± 0.03
Sa-Tri-Lhung-Klod remedy	9.17 ± 0.04	-	6.37 ± 0.30	-	1.03 ± 0.15	-	1.69 ± 0.09	2.99 ± 0.40

[a] The standard values of THP 2021, - Not reported

Table 3 Stability of STE and STW on the percentage of the remaining compounds and inhibitory effects of LPS-induced NO production during 180-day storage under accelerated conditions. Each value represents the mean ± SD (n = 3).

Sample	Day 0	Day 15	Day 30	Day 60	Day 90	Day 120	Day 150	Day 180
% Remaining compounds (%)								
STE:								
Brazilin	100.00 ± 0.00	67.01 ± 0.87*	89.31 ± 8.46*	93.82 ± 6.44	89.81 ± 2.42*	81.65 ± 7.50*	75.03 ± 2.73*	57.97 ± 6.70*
(3*S*)-1-(3,4-dihydroxyphenyl) -7-phenyl-(6*E*)-6-hepten-3-ol	100.00 ± 0.00	123.95 ± 2.14	91.73 ± 0.96	95.98 ± 10.97	104.35 ± 11.52	144.21 ± 3.45	94.20 ± 4.42	98.19 ± 0.72
Piperine	100.00 ± 0.00	125.07 ± 2.86	86.86 ± 1.78	91.27 ± 15.93	106.79 ± 11.44	116.89 ± 1.12	100.88 ± 2.38	113.95 ± 1.98
Pipernonaline	100.00 ± 0.00	119.70 ± 1.60	71.89 ± 2.01*	71.09 ± 12.79*	101.57 ± 10.71	110.36 ± 1.76	69.10 ± 0.66*	107.84 ± 2.65
(3*S*)-1,7-diphenyl-(6E)-6-hepten-3-ol	100.00 ± 0.00	131.01 ± 3.28	92.28 ± 1.41	93.20 ± 16.52	129.90 ± 8.53	124.72 ± 2.48	105.43 ± 1.48	125.10 ± 3.37
(3*R*)-1,7-diphenyl-(4*E*,6*E*)-4,6-heptadiene-3-ol	100.00 ± 0.00	81.45 ± 1.43*	65.37 ± 0.19*	65.60 ± 7.56*	77.73 ± 7.04*	86.09 ± 0.57*	78.81 ± 0.53*	80.71 ± 8.55*
Artocarpin	100.00 ± 0.00	133.48 ± 0.63	92.06 ± 4.40	92.19 ± 13.88	109.92 ± 5.68	122.36 ± 7.82	144.27 ± 11.13	106.16 ± 7.15
STW:								
Brazilin	100.00 ± 0.00	90.08 ± 1.46*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*
IC_{50} of NO production (µg/mL)								
STE	20.59 ± 0.06	23.56 ± 1.30*	23.96 ± 0.79*	24.28 ± 0.20*	24.75 ± 0.64*	24.74 ± 0.60*	25.48 ± 0.74*	25.61 ± 0.45*
STW	52.93 ± 1.55	> 100 *	> 100 *	> 100 *	> 100 *	> 100 *	> 100 *	> 100 *
Indomethacin	28.37 ± 0.28	-	-	-	-	-	-	-

* $p < 0.05$ compared with Day 0 in the same sample. – Not test

Discussion

The use of herbal medicines in Thai traditional medicine plays an important role in maternal health care during the post-partum period. It is interesting that the ST remedy is currently included in the NLEM and is used in obstetrics and gynecology to reduce inflammation, excrete amniotic fluid, and nourish the blood. The Thai Herbal Preparation Pharmacopoeia (THPP) provides five monographs on obstetrics and gynecology medicine remedies, including Fai-Ha-Kong, Fai-Pra-Lai-Kan, Lueat-Ngam, Pluk-Fai-That, and Pra-Sa-Phlai remedy.[6] However, the THPP lacks a monograph for the quality control and stability study of the ST remedy. This study provides new scientific evidence on the quality control and stability of ST remedy, following THP guidelines.The findings of this research can support the establishment of a quality specification for the ST remedy.

Quality control of ST remedy and plant components was investigated according to THP as follows: loss on drying, total ash, acid-insoluble ash, and extractive values. The standard requirements on quality control are only available for some plants listed in ST remedy in THP such as *Angelica sinensis*, *Carthamus tinctorius*, *Curcuma comosa*, *Jasminum sambac*, *Mesua ferrea*, *Mimusops elengi*, *Nelumbo nucifera*, *Piper longum*, *Piper nigrum*, *Piper ribesioides*, and *Piper sarmentosum*.[7] Loss on drying is the weight loss due to water and any other volatile matter that can be driven off. The standard quality value of a loss on drying in the THP exhibited *Carthamus tinctorius*, *Curcuma comosa*, *Jasminum sambac*, *Mesua ferrea*, *Mimusops elengi*, *Nelumbo nucifera*, *Piper ribesioides*, and *Piper sarmentosum* was not more than 14.0, 11.0, 11.0, 11.0, 16.0, 12.0, 8.0, and 10.0% w/w, respectively.[7] Total ash is what remains after the sample has completely burned, which is obtained from the plant tissue itself and extraneous matter (soil and sand) adhering to the plant surface. The total ash standards in THP of *Angelica sinensis*, *Carthamus tinctorius*, *Curcuma comosa*, *Jasminum sambac*, *Mesua ferrea*, *Mimusops elengi*, *Nelumbo nucifera*, *Piper longum*, *Piper nigrum*, *Piper ribesioides*, and *Piper sarmentosum* are not exceed 7.0, 15.0, 10.0, 8.0, 5.0, 7.0, 6.0, 7.5, 4.0, 9.0, and 14.0% w/w respectively.[7] Acid-insoluble ash is the portion of ash that is insoluble in acid. It is a part of the total ash produced when a test material is burned. An ash constitutes inorganic matter, but acid-insoluble ash consists mainly of silica.[12] The standard values of acid-insoluble ash of some herbs in THP such as *Angelica sinensis*, *Carthamus tinctorius*, *Curcuma comosa*, *Jasminum sambac*, *Mesua ferrea*, *Mimusops elengi*, *Nelumbo nucifera*, *Piper longum*, *Piper nigrum*, and *Piper sarmentosum* were set not more than 2.0, 5.0, 3.0, 1.5, 1.5, 3.0, 1.0, 0.4, 0.5, and 4.0% w/w respectively.[7] However, there is no specific requirement for ST remedy in THPP, the general requirements of loss on drying, total ash, and acid-insoluble ash for the herbal plants should not exceed 10%, 10%, and 2%, respectively.[11] According to the results, the ST remedy and all plant components have met the standard criteria of loss on drying, total ash, and acid-insoluble ash.

Extractive values of a medicinal plant are used to determine amounts of active constituents present in a given amount of plant material when extracted. This is typically done using a solvent to extract the active compounds from the plant material. The extractive values can be an important factor in determining the potency and effectiveness of herbal remedies and medicines. Tannins are commonly found in plants and they are important bioactive compounds reported to have several biological effects, including anti-inflammatory activity. Tannins are used as anti-inflammatory agents and have wound-healing potential.[13,14] In addition, tannins contribute directly to major organoleptic properties, in particular to taste attributes such as astringency and bitter taste.[15] ST contains astringent taste plants derived from the heartwood, namely *Artocarpus heterophyllus*, *Caesalpinia sappan*, *Maclura cochinchinensis*, and *Salacia chinensis*. The present results suggest that both STE and STW resulted in higher tannin concentrations than the coarse powder. As a result, the extraction process can enhance the tannin concentration. Unfortunately, ST remedy and plant components have not reported tannin content in THPP and THP.

Stability testing was performed to control the quality and storage methods to maintain its effectiveness following the ICH guidelines.[8] Previous research has reported on the chemical compounds of STE and STW. In addition,

STE and STW exhibited anti-inflammatory effects by inhibiting the production of nitric oxide (NO) and prostaglandin E_2 (PGE_2).[9] Additionally, among the compounds tested in previous research, Brazilin, Piperine, (3*S*)-1-(3, 4-dihydroxyphenyl)-7-phenyl-(6*E*)-6-hepten-3-ol, (3*S*)-1,7-diphenyl-(6*E*)-6-hepten-3-ol, and (3*R*)-1, 7-diphenyl-(4*E*,6*E*)-4,6-heptadiene-3-ol were effective against lipopolysaccharide (LPS)-induced NO production in RAW264.7 cells.[16-18] Stability testing from this study indicated that the percentage of the remaining compounds of STE storage under accelerated conditions remained constant from day 0 to day 180 and did not lose its inhibitory effect on NO production. In contrast, the percentage of remaining brazilin of STW was unstable and undetectable from day 30 and anti-inflammatory effects were not measurable. After STW storage under accelerated conditions at 40 ± 2 ºC, 75 ± 5% RH from day 15-180, its physical characteristics were moist and sticky, which differed from the fresh extract (day 0) as a completely dry powder. Humidity and temperature may cause STW to deteriorate and cause the brazilin content to degrade. Therefore, it can be concluded that STE can be stored at room temperature for up to two years, whereas STW requires refrigeration in order to extend its shelf life.

This study highlights the importance of quality control and stability testing for ST remedy. The findings can serve as a benchmark for future scientific research and manufacturing, ensuring consistent quality and shelf-life of ST remedy. It is essential to adhere to these standards to maintain the efficacy of the remedy for patient use.

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Compliance with Ethics Requirements

The experiments were approved by the Institute Biosafety Committee of Thammasat University (Number 065/2561) and performed under biosafety level 2.

Conflict of Interest

The authors declare that they have no conflict of interests.

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Author Contributions

J. Inprasit undertook all the laboratory experiments and wrote the draft manuscript with support from **S. Ruangnoo** and **A. Itharat**, who were J. Inprasit's advisors. **A. Itharat** is the project manager and finance provider for this project. **S. Makchuchit** examined and analyzed the results of anti-inflammatory activity. **W. Pipatrattanaseree** verified the analytical chromatographic conditions and HPLC methodology. **N. M. Davies** provided technical scientific input, helped write the initial manuscript, and revised the manuscript.

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Case Report

Challenging Cytological Findings of A Rare Warthin-like Papillary Thyroid Carcinoma: A Case Report

Jirayu Visanuruk

Abstract

Warthin-like papillary thyroid carcinoma (WLPTC) stands out as a particularly uncommon subtype within the spectrum of papillary thyroid carcinoma, known for its distinctive histological and cytological features. This presentation illuminates a case of WLPTC, highlighting an unusual microfollicular pattern and a restrained lymphoplasmacytic background, which makes it challenging to make a conclusive diagnosis.

Keywords: Papillary thyroid carcinoma, Warthin-like papillary thyroid carcinoma, Cytological findings, Case report

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Introduction

Papillary thyroid carcinoma (PTC) stands is out as the most prevalent malignant tumor affecting the thyroid gland, with various subtypes identified based on distinctive histological features. These subtypes encompass classic, encapsulated classic, infiltrative follicular, diffuse sclerosing, solid/trabecular, tall cell, columnar cell, hobnail, clear cell, spindle cell, Warthin-like, oncocytic, and PTC with fibromatosis/fasciitis-like/desmoid-type stroma.[1]

Among these, the Warthin-like subtype (WLPTC) emerges as a rare entity with unique histological characteristics.[2-3] Cytologically, WLPTC typically exhibits numerous papillae and dispersed oncocytic cells, accompanied by a lymphoplasmacytic background and characteristic PTC nuclear features.[4] This report presents a case featuring uncommon oncocytic cells displaying a microfollicular pattern in a patient who was later histologically diagnosed with WLPTC.

Case Presentation

A 35-year-old female presented with a painless right neck nodule that persists for a duration of one year. She exhibited an absence does not have of familial predisposition to malignancy and reported an unremarkable medical history. Revealed the physical examination unveiled a palpable right thyroid nodule measuring 2 × 2 cm, devoid without revealed of tenderness. Ultrasonographic assessments disclosed an enlarged dimension of the right thyroid gland, accompanied by a poorly-defined hypoechoic mass exhibiting mild increased vascularity, measuring 2.9 × 2.2 × 2.1 cm. The results of thyroid function tests demonstrated values within the established normal range. Subsequently, a clinician conducted a fine-needle aspiration (FNA) for the purpose of cytological evaluation.

The FNA cytological findings unveiled showed an increased heightened cellularity within smears, delineated by a predominant microfollicle and clusters with follicular cell patterns, accompanied by rare small lymphocyte impingement in the clean background. The follicular cells exhibited focal oncocytic metaplasia, characterized by abundant amphophilic cytoplasm, mild anisonucleosis, nuclear crowding, mild irregular nuclear contour, finely granular chromatin, and a small distinct nucleolus. The presence of nuclear grooves was an infrequent observation, while very rare intranuclear cytoplasmic pseudoinclusions (INCIs) were rarely detetected. The diagnostic categorization assigned these observations to 2nd edition Bethesda category IV: Suspicious for follicular neoplasm, with the corresponding commentary recognized that although architectural features suggested a follicular neoplasm, specific oncocytic-like and nuclear attributes introduced the prospect of an invasive follicular subtype of PTC, oncocytic subtype of PTC, or NIFTP. Definitive the oncolytic differentiation among these entities proved to be formidable task based on the cytological a formidable specimens. Consequently, the patient underwent a right thyroid gland lobectomy.

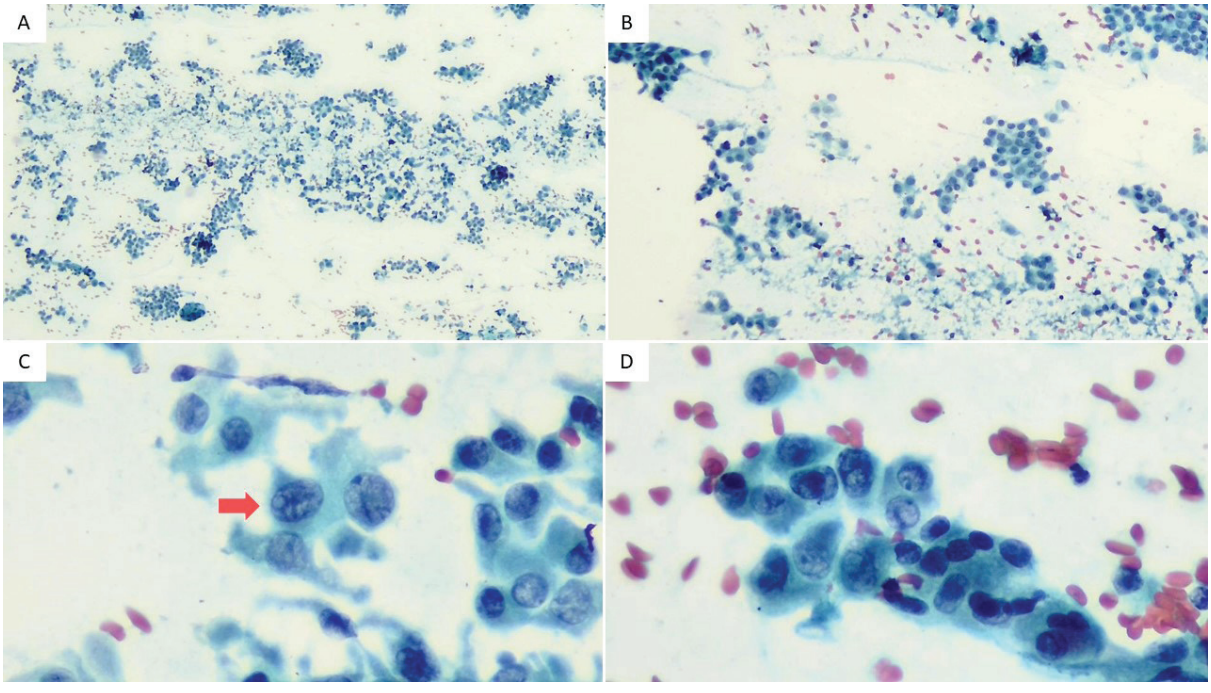

Figure 1 Cytological findings (A) Cytologically, low-power field shows hypercellularity with a predominantly microfollicles and cluster of follicular cells with clean background, (B) The follicular cell arranged in the clusters and microfollicles, (C-D) Some follicular cells exhibit anisonucleosis, nuclear atypia, and rare intranuclear cytoplasmic pseudoinclusions. (red arrow)

Macroscopically, the dimensions of the thyroid gland were quantified at 5.5 × 4.0 × 3.0 cm, with a corresponding weight of 13.0 grams. A serial sectioning process revealed a well-circumscribed, unencapsulated, firm, white-tan mass measuring 3.5 × 2.5 × 2.3 cm, predominantly occupied in the upper pole. Furthermore, the examination encompassed scrutiny of two isthmic lymph nodes.

The histopathological analysis unveiled a well-defined mass characterized by numerous papillae configurations lined by oncocytic cells, which presents a distinctive cell border, abundant eosinophilic granular cytoplasm, and nuclear clearing. Moreover, discernible features such as nuclear grooves and INCIs were readily observed. The papillae core exhibited conspicuous lymphoplasmacytic cells, impartinga visual resemblance to a Warthin tumor commonly found in the salivary gland. Significantly, indication of angioinvasion and lymphatic invasion were noted. Additionally, observed the presence of metastatic carcinoma was confirmed in two isthmic lymph nodes. The residual thyroidal parenchyma manifested diverse-sized follicles, accompanied by diffused lymphoplasmacytic cell aggregation and the formation of germinal centers.

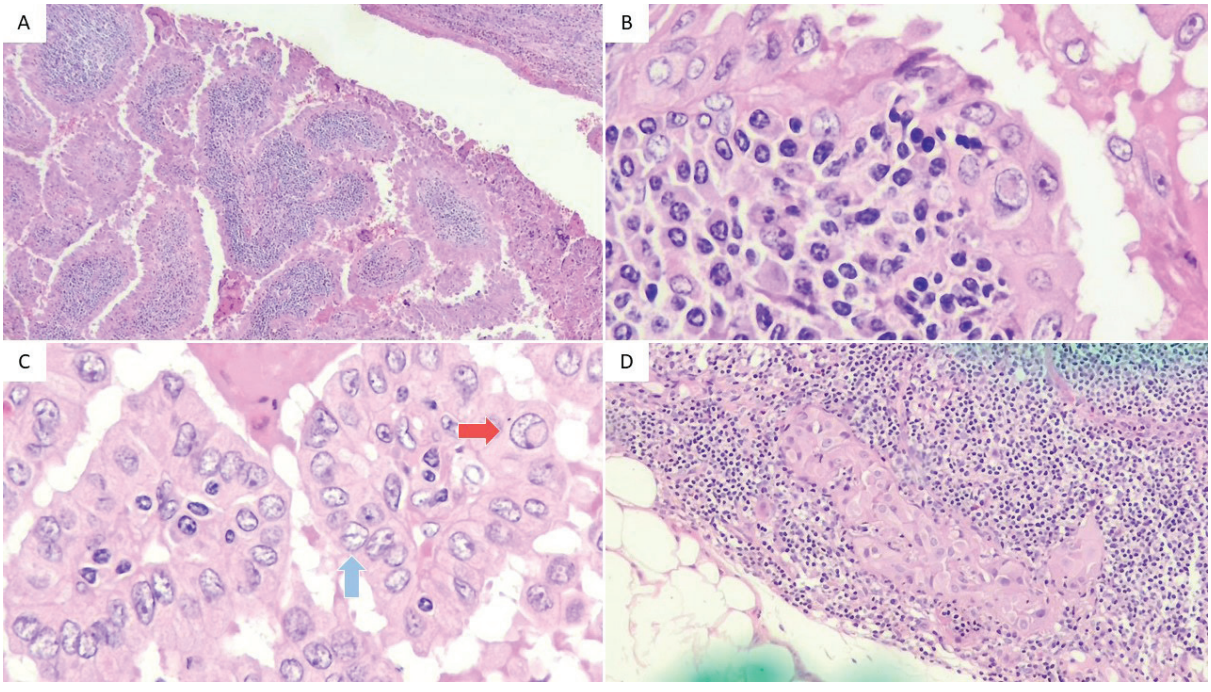

Figure 2 Histological findings (A) Microscopically, the tumor has a broad and hierarchy papillary configuration with dense lymphoid papillary cores, (B) The papillae are lined by dysplastic columnar cell showing oncocytic appearance and the lymphoid stroma comprises lymphoplasmacytic cells, (C) The dysplastic oncocytic cell shows some nuclear crowding, elongation, pleomorphism, nuclear groove (blue arrow), and INCIs (red arrow), (D) The isthmic lymph node reveals nests of metastatic cells.

The conclusive pathological diagnosis was a papillary thyroid carcinoma, precisely of the Warthin-like subtype, concomitant with two lymph node metastases. The pathological according to staging, as per the 8th edition AJCC,delineated the condition as pT2a N1. Subsequent to this determination, a complete thyroidectomy was executed, performed which elucidated chronic lymphocytic thyroiditis as the exclusive finding in the definitive histopathological examination. Following the surgical intervention, the patient underwent I131 radioiodine therapy. Currently for, she is undergoing routine follow-up, with a year having transpired, and is shows exhibiting favorable clinical outcome.

Discussion

Currently, the fifth WHO classification of Endocrine and Neuroendocrine tumors systematically classifies the subtypes of papillary thyroid carcinoma (PTC) into thirteen discrete subtypes, as previously delineated. The inception of Warthin-like papillary thyroid carcinoma (WLPTC) in the medical literature dates back to 1995 when Apel et al. formally introduced it.[2] This particular subtype acquired its nomenclature owing to its distinctive papillary configuration, characterized by the amalgamation of oncocytic cells displaying PTC nuclear features and a lymphoplasmacytic stroma, evoking the histological characteristics of the Warthin tumor commonly observed in the salivary gland. Furthermore, studies indicate that the prevalence of WLPTC is higher among females in the fourth decade of life.[5]

The cytological findings in WLPTC typically mirror those observed in classic PTC, characterized by an elevated presence of oncocytic cells and a discernable lymphoplasmacytic background.[4-5] However, it is imperativeto distinguish WLPTCL from other PTC subtypes that exhibit oncocytic cells alongside Hashimoto thyroiditis. In cases of polymorphic Hashimoto

thyroiditis, an abundance of polymorphous lymphoid cell populations is often evident, accompanied by oncocytic cells arranged in flattened sheets or isolated cells. In this specific case under consideration, the cytological examination unveils an unusually hypercellular smears, marked by numerous microfollicles and densely packed clusters of follicular cells manifesting focal oncocytic changes, questionable PTC nuclear features, and a constrained lymphoplasmacytic background.

The determination of reporting results, in adherence to the 2nd edition Bethesda system, pivots on distinguishing among CAT IV (subdivided into 2 categories): follicular neoplasm, and follicular neoplasm with Hurthle cell type, or CAT V: suspicious of PTC. Initially, the exclusion of CAT V was warranted due to the predominant microfollicle pattern and the presence of a few PTC-like nuclear features. Subsequently, CAT IV: follicular neoplasm, Hurthle cell type, ensued as only a focal oncocytic change was dismissed. Finally, the cytological report was documented as CAT IV: follicular neoplasm with a corresponding comment "Even though, the architectural features suggested a follicular neoplasm. There were some oncocytic-like and nuclear features that raise the possibility of an invasive follicular subtype of PTC, oncocytic subtype of PTC, or NIFTP. The definite distinction among these entities was not possible on the cytological specimens".

The complexities in this case arise from the limited presence of microfollicular patterns and INCIs, unlike what is typically observed in other previous studies.[3,5] However, definitively excluding the possibility of PTC still remains challenging. In order to preempt potential diagnostic inaccuracies, it is imperative for that cytologists and pathologists to exercise careful discernment when interpreting slides and formulating diagnoses within the framework of the Bethesda system. The inclusion of supplementary comments in diagnostic reports to explicate potential considerations proves to be of considerable significance. This practice not only amplifies the efficacy of communication with the operating physician but also functions as a proactive measure against erroneous diagnoses. Emphasizing the didactic purpose of this case report underscores the importance of proactively implementing measures to guarantee precise diagnoses and uphold the standards of optimal patient care.

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Case Report

Hypertrophic Pachymeningitis from Neuro-Behçet's Disease: A Case Report

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Abstract

A 26-year-old female presented with visual loss for 10 days from optic neuritis, which had recurred in the fellow eye one year apart. Neuroimaging, Pathergy test and skin biopsy results supported the diagnosis of neuro-Behçet's disease. The patient was successfully treated with pulse methylprednisolone followed by prednisolone and immunosuppressive agents.

Keywords: Optic Neuritis, Pachymeningitis, Behçet's Disease

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Introduction

Hypertrophic pachymeningitis is a rare inflammatory meningeal process, which involves the dura mater and causes diffuse or local dura thickening. It can be caused by various etiologies such as infection, neoplasm, inflammation, or idiopathic. Clinical features include headache, optic neuropathy, cranial nerve palsy, stroke, seizure, and intracranial hypertension.[1] Behçet's disease is a chronic recurrent multiple-organ inflammatory disorder. Neuro-Behçet's disease is caused by vasculitis-induced thrombosis, while hypertrophic pachymeningitis is rare.[2] Behçet's disease is a rare disease, and hypertrophic pachymeningitis from presumed Behçet's disease is a very rare condition. Previous studies reported cases of hypertrophic pachymeningitis from Neuro-Behçet's disease with bilateral anterior uveitis, benign intracranial hypertension with papilledema, and cranial nerve palsies.[3-5] Opticneuropathy is an uncommon ocular involvement in Behçet's disease and optic neuritis has rarely been reported in Behçet's disease. The authors report an unusual case of unilateral optic neuritis from hypertrophic pachymeningitis with sequential involvement of the other is in accordance with fellow eye one year later. This study followed the tenets of the Declaration of Helsinki for research involving human subjects in all subjects. The study protocol was approved by our Institutional Ethics Committee (approval number MTU-EC-OP-0-074/66). Informed consent was obtained from the patient.

Case Report

A 26-year-old female presented with acute headache and progressive visual loss in the right eye for 10 days. The initial best corrected visual acuity (BCVA) was 10/200 OD and 20/30 OS. The intraocular pressure was 16, 19 mm Hg. Ocular examinations were normal OU. Pupils test revealed a relative afferent pupillary defect (RAPD) positive grade I in the right eye. The fundus examination revealed normal background fundus and the optic disc appeared pink with sharp margins and a cup-to-disc ratio of taken at 0.3 in both eyes (fundus photo was not done in the first visit). The color vision was normal and the visual field revealed a right central scotoma and a left normal left visual field. We suspected retrobulbaroptic neuropathy and we performed Optical coherence tomography (OCT) for a baseline to assess the retinal nerve fiber layer (RNFL) the loss of time. OCT showed a normal average peripapillary RNFL thickness in both eyes. Neurological examinations revealed no focal deficits. Other systemic symptoms were negative.

The following laboratory analyses were unremarkable: complete blood count, fasting blood sugar, lipid profiles, coagulation assay, venereal disease research laboratory (VDRL), treponema pallidum hemagglutination assay (TPHA), hepatitis panel, anti-HIV test, thyroid function test (TFT), rheumatoid factor (RF), antinuclear antibody (ANA), anti-double-stranded DNA (anti-dsDNA), antineutrophil cytoplasmic antibodies (ANCA), and antibodies to aquaporin-4 (AQP4-IgG). However, anti-aquaporin-4 high levels of blood tests showed high erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels (20 mm/hr and 26.69 mg/dL). Contrast-enhanced magnetic resonance imaging (MRI) of the brain and orbit showed faint hyperintensity with focal enhancement at the right intracanalicular optic nerve with a relative enhancement of the adjacent optic nerve sheath complex at the right optic canal, and thin dural thickening along the right anterior clinoid process. MRI of the brain also showed multifocal dural thickening along bilateral parietooccipitotemporal convexities, bilateral tentorium cerebelli, and posterior fossae, up to 0.5 cm in maximal thickness, indicating possible idiopathic pachymeningitis or IgG-4 related disease (Figure 1). Lumbar puncture revealed normal cerebrospinal fluid (CSF) pressure, protein, sugar, cell count, gram stain and culture. Additional serum IgG-4 testing was normal. While waiting for laboratory and MRI results and before starting steroids, her right visual acuity was improved to 20/30 at one month following after the initial visit. Repeat visual field testing revealed an improvement of the right central scotoma and resolution of the right central scotoma in the third clinical visit. We suggested steroid treatment, but the patient had spontaneous resolution without treatment, and then she was lost to follow-up during the coronavirus pandemic.

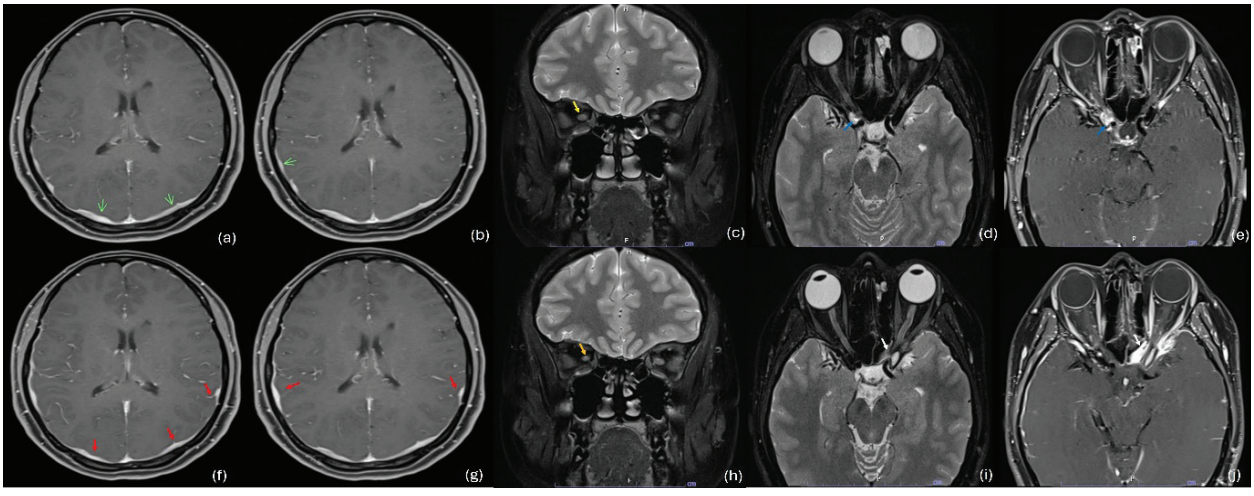

Figure 1 Contrast-enhanced MRI brain and orbit with comparison between the 1st episode (a-e) and 2nd episode (f-j): (a,b) axial view T1FS shows multifocal dural thickening along bilateral parietooccipitotemporal convexities (green arrow), (c) coronal view T2FS shows the right optic nerve (yellow arrow), (d) axial view T2FS shows a faint hyperintense right optic nerve (blue arrow), (e) axial view T1 FS with gadolinium shows focal enhancement at the right intracanalicular optic nerve with an enhancement of the optic nerve sheath and thin dural thickening along the right anterior clinoid process (blue arrow), (f, g) progression of multifocal dural thickening (red arrow), (h) a decrease in size of the right optic nerve (orange arrow), (i) a faint hyperintense left optic nerve (white arrow), (j) focal enhancement is noted at the left intracanalicular optic nerve with an enhancement of the optic nerve sheath, and thin dural thickening along the left anterior clinoid process (white arrow).

One year later, she experienced of acute headaches and visual loss in the left eye for one day. She was earlier vaccinated with an inactivated coronavirus vaccine one week prior. BCVA was 20/20 OD and 5/200 OS. Slit-lamp examinations revealed a normal anterior segment with RAPD positive grade I OS. Fundus examination showed temporal pallor of the right optic disc and mild swelling hyperemic left optic disc. OCT optic nerve head images show thinning of the temporal RNFL in the right eye and increased peripapillary RNFL thickness of the left eye (Figure 2). She had dyschromatopsia OS and visual field evaluation revealed a right normal central visual field and a left generalized depression. Left optic neuritis was diagnosed. MRI of the brain and orbit showed faint hyperintensity with focal enhancement at the left intracanalicular optic nerve in with a relative enhancement of adjacent optic nerve in the sheath complex at the left optic canal, and thin dural thickening along the left anterior clinoid process involving left orbital apex and anterior aspect of the left cavernous sinus. It also showed multifocal dural thickening along bilateral cerebral convexities, bilateral tentorium cerebelli, and posterior fossa (Figure 1). The overall progression of these lesions probably suggested an IgG4-related disease or other inflammatory diseases. Lumbar puncture revealed normal CSF profiles.

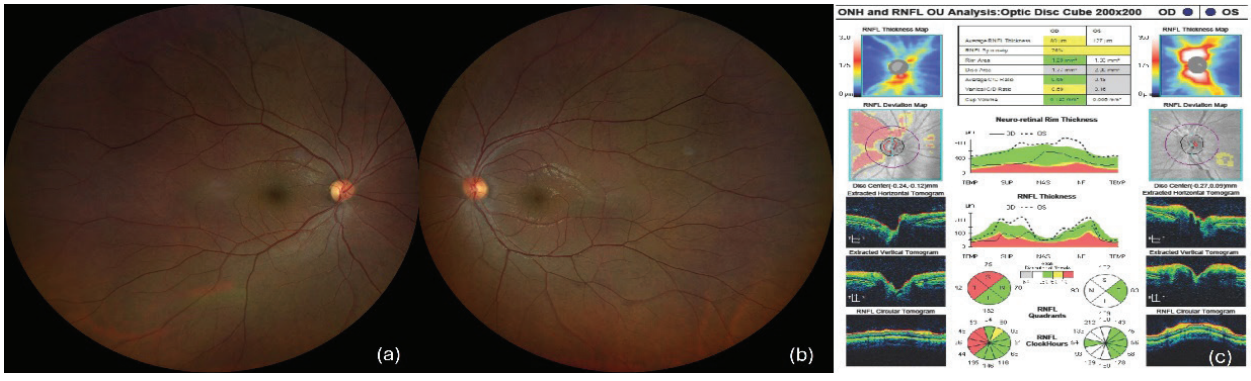

Figure 2 Optic disc photographs show temporal pallor of the right optic disc (a) and mild swelling hyperemic left optic disc (b), OCT optic nerve head images show thinning of the temporal RNFL in the right eye and increased peripapillary RNFL thickness of the left eye (c).

She had papulopustules on the extremities and vulva. We consulted a dermatologist, and rheumatologist. Repeat laboratory analyses before steroid treatment were unremarkable. ESR and CRP were used as markers to monitor the inflammatory levels. The patient still had elevated ESR and CRP (13 mm/hr and 14.26 mg/dL). Her skin pathergy test was positive. We did not send human leukocyte antigen typing B51 (HLA-B51), but we performed a skin biopsy on the forearm and the vulva, in which histopathological findings showed the presence of nuclear dust in the superficial perivascular area and deep perivascular infiltrate of lymphohistiocytes with some polymorphonuclear neutrophils (PMN) (Figure 3). We proposed the diagnosis of neuro-Behçet's disease. The patient received intravenous methylprednisolone (IVMP) for 3 days, followed by 1 mg/kg/day (MKD) of oral prednisolone. One week later, BCVA improved to 20/20 OS. Prednisolone was decreased to a dose of 50 mg/day and tapered off within 8 weeks. The patient received colchicine 0.5-2 mg/day and azathioprine 2-2.5 mg/kg/day to control inflammation and prevent relapse. ESR and CRP decreased to normal levels. Papulopustular skin and vulva lesions improved. Her headache and visual symptoms have not recurred during an 18-month follow-up.

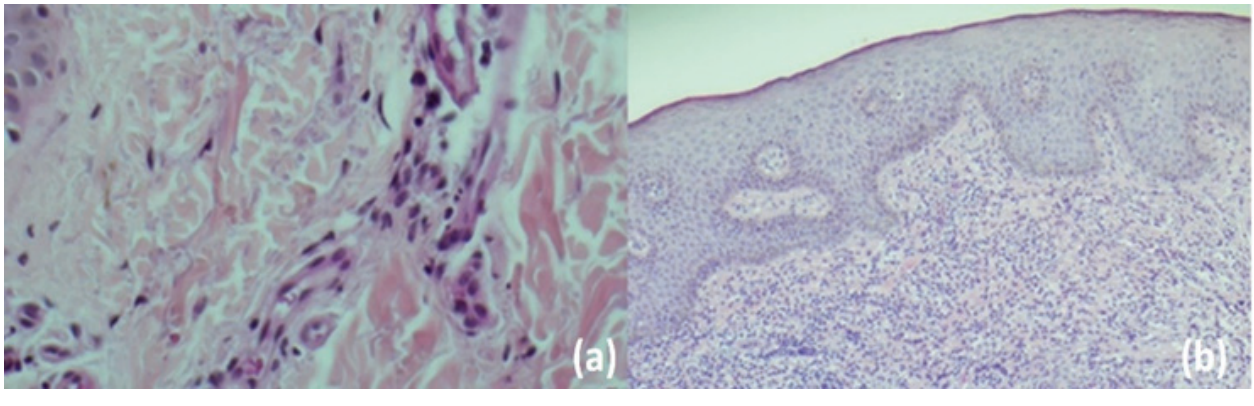

Figure 3 A histopathologic examination reveals (a) the presence of nuclear dust in the superficial perivascular area of the forearm, (b) psoriasiform and spongiotic dermatitis with hypergranulosis; irregular acanthosis; lymphocytic and neutrophilic exocytosis; dense superficial perivascular and interstitial infiltrate with a mildly dense deep perivascular infiltrate of lymphohistiocytes with some PMNs, without demonstrable ulcer or viral cytopathic changes of the vulva.

Discussion

Behçet's disease is an uncommon cause of hypertrophic pachymeningitis. Yoon et al. reported the first case presenting with blurred vision and chronic headaches from hypertrophic pachymeningitis in neuro-Behçet's disease.[3] He had anterior uveitis, skin lesions, recurrent oral ulcers, pachymeningitis, and a positive pathergy test. His patient had a negative HLA-B51 test and did not undergo a dura biopsy, but his patient was diagnosed with Behçet's disease based on the areas of involvement (anterior uveitis, skin lesion, recurrent oral ulcer, pachymeningitis, and positive pathergy test). Previous studies found that HLA-B51 was highly associated with Behçet's disease, especially in the Silk Road countries where reports have found a prevalence of up to 50-80%.[6,7]

In the first episode, the clinical findings of our patient were suggestive of retrobulbar optic neuropathy with headache. An initial differential diagnosis of retrobulbar optic neuritis, or compressive/infiltrative optic neuropathy was made. We evaluated the patient as an atypical optic neuritis and performed neuroimaging to exclude compressive or infiltrative lesions, but the MRI findings suggested inflammatory optic neuropathy from adjacent dural disease. Hypertrophic pachymeningitis can be diagnosed with characteristic the MRI findings, it presents as a localized or diffuse thickening of the dura, where the inflammatory process often causes abnormal enhancement in those areas.[8] Dural biopsy makes a definitive diagnosis, but sometimes there is a limitation to performing dural biopsy, lumbar puncture and blood tests to exclude secondary hypertrophic pachymeningitis may be needed. In this case, we diagnosed hypertrophic pachymeningitis as idiopathic in the first episode because our incomplete investigations could not provide clues to the underlying systemic diseases. Prednisone at a dose 1 mg/kg/day used for often employed as the first-line treatment of idiopathic hypertrophic pachymeningitis. However, our patient had spontaneous improvement, and previous literature has also reported spontaneousresolution of idiopathic hypertrophic pachymeningitis without treatment.[9]

In the second episode, the clinical finding suggest of anterior optic neuropathy. A possible differential diagnosis of post-vaccination optic neuritis or recurrent hypertrophic pachymeningitis was made. Repeated MRI findings suggested optic neuritis from adjacent sinusitis or dural disease in the fellow eye and suspected worsening of the meningeal inflammatory process. Our further investigation included a planned dural biopsy because we suspected a secondary cause. Fortunately, the patient had papulopustular skin lesions on the trunk and extremities which are the most common cutaneous manifestation, and in the common distribution, of Behçet's disease.[10,11] Biopsy of the skin and genitalia is less invasive than a dura biopsy histopathological. A histopathologic examination of the biopsied lesions revealed lymphohistiocytic and neutrophilic inflammatory infiltration at perivascular areas, which had similar features described in the previous histopathological study of Behcet's disease.[12] The pathergy test is one of the diagnostic criteria, and recent studies have revealed a rate of approximately pathergy positivity of about 45%.[13] The majority of patients with neurological symptoms have been reported without ocular involvement. Our case did not have uveitis, retinal vasculitis, or any signs of intraocular inflammation. According to the new international criteria for the diagnosis of Behçet's disease (ICBD),[14] ICBD has a sensitivity of 98.2% and a specificity of 95.6% if the total score is greater than or equal to 4 points. We did not investigate HLA-B51, but we diagnosed neuro-Behçet's disease based on the ICBD score. (genital aphthosis (2 points), skin lesions (1 point), optic neuritis (2 points), neurological manifestations (1 point), and a positive pathergy test (1 point)).

A previous study by the Japanese National Research Committee for Behçet's disease recommended moderate to high dose corticosteroids in acute or subacute attacks of neuro-Behçet's disease. Prednisolone $\geq$ 20 mg/day may be given first and tapered over two to three months. Pulse therapy may be considered if the patient dose not response.[15] Hirohata et al. recommended starting a colchicine dose of 1.0-2.0 mg/day for 5 years to prevent long-term relapse, while prednisolone should be decreased gradually.[16]A previous study by the European League Against Rheumatism (EULAR) Standing Committee for Behçet's disease recommended pulsed IVMP and azathioprine as first-line therapy for neurological involvement, and they considered colchicine as first-line therapy for

oral/genital ulcers, papulopustular, and acne-like lesions.[17] In the present study, combined colchicine and azathioprine are possibly effective in for the prevention of relapse.

In summary, hypertrophic pachymeningitis, resulting in optic neuritis, is a rare presentation of a neuro-Behcet disease. Tissue biopsy is essential for the diagnosis combined with clinical signs and symtoms is essential for diagnosis. High-dose corticosteroids are fundamental in such cases. Our multidisciplinary teamwork demonstrated an uncommon biopsy-proven case of neuro-Behçet's disease, with neuroimaging findings on presentation suggestive of hypertrophic pachymeningitis.

Acknowledgments

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Conflict of Interest: All authors report no conflicts of interest relevant to this article.

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Brief Research

Factors Influencing Problematic Sleep Among Preschool Children with ADHD: Brief Research

Prakasit Wannapaschaiyong[1*], Sureelak Sutchritpongsa[1]

Abstract

This study aimed to examine the prevalence of sleep disturbances and related factors among preschool children with ADHD. Our results showed that the prevalence of sleep disturbances was 40%. Factors associated with problematic sleep include combined-type ADHD, higher severity of ADHD symptoms, the use of taking methylphenidate, higher behavioral difficulties, and no bedtime routine.

Keywords: ADHD, Preschool children, Sleep disturbances

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Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a common neurodevelopmental disorder in children and adolescents with a 5.3% prevalence.[1] According to the Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-5), ADHD symptoms include inattention, hyperactivity, and impulsiveness, which affect daily functions and learning processes.[2] Currently, pediatricians prefer to diagnose ADHD in the preschool period so that preschoolers with ADHD receive early intervention, which will lead to a reduction in negative consequences such as behavioral and emotional problems in the school-age period.[2]

Sleep problems are common in children with ADHD. According to a study by Owens (2009), 50% of school-aged children with ADHD have sleep problems, which is more than those without ADHD (25%).[3] Many factors, including the intrinsic features of ADHD, psychiatric comorbidities, and the influence of ADHD medications cause sleep disturbances in these children.[4] Many studies have shown the relationship between poor sleep quality and increased severity of ADHD symptoms and behavioral problems, including irritability, aggressive behavior, and conduct problems, in school-aged children with ADHD.[4,5] Moreover, increasing behavioral problems and severity of ADHD symptoms from poor sleep quality may lead to negative long-term consequences, including interpersonal problems in interpersonal relationships problems, peer rejection, and future academic and work failures.[6] Therefore, if sleep problems in children with ADHD can be reduced, negative behavioral sequences can also be reduced. Especially preschool-aged children, their self-regulation abilities are not yet fully developed at that age.[7] Thus, sleep disturbances in preschoolers with ADHD may cause behavioral and emotional problems more easily than in older children and for sleep adolescents. Screening sleep problems in preschool-aged children with ADHD and identifying factors that affect their sleep are crucial to caring for these children. Only one previous study, Stickley's study (2021), found that the prevalence of sleep problems in preschool children with ADHD was as high as 83.6%. The most common sleep problems in these children included awakening at night (59.6%), nightmares (29.9%), and snoring (22.6%).[8] However, currently, there is no study to determine the related factors of sleep problems among preschool children with ADHD. Therefore, this research was developed to narrow the knowledge gap in this field.

Methods

Study Design and Participants

The secondary data from Wannapaschaiyong's unpublished cross-sectional study, which investigated the relationship between sleep disturbances and emotional/behavioral difficulties in preschoolers with ADHD, were used in this study. The 80 preschoolers aged 4-6 years were recruited from the child development clinic at Siriraj Hospital between October and December 2023. Developmental and behavioral pediatricians diagnosed and classified all participants according to DSM-5 criteria. Participants who had global developmental delay and autism spectrum disorder were excluded.

Data Collection

The original study (Protocol number 719/2023) was approved by the Siriraj Institutional Review Board. Caregivers of eligible participants were recruited and informed about the original protocol. After providing informed consent, they completed the paper-based questionnaires, including the demographic information form, the Children's Sleep Habit Questionnaire (CSHQ), and the Strengths and Difficulties Questionnaire (SDQ).

Research Instruments

1. Demographic information form

The participant's demographic characteristics, including sex, bedtime environments, bedtime routine, type and severity symptoms of ADHD, comorbidities, and treatment modalities they received.

2. The Children's Sleep Habits Questionnaire, Thai-version (CSHQ-Thai)[9]

The CSHQ-Thai was used to assessed sleep characteristics and problem in children aged 4-10 years. This questionnaire comprises 33 sleep habit questions, each scored on a three-point Likert scale ranging from "usually" to "rarely." A total score greater than 41 points is evaluated characteristics and problems of sleep. This tool has strong internal consistency (Cronbach's alpha coefficients = 0.83).

3. Strengths and Difficulties Questionnaire, parent rating – Thai version (SDQ-parent rating)[10]

Behavioral difficulties were screened with SDQ-parent rating. The 25 items in this questionnaire evaluated the participant's five aspects of behaviors, including emotional symptoms, hyperactivity, conduct problems, peer relationship problems, and prosocial behaviors. Their parents were required to rate on a 3-point Likert scale: 0 = "not true," 1 = "somewhat true," and 2 = "definitely true." The four behavioral domains of hyperactivity, emotional symptoms, conduct problems, and peer relationship problems were then used to calculate the overall difficulty score. Participants assessed greater than having a total difficulty score exceeding 16 are considered to have significant behavioral difficulties. SDQ had an overall Cronbach's alpha internal consistency of 0.7, exhibiting a sensitivity and specificity of 0.63 and 0.95, respectively.

Statistical Analyses

A descriptive analysis was performed to calculate the frequency and percentage in this study. Chi-square and Fisher's exact test were used to compare the related factors between participants with and without problematic sleep. This study used IBM SPSS version 25.0 (SPSS Inc., Chicago, USA) for analyses.

Results

80 preschool children, including 60 boys (75%) and 20 girls (25%) were studied. The mean age of participants was 5 ± 0.61 years. More than one third of the participants (40%) had clinically significant sleep disturbances. In Table 1, the proportion of participants diagnosed with combined presentation had more sleep problems than those with hyperactive and impulsive presentation ($p = 0.002$). 75% of participants without sleep problems had mild severity of ADHD symptoms. On the other hand, 87.5% of participants with problematic sleep had moderate to severe ADHD symptoms. When comparing the participants with and without sleep disturbances, the severity of ADHD symptoms was significantly different ($p < 0.001$). Nearly 60% of participants with sleep problems were treated with methylphenidate, which was different from those without sleep problems (2.08%) ($p < 0.001$).

Almost all participants who showed behavioral difficulties from the SDQ assessment had sleep problems. When comparing the participants with problematic sleep and those without sleep problems, the proportion of participants with behavioral difficulties is significantly different ($p < 0.001$). In addition, compared to the participants with sleep problems, the proportion of participants without sleep disturbances who had bedtime routines was higher ($p < 0.001$). On the other hand, the bedroom environment, such as temperature and exposure to light and sound during the night, did not show a difference between participants with and without problematic sleep.

Table 1 Comparison of problematic sleep and related factors among preschool children with ADHD

Characteristics	**Participants without problematic sleep (n = 48)**	**Participants with problematic sleep (n = 32)**	***P*-value**
Sex			0.292
Boy	34 (70.83)	26 (81.25)	
Girl	14 (29.17)	6 (18.75)	
ADHD type			0.002*
Hyperactive/Impulsive	39 (81.25)	15 (46.88)	
Combined presentation	9 (18.75)	17 (53.12)	
Severity of ADHD			< 0.001*
Mild	36 (75)	4 (12.5)	
Moderate	11 (22.92)	24 (75)	

Table 1 Comparison of problematic sleep and related factors among preschool children with ADHD (cont.)

Characteristics	Participants without problematic sleep (n = 48)	Participants with problematic sleep (n = 32)	*P*-value
Severe	1 (2.08)	4 (12.5)	
Pharmacological therapy			< 0.001*
No	31 (64.59)	7 (21.88)	
Methylphenidate	1 (2.08)	19 (59.38)	
Risperidone	16 (33.33)	6 (18.75)	
Behavioral difficulties			< 0.001*
Not significant difficulties	47 (97.92)	8 (25)	
Significant difficulties	1 (2.08)	24 (75)	
Bedtime routine			< 0.001*
No	21 (43.75)	31 (96.88)	
Yes	27 (56.25)	1 (3.12)	
Bedroom temperature (Parents' perception)			1.000
Normal	45 (93.75)	30 (93.75)	
Cold	3 (6.25)	2 (6.25)	
Exposed light in bedroom			0.400
No	41 (85.42)	25 (78.12)	
Yes	7 (14.58)	7 (21.88)	
Exposed sound in bedroom			1.000
No	47 (97.92)	31 (96.88)	
Yes	1 (2.08)	1 (3.12)	

Data presented as number (percentage)
Abbreviations: ADHD = Attention Deficit Hyperactivity Disorder
*significant with level of $P < 0.05$

Discussion

This study found that 40% of preschoolers with ADHD had significant sleep disturbances. Our result was less than the Stickley's study (2021), which found that of the prevalence of sleep problems among preschool children with ADHD was as high as 83.6%.[8] Our small population, collected from a single center, can explain this inconsistency, which may be the reason for the low prevalence rate in this study. In addition, Stickley's research did not use the standard questionnaire to ask about sleep problems, which may lead to overestimating the prevalence result.

Factors associated with significant sleep disturbances among preschool children with ADHD include type and severity of ADHD, taking methylphenidate, considerable behavioral difficulties, and no bedtime routine. Our results are consistent with previous studies that found that combined-type ADHD and higher severity symptoms were associated with sleep disturbances.[4] Children with combined-type ADHD may have more significant sleep problems than those who have predominantly inattentive or hyperactive-impulsive type because children with combined-type ADHD have more impaired regulation of brain activity, which leads to delayed circadian rhythm with a later onset of melatonin production.[11] In addition, the higher the severity of ADHD symptoms, the worse the function related to brain networks, which leads to more severe sleep problems.[11] More behavioral difficulties are another symptom that reflects the deterioration of behavioral and emotional control caused by the worsening prefrontal cortex function.[11]

The use of methylphenidate use, which is the first-line treatment for children with ADHD, results in sleep difficulties.[4] Although the United States Food and Drug Administration (FDA) approves methylphenidate for the treatment of ADHD in patients older than 6 years, current guidelines and studies recommend the use of this medication for the treatment of children under six who continue to have symptoms of ADHD and seriously impaired daily living activities and social function, after intensive treatment with parent management training and behavioral modification.[12,13] However, younger children tend to have a more significant response to psychostimulants than older children and adolescents and therefore, younger children are more likely to experience medical side effects, including insomnia and prolonged sleep latency.[14] This data supports our findings that the use of psychostimulants in these children may be a significant cause of sleep disturbances.

Whereas psychostimulants can interfere with sleep, implementing a consistent bedtime routine can ameliorate sleep problems because it supports children in managing themselves calmly before bed, creating a regular sleep schedule, and promoting falling asleep independently.[15] Our results are consistent with studies by Mindell (2009)[16] and Henderson and Jordan (2010),[17] which found that having adaptive bedtime routines in preschool children is associated with better sleep quality, longer duration of sleep at night, and decreased bedtime resistance. Therefore, preschool children with ADHD, who are more prone to sleep difficulties, are likely to benefit significantly from a regular bedtime routine.

Our study has some limitations. First, this study is based on secondary data analysis of the original research investigating the relationship between sleep problems and behavioral problems in preschool children. Some potential variables related to sleep problems, including the dose and duration of receiving medication, and demographic characteristics and parenting practices of primary caregivers, were not collected. Therefore, further research should also collect these variables. Second, the number of participants in our study was small. In a sigle addition, our participants were recruited from single center, which may have selective bias and did not represent all preschool-aged children with ADHD. Large sample sizes and multicenter studies should be investigated. Finally, this cross-sectional study design cannot determine the causal relationship between related factors and sleep disturbances. Thus, longitudinal studies should be conducted in the future.

In conclusion, almost half of preschoolers with ADHD are likely to experience sleep problems. Therefore, identifying sleep problems and related factors in these children should always be checked and addressed. The implementation strategies that can reduce these children's sleep problems include providing effective treatment for ADHD, appropriate management of behavioral difficulties, avoiding the use of methylphenidate without appropriate indications, and promoting a regular bedtime routine.

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Compliance with Ethics Requirements All procedures performed in this study involving human participants were in accordance with the ethical standards of the Siriraj Institutional Review Board (SIRB), Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand (Protocol number: 719/2023) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of Interest The authors declare no conflict of interest regarding the contents and publication of this article.

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Author Contributions All authors approved the final article. The authors involved in the study are as follows; Prakasit Wannapaschaiyong: Conceptualization, Methodology, Investigation and data collection, Writing-Original draft. Sureelak Sutchritpongsa: Writing-Review and editing.

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Acknowledgments

Financial support. Information on financial support should be provided by authors. The Acknowledgments section should list all sources of financial support for the work, including any financial arrangement with a company whose product is related to the study. If there was no financial support, that too should be stated. Acknowledgments, including grant support, should be placed after the text.

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Journal article (examples)

1. Pittet D, Simon A, Hugonnet S, Pessoa-Silva CL, Sauvan V, Perneger TV. Hand hygiene among physicians: performance, beliefs, and perceptions. *Ann Intern* Med. 2004;141:1-8.
2. Camins BC, Richmond AM, Dyer KL, et al. A crossover intervention trial evaluating the efficacy of a chlorhexidine-impregnated sponge in reducing catheter-related bloodstream infections among patients undergoing hemodialysis. *Infect Control Hosp Epidemiol.* 2010;31:1118-1123.

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3. Figueroa P, Johanssen KL, Price FG, et al. Outbreak of Acinetobacter infection in a neonatal intensive care unit. *Pediatr Infect Dis J* (in press).

Paper presented at a professional meeting (example)

4. Chen LF, Freeman JT, Sexton DJ, Choi YI, Anderson DJ. NHSN definition of laboratory-detected BSI is overly sensitive for Enterococcus. In: Program and abstracts of the 19th Annual Scientific Meeting of the Society for Healthcare Epidemiology of America (SHEA); March 18-22, 2009; San Diego, CA. Abstract 359.

Book (example)

5. Heoprich PD. *Infectious Diseases. 2nd ed.* New York, NY: Harper & Row; 1977.

Chapter in a book (example)

6. Schaffner W. Psittacosis: ornithosis, parrot fever. In: Beeson PB, McDermott W, Wyngaarden JB, eds. *Cecil Textbook of Medicine. 15th ed.* Philadelphia, PA: W. B. Saunders; 1979:336-338.

Web page (example)

7. Clinical laboratory fee schedule. Centers for Medicare and Medicaid Services website. https://www.cms.gov/ClinicalLabFeeSched/02_clinlab.asp#TopOfPage. Published 2010. Accessed April 2, 2010.

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Example of Tables:

Table 2 Inhibitory effects of *E. elatior* extracts on nitric oxide, PGE2 and TNF-α production from RAW264.7 cells.

Sample	Inhibitory effects (IC_{50}; μg/mL)		
	Nitric oxide	PGE_2	TNF-α
EEE	22.59 ± 3.33*	66.56 ± 14.59*	> 100*
EE95	16.78 ± 7.21*	45.26 ± 9.28*	> 100*
EE70	16.36 ± 4.19*	> 100*	> 100*
EE50	96.09 ± 12.1*	> 100*	> 100*
EEDec	> 100*	> 100*	> 100*
L-NAME	6.69 ± 1.95 (0.029 ± 0.008 μM)	NA	NA
Indomethacin	NA	9.31 ± 3.23 (0.026 ± 0.009 μM)	NA
Dexamethasone	NA	NA	35.73 ± 15.31 (0.091 ± 0.039 μM)

*Significant difference from the positive control (*p*-value <0.05) analyzed by ANOVA with Dunnett post-hoc analysis.

Table 3 Univariable risk regression of the hearing loss

Characteristics	**Risk ratio**	**95% CI**	***P*-value**
Congenital hypothyroidism	2.00	1.09 - 3.67	.0286
Ototoxic	2.37	1.34 - 4.19	.003
Sepsis	3.11	1.74 - 5.58	.0007

Abbreviations: CI, confidence interval

Figure

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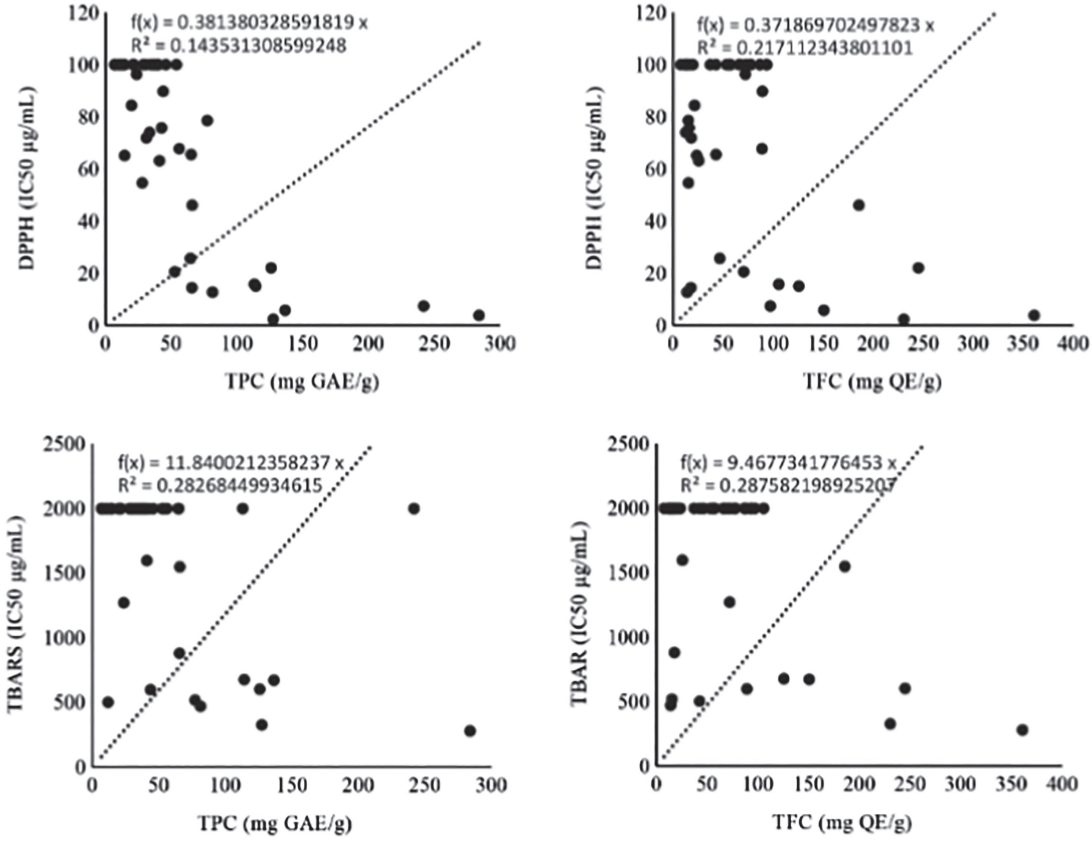

Figure 1 The Pearson statistical correlation scatters plot of the linear relationship between (A) DPPH and TPC, (B) DPPH and TFC, (C) TBARS and TPC, and (D) TBARS and TFC.

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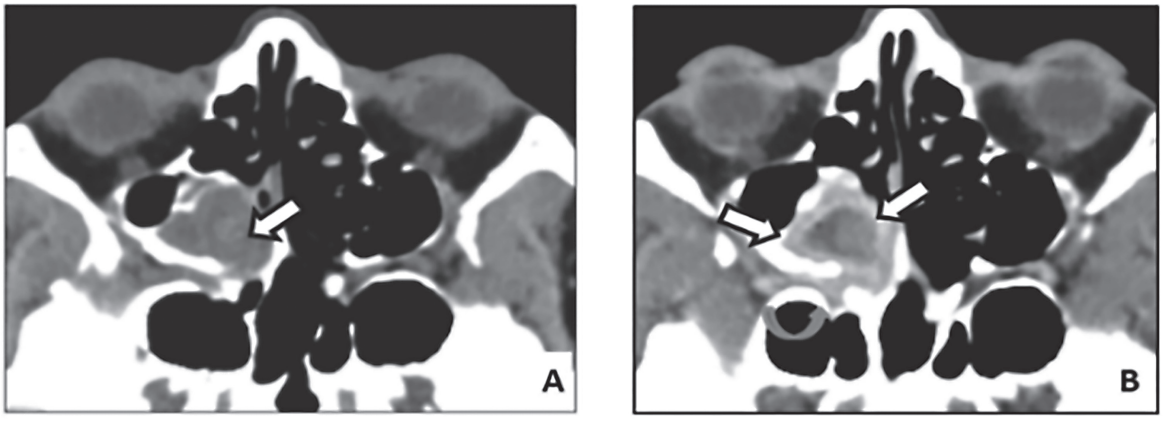

Figure 4 A and B: 58-year-old man with fungal ball at right ethmoid sinus was interpreted as inflammatory lesion. There is a hyperattenuating content (open arrow in A) with mucosal thickening at right posterior ethmoid sinus (open arrow in B) with fat haziness and increased enhancement at right PPF (curve arrow in B).