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RESEARCH ARTICLE

Exploring Biological Activities of a Thai Traditional Remedy Called "Ruean-Mhoon-Nok" and its Plant Ingredient Extracts for the Treatment of Dermatitis

Atchanika Taingthum[1], Sumalee Panthong[2], Puritat Kanokkangsadal[2] and Intouch Sakpakdeejaroen[2,*]

[1]*Student of Doctor of Philosophy in Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Pathum Thani, 12120, Thailand;* [2]*Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Pathum Thani, 12120, Thailand*

Abstract: ***Background*:** Ruean-Mhoon-Nok (RMN) remedy has been used to treat skin inflammatory diseases (*e.g.*, dermatitis and psoriasis). However, its bioactivities related to traditional use remain unclear.

***Objective*:** To investigate the biological activities related to dermatitis treatment of the RMN and its plant ingredient extracts, including the determination of bioactive compounds and stability study.

***Methods*:** *in vitro* anti-inflammatory activities were assessed through the inhibition of NO using Griess reagent in RAW 264.7 cells, as well as IL-6 and TNF-α production using an ELISA test kit. The anti-allergic activity was performed *via* degranulation assay in RBL-2H3 cells. The microtiter plate-based antibacterial assay was used to assess MIC and MBC. The bioactive compound in the RMN extract was measured by HPLC, while its stability was evaluated under accelerated storage conditions.

***Results*:** The RMN extract exhibited a potential inhibitory effect on NO and IL-6 production, while it had a limited effect on inhibition of β-hexosaminidase release. In addition, the RMN extract displayed antibacterial activity against Gram-positive bacteria, including *S. epidermis*, *S. aureus,* and MRSA. Among individual plants, *Piper wallichii* extract displayed outstanding results in all assays compared to the others. The HPLC results confirmed that hydroxychavicol is a major RMN extract constituent, demonstrating potent inhibitory activity on NO and IL-6 productions. However, the RMN extract was unstable when stored under accelerated conditions.

***Conclusion*:** The RMN remedy and its bioactive compound, hydroxychavicol, have highly promising anti-inflammatory and anti-bacterial properties that might support its traditional use. However, further investigations related to the pathogenesis of dermatitis are required, including preclinical and clinical studies.

ARTICLE HISTORY

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1. INTRODUCTION

Dermatitis is an inflammatory skin condition that occurs in the epidermis and dermis, arising from dysfunction of the dermatologic barrier caused by a combination of endogenous (*e.g.*, genetic and immune system) and exogenous (*e.g.*, environment, allergens, and pathogens) factors [1]. Generally, dermatitis symptoms are primarily characterized by scaly skin, pruritic, erythema, and vesicular lesions, depending on the severity and stage of the disease [2]. Several pro-inflammatory mediators, such as nitric oxide (NO), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), have been reported to be associated with the progression of acute and chronic dermatitis [3, 4]. In Thailand, dermatitis accounted for 31.3% of skin diseases found in a primary care area over five years (2015 to 2019). Recently, it has been reported that dermatitis contributed to

* Address correspondence to this author at the Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Pathum Thani, 12120, Thailand; Tel: (+66) 29269749; E-mail: intouch@tu.ac.th

the highest disability-adjusted life years (DALYs) among skin and subcutaneous diseases worldwide, affecting patients' quality of life [5].

Topical corticosteroids are commonly used as a first-line treatment of dermatitis. In addition, daily application of emollients (moisturizers) is also recommended to prevent skin dryness, while specific drugs such as antihistamines and antibiotics are the treatment options in some cases. Although topical corticosteroids can be highly effective in a short period, their long-term use can affect the skin's repairing function, causing skin fragility and striae [2]. In recent years, traditional and herbal medicines have gained considerable attention as potential treatments for dermatitis owing to a wide range of pharmacological activities, especially anti-inflammation, anti-allergy, and antimicrobial.

Ruean-Mhoon-Nok (RMN) remedy, a Thai traditional medicine (TTM), has been used as a topical preparation for the treatment of skin inflammatory diseases, including dermatitis and psoriasis [6]. This remedy consists of nine plant ingredients in equal ratio: the leaves of *Casearia grewiifolia* Vent. (CG), the leaves of *Crateva religiosa* G. Forst. (CR), the leaves of *Crateva adansonii* DC. (CA), the leaves of *Piper wallichii* (Miq.) Hand.-Mazz. (PW), the leaves of *Datura metel* L. (DM), the leaves of *Persicaria chinensis* (L.) H. Gross (PC), the aerial parts of *Pouzolzia zeylanica* (L.) Benn. (PZ), the aerial parts of *Gonostegia pentandra* (Roxb.) Miq. (GP), and the rhizomes of *Alpinia galanga* (L.) Willd. (AG). Currently, some individual plant components of the RMN remedy have demonstrated a potential capability to inhibit pro-inflammatory cytokines, degranulation of mast cells, and skin pathogenic bacteria [7-13]. Our previous study revealed a promising *in vitro* antioxidant ability of the 95% ethanolic extract of RMN remedy. Moreover, the extract also contained high phenolic and flavonoid contents [14]. These results might support the traditional use of RMN remedy as oxidative stress has been proposed as a risk factor in the pathogenesis of skin inflammation diseases. Therefore, in this study, we further investigated the capability of RMN remedy extract for the treatment of dermatitis, including anti-inflammatory, anti-allergic, and antimicrobial activities. In addition, we also determined the bioactive constituent of RMN remedy, as well as evaluate its stability under accelerated storage conditions.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium (MEM), Fetal bovine serum (FBS), Penicillin-Streptomycin (P/S) (10,000 U/mL), and 0.4% Trypan blue solution were purchased from Gibco, USA. Albumin bovine fraction V powder, anti-dinitrophenyl bovine serum albumin (DNP-BSA), DNP-specific IgE (monoclonal anti-DNP), 4-Nitrophenyl N-acetyl -β-D-glucosaminide (p-NAG), lipopolysaccharide (LPS) from *E. coli* O55:B5, sulfanilamide, phosphate-buffered saline (PBS) and N-(1-Naphthy) ethylenediamine dihydrochloride, 3-(4,5- dimethylthiazol -2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), and all other chemicals that are not specifically mentioned below were purchased from Sigma Aldrich, Germany. Hydroxychavicol, diphenhydramine, and betamethasone were obtained from TCI, Japan. TNF-α and IL-6 ELISA kits were purchased from R&D Systems, USA. Dimethyl sulfoxide (DMSO) and 95% ethanol were purchased from RCI Labscan, Thailand.

2.2. Preparation of RNK Remedy and its Plant Ingredient Extracts

2.2.1. Plant Materials, Collection, and Identification

The reference cultivated plant ingredients of the RMN remedy, which include *A. galanga*, *C. grewiifolia*, *C. adansonii, C. religiosa*, *D. metel*, *G. pentandra*, *P. chinensis*, *P. wallichii*, and *P. zeylanica*, were purchased from several herbal shops in Thailand in May and June 2020. The plants were identified by an herbal medicine expert from the Department of Thai Traditional and Alternative Medicine (DTAM), and the voucher specimens (Table **1**) were deposited at the herbarium of Thai Traditional Medicine Research Institute, DTAM, Bangkok, Thailand.

2.2.2. Preparation of Crude Extracts

All plant materials were cleaned, dried, and ground into coarse powder. The RMN remedy was prepared by mixing each plant in equal portions and extracted by maceration with 95% ethanol for 3 days. The extracts were filtrated through Whatman No.1 filter paper and then concentrated under reduced pressure using a rotary evaporator. The maceration process was repeated twice (a total of 3 times). Finally, the extracts were combined and dried in a vacuum dryer. Plant ingredients were extracted by maceration with 95% ethanol in the same manner as the RMN remedy. All extracts were stored at -20 °C for further experiments.

2.3. Anti-inflammatory Activity

2.3.1. Cell culture

The RAW264.7 murine macrophage cell line obtained from the American Type Culture Collection (ATCC TIB-71) was used in this study. Cells were grown as a monolayer in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin. Cells were cultured at 37°C in an incubator with a 5% carbon dioxide (CO_2) humid atmosphere.

2.3.2. Inhibition of NO Production in LPS-stimulated RAW 264.7 Cells

The inhibition of NO production assay was evaluated by measuring the concentration of the end product, nitrite (NO^{2-}), using the Griess reagent (containing 0.1% *N*-(1-Naphthy) ethylenediamine dihydrochloride and 1%

Table 1. General information on plant ingredients of the RMN remedy.

Plant Species	Code	Family	Voucher Specimen Number	Part Used	Source
Alpinia galanga (L.) Willd.	AG	Zingiberaceae	TTM 0005447	Rhizomes	Nakhon Pathom
Casearia grewiifolia Vent.	CG	Salicaceae	TTM 1000658	Leaves	Chachoengsao
Crateva adansonii DC.	CA	Capparaceae	TTM 1000660	Leaves	Chon Buri
Crateva religiosa G. Forst.	CR	Capparaceae	TTM 1000659	Leaves	Samut Sakhon
Datura metel L.	DM	Solanaceae	TTM 0005448	Leaves	Ayutthaya
Gonostegia pentandra (Roxb.) Miq.	GP	Urticaceae	TTM 1000662	Aerial parts	Samut Sakhon
Persicaria chinensis (L.) H. Gross	PC	Polygonaceae	TTM 1000664	Leaves	Samut Sakhon
Piper wallichii (Miq.) Hand.-Mazz	PW	Piperaceae	TTM 1000663	Leaves	Nakhon Pathom
Pouzolzia zeylanica (L.) Benn.	PZ	Urticaceae	TTM 0005449	Aerial parts	Samut Sakhon

sulfanilamide in 2.5% phosphoric acid solution) [15, 16]. Briefly, the RAW 264.7 cells were seeded into a 96-well plate at a density of $1x10^5$ cells/well and grown for 24 hours in an atmosphere of 37 °C with 5% CO_2 to allow for cell attachment. After that, the medium was removed and replaced with 100 µL of fresh medium containing 10 ng/mL of LPS. Cells were then treated with 100 µL of tested samples at various concentrations. After 24 hours, 100 µL of supernatant was transferred to another 96-well plate and mixed with the same volume of Griess reagent. The optical density was measured at a wavelength of 570 nm. The results were expressed as percentage inhibition according to the equation below. Dose-response curves were fitted to obtain the inhibitory concentration for 50% (IC_{50}) of NO production. Betamethasone was used as a positive control.

$$\%Inhibition = \frac{OD\ control - OD\ sample}{OD\ control} \times 100$$

2.3.3. Inhibition of IL-6 and TNF-α Production in LPS-stimulated RAW 264.7 Cells

The inhibition of IL-6 and TNF-α production was determined using ELISA kits (R&D Systems, USA), as described in the manufacturer's instructions [16]. The RAW264.7 cells were grown and then treated with LPS and tested samples in the same manner described in the previous section. After 24 hours of treatment, 100 µL of supernatant was collected to measure IL-6 and TNF-α production. To do so, a 96-well plate was coated with mouse IL-6 or TNF-α captured antibodies overnight at 4 °C. The plate was then washed three times with wash buffer and blocked with 5% bovine serum albumin in PBS for 2 hours at room temperature. After blocking, 100 µL of supernatants were added into each well and incubated at room temperature for 2 hours, followed by the washing step. Subsequently, streptavidin-HRP was added and incubated for 30 minutes (with protection from light) before repeating the washing step. After that, the TMB (3,3′,5,5′-Tetramethylbenzidine) substrate was added and incubated for 30 minutes. Finally, 50 µL of sulfuric acid solution (H_2SO_4, 1M) was added to stop the reaction. The optical density was measured at a wavelength of 450 nm. The IL-6 and TNF-α production was calculated from the absorbance of each sample by correlating with the standard curve of recombinant protein. The results were expressed as percentage inhibition of IL-6 and TNF-α production according to the equation below. Dose-response curves were fitted to obtain the inhibitory concentration for 50% (IC_{50}) of IL-6 and TNF-α production.

$$\%Inhibition = \frac{Production\ of\ control - Production\ of\ sample}{Production\ of\ control} \times 100$$

2.4. Inhibition of β-hexosaminidase Release from RBL-2H3 Cells

The RBL-2H3 basophilic leukemia cell line was used in the degranulation assay [15]. Cells were cultured in MEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin. To perform a degranulation assay, the RBL-2H3 cells were seeded into a 24-well plate at a density of $2x10^5$ cells/well and incubated at 37°C in 5% CO_2 for 2 hours. Cells were then sensitized by adding 40 µL of DNP-specific IgE (5 µg/mL) and incubated at 37°C in 5% CO_2 for 24 hours. After incubation, the supernatant was removed and washed twice with buffer A (400 µL/well), followed by adding 160 µL of buffer A into each well and incubating for 10 minutes. Cells were then treated with 20 µL of tested samples at various concentrations and incubated for 10 minutes. Subsequently, 20 µL of antigen DNP□BSA (0.1 mg/mL) was added and incubated for 20 minutes. After that, 50 µL of supernatant was transferred to a 96-well plate before reacting with PNAG (50 µL/well) for 2 hours. Finally, 200 µL of sodium carbonate solution (Na_2CO_3, 0.1 M) was added to stop the reaction. The optical density was then measured at a wavelength of 405 nm. The results were expressed as percentage inhibition of β-hexosaminidase release (as a marker of degranulation) according to the equation below. Diphenhydramine was used as a positive control.

$$\% \, Inhibition \, of \, degranulation = \left[1 - \frac{(OD \, sample - OD \, blank)}{(OD \, control - OD \, blank)}\right] \times 100$$

2.5. Antibacterial Activity

2.5.1. Tested Microorganism

A total of four bacterial species were obtained from the National Institute of Health of Thailand, including three species of Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, methicillin-resistant *Staphylococcus aureus* (MRSA) DMST 20651, and *Staphylococcus epidermis* ATCC 12228) and one species of Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 9027). All microorganisms were cultured on nutrient agar (Difco, USA).

2.5.2. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC value of RMN and its plant ingredient extracts was evaluated using the microtiter plate-based antibacterial assay [16, 17]. A stock solution of extract (500 mg/mL in ethanol) was diluted with Mueller-Hinton broth (MHB) to a maximum concentration of 10 mg/mL, followed by 2-fold serial dilution with MHB to obtain different concentrations ranging from 0.078 to 10 mg/mL. The tested samples (50 µL/well) were then transferred to a sterile 96-well plate. Bacteria that had been cultured on nutrient agar at 37°C for 24 hours were adjusted with MHB to obtain 0.5 McFarland turbidity standard and further diluted to 1/200 with MHB. The bacterial suspension (50 µL) was then added into each well and incubated at 37°C for 20 hours. After incubation, 10 µL of resazurin (1 mg/mL) was added into each well and incubated for 2 hours. The MIC value was defined as the lowest concentration of tested samples that did not change resazurin color. All tested samples with no color change of resazurin were streaked on nutrient agar and incubated at 37°C for 24 h. The lowest concentration of tested samples exhibiting no bacterial growth was considered as the MBC value. Ampicillin, norfloxacin, and vancomycin were used as positive controls.

2.6. Determination of Bioactive Constituent of RMN Remedy Extract using the HPLC Technique

The bioactive constituent of RMN extract was determined using an HPLC system (TSP Spectra System P4000) with a UV-visible detector (TSP Spectra System UV2000) and automatic injector (TSP Spectra System AS3500). Data were analyzed with ChromQuest 5.0 software (Thermo Fisher Scientific Inc., USA). The RMN extract was dissolved in methanol (10 mg/mL) and injected into a reversed-phase column, ZORBAX Eclipse XDB-C18 (4.60 x 250 mm, 5 µm), with a guard column of the same material. The mobile phases are composed of water (A) and acetonitrile (B) with gradient elution as follows: 0 min, 90:10; 10 min, 90:10; 30 min, 70:30; 45 min, 60:40; 53 min, 95:5; 60 min, 95:5; 60.1 min, 90:10; 65 min, 90:10. The flow rate was 1 mL/min with UV detection at a wavelength of 210 nm. The operating temperature was maintained at room temperature. According to our previous study (unpublished data), hydroxychavicol is the major constituent of RMN extract derived from *Piper wallichii*. Thus, this compound was used as a marker of the RMN remedy. The content of hydroxychavicol in the RMN extract was calculated by correlating the peak area with the standard calibration curve of hydroxychavicol.

2.7. Stability Study of RMN Remedy Extract Under Accelerated Condition

The stability study was performed according to the Association of Southeast Asian Nations (ASEAN) guidelines [18]. The RMN extract (50 mg) was placed in a well-closed container (glass vial) and stored in a climatic chamber under accelerated conditions at 40 ± 2 °C with 75 ± 5% relative humidity (RH) for 6 months (180 days). At specific time points (0, 30, 60, 90, 120, 150, and 180 days), the sample vials were taken and analyzed for anti-inflammatory activity using the inhibition of NO production assay, while the bioactive constituent was determined using HPLC.

2.8. Statistical Analysis

All experiments were performed in triplicate and expressed as mean ± standard error of the mean (SEM). Statistical analysis was assessed using the student's t-test or one-way analysis of variance analysis (ANOVA), followed by Dunnett's multiple comparison test. Differences were considered statistically significant for *p*-values lower than 0.05.

3. RESULTS

3.1. The RMN Remedy and its Plant Ingredient Extracts Inhibited Inflammatory Mediators in LPS-stimulated RAW 264.7 Cell

The RMN remedy and its plant ingredient extracts were investigated for their inhibitory effect on inflammatory mediator production in LPS-stimulated RAW 264.7 cells. As shown in Fig. (**1A**), the RMN extract (IC_{50}: 15.19 ± 1.57 µg/mL) and hydroxychavicol (IC_{50}: 19.73 ± 0.70 µg/mL) exhibited the inhibitory effect on NO production, but slightly lower than betamethasone (IC_{50}: 9.08 ± 1.65 µg/mL). Among all plant ingredient extracts, PW extract (IC_{50}: 10.75 ± 0.75 µg/mL) showed the most potent inhibition activity on NO production, followed by AG and DA extracts (IC_{50}: 11.43 ± 2.27 µg/mL for AG and 18.20 ± 0.96 µg/mL for DA).

For IL-6 mediator, the RMN extract (IC_{50}: 3.70 ± 0.10 µg/mL) exhibited the highest inhibitory effect on IL-6 production, as shown in Fig. (**1B**). Moreover, the results showed that hydroxychavicol (IC_{50}: 8.42 ± 0.17 µg/mL) could inhibit IL-6 production, better than betamethasone (IC_{50}: 28.57 ± 0.85 µg/mL). Interestingly, most plant ingre-

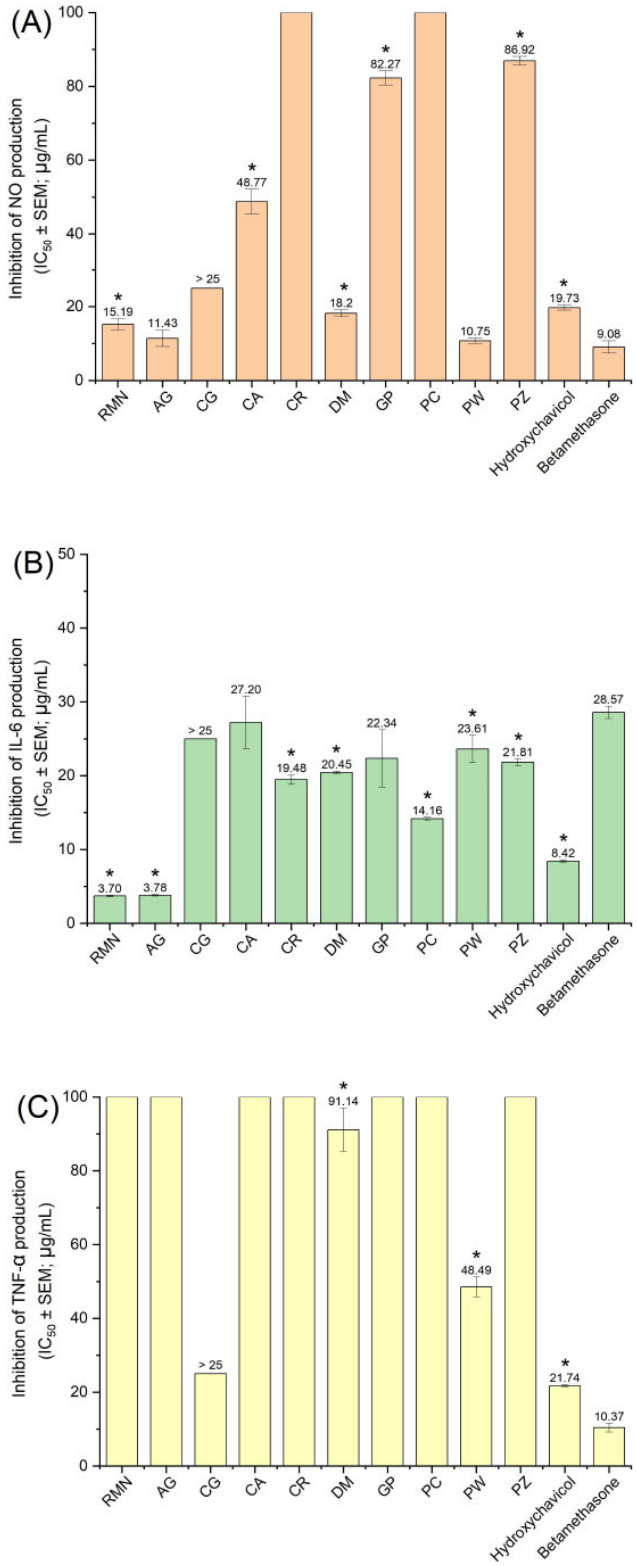

Fig. (1). Inhibitory effect of the RMN remedy and its plant ingredient extracts on NO (**A**), IL-6 (**B**), and TNF-α (C) production in LPS-stimulated RAW 264.7 cells (n = 3) (*: $p<0.05$ *vs* Betamethasone). Note: The maximum concentration of CG extract that showed non-toxic on RAW264.7 cells was 25 µg/mL. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

Table 2. Inhibitory effect of the RMN remedy and its plant ingredient extracts on β-hexosaminidase release from RBL-2H3 cells (*n*=3) (*: *p*<0.05 vs diphenhydramine).

Sample	% Inhibition on β-hexosaminidase Release at various concentrations (μg/mL)				IC_{50} (μg/mL) [Mean ± SEM]
	1	10	50	100	
RMN	-	-	-	44.41 ± 0.37	> 100
AG	-3.13 ± 1.38	9.16 ± 3.82	71.44 ± 0.66	91.91 ± 4.96	34.45 ± 1.98*
CG	-	-	-	6.71 ± 3.78	> 100
CA	-	-	-	19.98 ± 2.48	> 100
CR	-	-	-	24.25 ± 9.56	> 100
DM	-	-	-	21.68 ± 3.27	> 100
GP	-	-	-	17.34 ± 7.42	> 100
PC	-	-	-	38.25 ± 1.45	> 100
PW	-2.80 ± 0.72	35.20 ± 0.83	72.69 ± 1.14	94.83 ± 1.86	14.79 ± 0.34*
PZ	-	-	-	17.91 ± 4.90	> 100
Hydroxychavicol	-1.92 ± 3.15	36.37 ± 2.01	68.34 ± 1.67	86.72 ± 2.41	14.40 ± 0.56*
Diphenhydramine	-3.23 ± 0.62	7.21 ± 2.70	50.63 ± 0.94	86.35 ± 1.52	48.91 ± 1.38

dient extracts also exhibited high IL-6 inhibitory activity (IC_{50} values ranging from 3.78 ± 0.11 to 27.20 ± 3.57 μg/mL). However, this was not the case for GC extract (IC_{50}: > 25 μg/mL), which showed toxicity to RAW 264.7 cells at a concentration over 25 μg/mL (data not shown).

By contrast, the RMN remedy and some plant ingredient extracts did not affect TNF-α production at all tested concentrations, except PW and DM extracts (IC_{50}: 48.49 ± 2.74 μg/mL for PW and 91.14 ± 5.89 μg/mL for DM), which exerted a limited inhibitory effect on TNF-α production, as shown in Fig. (**1C**). Meanwhile, hydroxychavicol (IC_{50}: 21.74 ± 0.30 μg/mL) could suppress TNF-α production, but slightly lower than betamethasone (IC_{50}: 10.37 ± 1.18 μg/mL).

3.2. Effect of RMN Remedy and its Plant Ingredient Extracts on Inhibition of β-hexosaminidase Release from RBL-2H3 Cells

Among all the tested samples, PW extract (IC_{50}: 14.79 ± 0.34 μg/mL) exhibited the most potent antiallergy activity in a dose-dependent manner, followed by AG extract (IC_{50}: 34.45 ± 1.98 μg/mL), as shown in Table **2**. In addition, hydroxychavicol also had high antiallergy activity similar to PW (IC_{50}: 14.40 ± 0.56 μg/mL), which is better than diphenhydramine (IC_{50}: 48.91 ± 1.38 μg/mL). However, the RMN extract (% inhibition at 100 μg/mL: 44.41 ± 0.37%) only exerted a limited effect on the inhibition of β-hexosaminidase release from RBL-2H3 cells, as well as other plant components (% inhibition at 100 μg/mL ranging from 6.71 to 38.25%).

3.3. Antimicrobial Effect of RMN Remedy and its Plant Ingredient Extracts

The RMN and some plant ingredient extracts displayed antibacterial activity against all tested bacteria, as shown in Table **3**. The results revealed that the RMN extract could inhibit three Gram-positive bacteria, including *S. epidermis* (MIC and MBC values of 5 mg/mL), *S. aureus* (MIC and MBC values of 5 mg/mL), and MRSA (MIC values of 2.5 mg/mL and MBC values of 5 mg/mL). Among all the ingredient extracts, PW extract exhibited the highest antibacterial activity against all Gram-positive bacteria (MIC and MBC values of 0.625 mg/mL). Meanwhile, AG and DA extracts also had an effect against Gram-positive bacteria (MIC and MBC values about 2.5–5 mg/mL). For Gram-negative bacteria, only PW extract possessed antibacterial activity against *P. aeruginosa* with the same MIC and MBC values of 2.5 mg/mL. Moreover, hydroxychavicol also exhibited a potent antibacterial effect against all tested bacterial strains (MIC and MBC values about 0.312–2.5 mg/mL), similar to PW extract. However, positive antibacterial drugs appeared to be more susceptible to all tested bacteria than these extracts.

3.4. Quantitative Analysis of Hydroxychavicol in RMN Remedy Extract

It is seen from the HPLC chromatograms that hydroxychavicol is a major constituent of the RMN extract (19.88 ± 0.20 mg/g of extract), as shown in Fig. (**2**). This result supported our previous data that hydroxychavicol is derived from *Piper wallichii* (122.93 ± 1.04 mg/g of extract).

3.5. Stability of RMN Remedy Extract

Due to the promising anti-inflammatory activity of RMN extract, it was selected for stability assessment under accelerated conditions. The RMN extract displayed moderate stability when stored at high temperature (40 ± 2 °C) and humidity (75 ± 5%), as shown in Figs. (**3** and **4**). The inhibitory effect on NO production of RMN extract appeared to be decreased due to a significant increase of its IC_{50} value during the experiment (from 22.88 ± 1.47 μg/mL at Day 0 to 63.32 ± 0.19 μg/mL at Day 180). In terms of bioactive constituent, the amount of hydroxychavicol in

Table 3. The MIC and MBC values (mg/mL) of the RMN remedy and its plant ingredient extracts against four species of bacteria.

Sample	*S. Epidermis*		*S. Aureus*		MRSA		*P. Aeruginosa*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
RMN	5	5	5	5	2.5	5	>5	>5
AG	2.5	5	5	5	5	5	>5	>5
CG	>5	>5	>5	>5	>5	>5	>5	>5
CA	>5	>5	>5	>5	>5	>5	>5	>5
CR	>5	>5	>5	>5	>5	>5	>5	>5
DM	2.5	2.5	5	5	2.5	2.5	>5	>5
GP	>5	>5	>5	>5	>5	>5	>5	>5
PC	>5	>5	>5	>5	>5	>5	>5	>5
PW	0.625	0.625	0.625	0.625	0.625	0.625	2.5	2.5
PZ	>5	>5	>5	>5	>5	>5	>5	>5
Hydroxychavicol	0.312	0.312	0.312	0.312	0.312	0.312	1.25	2.5
Ampicillin	0.00625	0.0125	0.0015625	0.0015625	-	-	-	-
Vancomycin	-	-	-	-	0.0001953	0.0003906	-	-
Norfloxacin	-	-	-	-	-	-	0.0000976	0.0003906

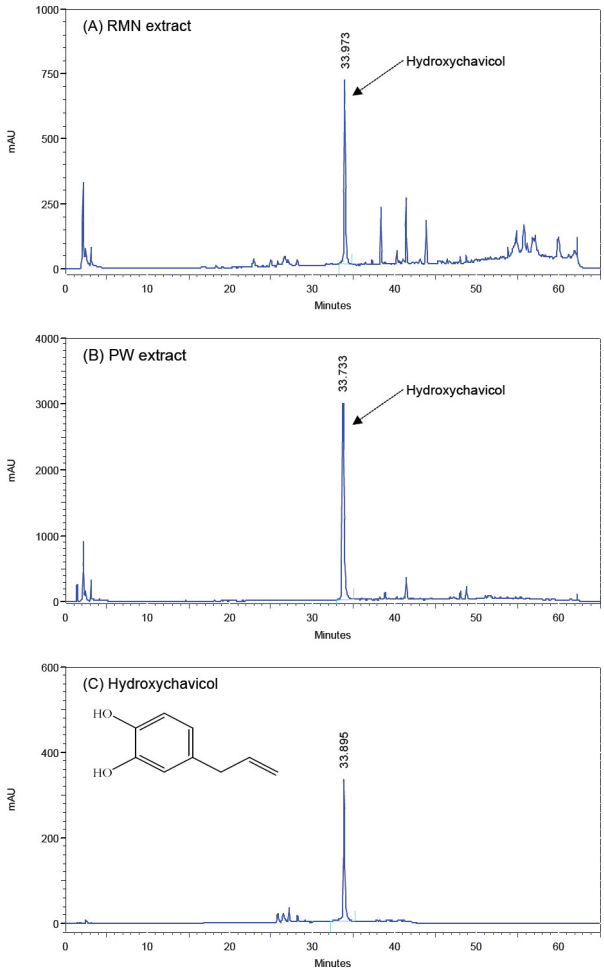

Fig. (2). HPLC chromatograms of RMN (**A**) and PW (**B**) extracts at 10 µg/mL, and hydroxychavicol (C) at 200 µg/mL. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

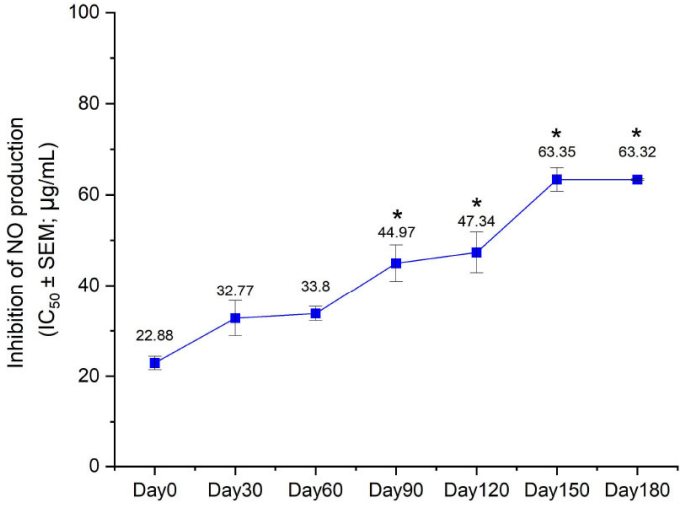

Fig. (3). Inhibitory effect of the RMN extract on NO production after storage under accelerated conditions for 6 months (n = 3) (*: $p<0.05$ vs Day0). *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

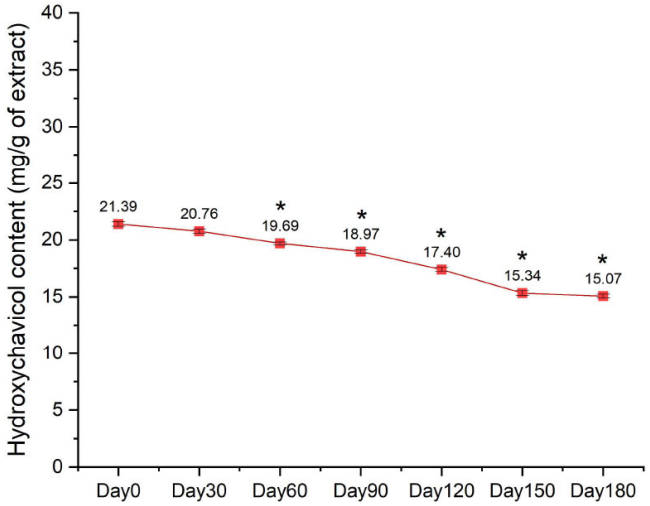

Fig. (4). The amount of hydroxychavicol remained in the RMN extract after storage under accelerated conditions for 6 months (n = 3) (*: $p<0.05$ vs Day0). *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

RMN extract was also found to decrease over time (from 21.39 ± 0.20 mg/g of extract at Day 0 to 15.07 ± 0.17 mg/g of extract at Day 180). These results indicated that storage conditions significantly impacted both anti-inflammatory activity and the bioactive constituent of RMN extract.

4. DISCUSSION

Dermatitis is an inflammatory skin condition associated with dermatologic barrier dysfunction and inflammatory processes in the epidermis and dermis. Commonly, dermatitis may be entirely endogenous (constitutional) or exogenous (contact) and also aggravated by the presence of pathogens such as *S. epidermidis* and *S. aureus* [1]. It is well established that NO is one of the key mediators associated with the progression of acute and chronic cutaneous inflammations. In both atopic dermatitis and allergic contact dermatitis, overproduction of NO is found in the dermis due to high expression levels of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) [4]. NO could affect several biological phenomena during the development of skin inflammation. For example, this molecule can cause inflammatory infiltration and skin edema, resulting from increased vascular permeation and leukocyte migration [19]. Moreover, it can stimulate pro-inflammatory cytokine production, such as IL-6 and TNF-α, which also play crucial roles in mediating the progression of inflammation in the skin [3]. Therefore, inhibiting the production of these pro-inflammatory cytokines may reduce inflammation in dermatitis.

In this study, to our knowledge, we have demonstrated for the first time that the RMN remedy extract could inhibit some pro-inflammatory cytokines related to skin inflammation. The RMN extract and its major constituent, hydroxychavicol, have shown a promising anti-inflammatory effect, as evidenced by similarly inhibited NO production compared to betamethasone. Regarding specific plant ingredients, PW, AG, and DM extracts displayed outstanding inhibitory effects on NO production. Furthermore, the inhibitory effect on IL-6 production of the RMN extract and hydroxychavicol is even better than positive control by 7.7-fold and 3.4-fold, respectively. Likewise, most plant ingredient extracts were shown to be good inhibitors against IL-6 production. Their activities are similar to or more potent than the standard drug, especially AG extract (7.6-fold higher than betamethasone). However, only PW extract and hydroxychavicol have been shown to inhibit TNF-α production, unlike the RMN and other plant ingredient extracts, whose activity did not affect the tested concentration.

The anti-inflammatory activity of some plant ingredients in the RMN remedy has previously been investigated using various experimental models with similar outcomes compared to our result. For instance, Matsuda and colleagues reported the inhibitory activity on LPS-stimulated NO production of *Alpinia officinarum* extracted with 80% aqueous acetone with the IC_{50} value of 35 μg/mL [9]. Besides, it was found that a diarylheptanoid compound, 7-(4′-hydroxy-3′-methoxyphenyl)-1-phenylhept-4-en-3-one (HMP), isolated from *Alpinia officinarum* rhizome (at a tested concentration of 25 μM), significantly inhibited NO, IL-1, and TNF-α production in LPS-stimulated RAW 264.7 murine macrophages model by 50.35, 84.91, and 43.16%, respectively [8]. In another work, Yang and colleagues isolated compounds from the leaves of DA (daturafolisides A, daturafolisides B, daturafolisides C, daturafolisides D, daturafolisides F, and daturataurin B) and found that these compounds displayed inhibitory effects on NO production in RAW 264.7 cells stimulated by LPS with IC_{50} values in the range of 17.7 to 71.2 μM [10]. The same research group also studied the anti-inflammatory activity of the semi-purified extract of DA leaves (52 and 104 mg/kg body weight for 7 days) using an imiquimod-induced psoriasis-like dermatitis mouse model and found that it could inhibit several inflammatory cytokines production, including IL-1β, IL-2, IL-6, IL-10, IL-12, IL-17, IL-22, IL-23, TNF-α, monocyte chemotactic protein 1 (MCP-1) and interferon-γ (IFN-γ) [13]. Hossen and colleagues also reported the inhibitory activity of PC extracted with methanol. They found that the extract at a concentration of 300 μg/mL could reduce IL-1β, IL-6, and TNF-α production in the LPS-stimulated RAW 264.7 murine macrophage model [11].

An allergen or irritant (*e.g.*, pollen, pet dander, food, cosmetics, fragrance, detergents, drugs, and dust mites) is one of the factors that cause dermatitis. Frequent exposure to these substances triggers an allergic response, causing the degranulation of basophils and mast cells, followed by the release of pro-inflammatory and vasoactive mediators (*e.g.*, β-hexosaminidase, histamine, prostaglandins, and leukotrienes) [20]. Our results indicated that PW and AG extracts were the only two plant ingredients that exhibited a strong antiallergic effect against the degranulation of RBL-2H3 cells, better than the standard drug (diphenhydramine). Likewise, hydroxychavicol also exhibited potent antiallergy activity similar to the extract, suggesting that it might be an active compound representing the antiallergy activity of PW extract. Unfortunately, the RMN extract showed a limited inhibition on the degranulation of RBL-2H3 cells, which may be due to low levels of bioactive compounds, especially hydroxychavicol, to exert the desired effect. A similar outcome of AG was also reported by Matsuda and colleagues, who revealed that the 80% aqueous acetone extract of AG rhizome extract decreased degranulation by inhibiting the release of β-hexosaminidase from RBL-2H3 cells with an IC_{50} value of 19 μg/mL. Besides, two isolated compounds from the extract, 10′S-1′-acetoxychavicol acetate, and 10′S-1′-acetoxyeugenol acetate, exhibited potent inhibitory activity with IC_{50} values of 15 and 19 mM, respectively. In a mouse model, they also found that these isolated compounds could inhibit the release mediated by passive skin anaphylaxis reactions [7]. However, to our knowledge, this is the first report on the antiallergy activity of PW. We could not find any studies reporting its activity to allow a comparison with our findings.

Another factor that causes dermatitis is a human pathogenic bacterium. Several studies have reported that the pathogenesis of dermatitis is correlated with some bacterial infections such as *S. epidermidis*, *S. aureus*, MRSA, and *P. aeruginosa*. These pathogens can promote inflammation and stimulate the immune system, leading to a fast progression and severity of the disease [21-23]. Our experiment demonstrated that PW extract and hydroxychavicol could efficiently inhibit all the bacterial species tested. Furthermore, their bactericidal effect was the most pronounced in Gram-positive bacteria, including *S. epidermidis*, *S. aureus,* and MRSA, whose bacteria are commonly colonized on the skin lesions of patients with mild to moderate eczema and atopic dermatitis [24]. A similar finding was also reported by Nongmai and colleagues [25]. In their study, the crude n-hexane, ethyl acetate, and methanol extracts from the stems and leaves of PW possessed antibacterial activity against *S. aureus* with the same MIC values of 1.28 mg/mL. In addition, it was reported that hydroxychavicol exhibited a time- and concentration-dependent killing effect against *Escherichia coli* [12]. The mechanism of hydroxychavicol influenced the antimicrobial properties was also investigated. Hydroxychavicol was found to induce bacterial cell death through superoxide generation, which finally causes oxidative DNA damage [26]. In other studies, hydroxychavicol has been reported to disrupt the cell membrane integrity and biofilm growth generated by *Trichophyton mentagrophytes* and *Candida parapsilosis* [27]. Furthermore, our results showed that the RMN, AG, and DA extracts could inhibit all tested Gram-positive

bacteria, but their effect appears weaker than the PW extract. Similarly, it was shown that the methanolic extract of DM leaves at a concentration of 100 mg/mL exhibited antimicrobial activity against *S. aureus*, *Steptococcus salivarious,* and *Steptococcus mutans,* with the zone of inhibition varying from 10–12 mm [28]. Likewise, the extract from AG has been reported to inhibit many types of pathogens, including *S. aureus*, *P. aeruginosa*, *S. epidermis*, *E. coli*, *Salmonella typhimurium*, and *Klebsiella pneumoniae,* with MIC and MBC values ranging from 0.04–0.64 mg/mL and 0.08–2.56 mg/mL, respectively, slightly lower than those obtained in our study [29-31].

The amount of a bioactive compound is an important parameter that affects the biological activity of medicinal plants and preparations. This parameter relies on several factors, for example, the genetic profile of plants, growth environmental conditions, phenological stage, parts used, harvesting processes, and extraction procedures [32, 33]. As expected, our HPLC results indicated that hydroxychavicol is a major constituent of the RMN extract, supporting our hypothesis that it is derived from PW. Also, the strong anti-allergic and antimicrobial activities of PW extract might be explained by the fact that it contained a high content of hydroxychavicol, which was respectively 6.2-fold higher than that observed in the RMN extract. This result confirms the influence of hydroxychavicol on the biological activities of RMN remedy. Similarly, Zamakshshari and colleagues suggested that the inhibitory effect of *Piper betle*leaf extract against Gram-positive bacteria correlates with hydroxychavicol content [34].

The stability of herbal drugs and products is another essential factor that affects their quality and safety. Several drug regulatory agencies like ASEAN suggest that it is necessary to carry out proper stability testing on plant extracts (as a drug substance) and herbal products. This assessment helps to understand better how phytochemicals and bioactivities of herbal products change over time and establishes their shelf life and storage conditions [35, 36]. In this study, the RMN extract was found to be stable under accelerated storage conditions at 40 ± 2 ºC and 75 ± 5% RH for at least 2 months. After that, the level of hydroxychavicol in the extract significantly decreases each month, with 70% remaining after 6 months of storage. As for the inhibitory effect on NO production, the RMN extract exhibited a gradual increase in the IC_{50} value by about 3-fold compared to the initial value. This may be attributed to the decrease of hydroxychavicol, confirming the correlation between this phenolic compound and the bioactivity of the RMN remedy. Several factors have been reported to involve the degradation and instability of phenolic compounds, leading to a decrease in their biological activities. For example, Kaur and colleagues found that the total phenolic content of*Centella asiatica* extract was decreased when stored at high temperature (40 °C) and humidity (75% RH) [35].

Similarly, the degradation of phenolic compounds in grape cane extracts stored at 40 °C was faster than at room temperature (25 °C) and also catalyzed by light. Therefore, these could be the reasons for the instability of the RMN extract under accelerated storage conditions. By contrast, a study by Ali and colleagues revealed that about 95% of phenolic constituents in *P. betle*leaf extract were preserved when stored under low temperatures (5 °C in dark conditions for 180 days of storage), including its antioxidant activity. Among individual phenolic compounds in the extract, hydroxychavicol exhibited the best stability without degradation [37], thus emphasizing the crucial need for an appropriate storage condition for the RMN remedy.

CONCLUSION

To our knowledge, this current study is the first report to reveal the potential bioactivity related to skin inflammation diseases of the RMN remedy extracted with 95% ethanol, including anti-inflammatory and anti-bacterial properties. Among its plant components, PW extract displayed the highest capability compared to the others. Hydroxychavicol, derived from PW, was found to be the bioactive compound of this remedy, demonstrating potent inhibitory activity on NO and IL-6 productions. Therefore, these promising results support the relationship between taste theory and the ethnopharmacological use of the RMN remedy to treat dermatitis. In addition, it should be noted that storage at low temperatures in dark conditions may be suitable for the RMN remedy as it is sensitive to high temperatures and humidity. Further investigations related to the pathogenesis of dermatitis are also recommended, including preclinical and clinical studies.

LIST OF ABBREVIATIONS

RMN = Ruean-Mhoon-Nok remedy

TTM = Thai Traditional Medicine

NO = Nitric Oxide

IL-6 = Interleukin-6

TNF-α = Tumor Necrosis Factor-alpha

HPLC = High-performance Liquid Chromatography

IC_{50} = Inhibition Concentration at 50%

MIC = Minimum Inhibitory Concentration

MBC = Minimum Bactericidal Concentration

AUTHORS' CONTRIBUTION

It is hereby acknowledged that all authors have accepted responsibility for the manuscript's content and consented to its submission. They have meticulously reviewed all results and unanimously approved the final version of the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data and supportive information are available within the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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