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Original Research Article

Anti-inflammatory and antiprotozoal effect of *Hedyotis diffusa* and *Scutellaria barbata*

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Abstract

Purpose: *To investigate the anti-inflammatory and antiprotozoal effects as well as chemical profile of ethanol extracts of Hedyotis diffusa (Rubiaceae) (HDE) and Scutellaria barbata (Lamiaceae) (SBE).*
Methods: *Dried whole plants collected in Vietnam were extracted with ethanol (30 and 96 %) by maceration for 4 weeks. The resulting extracts obtained after evaporating the solvent were stored in a refrigerator at 4 ^{o}C. Mouse carrageenan-induced inflammation and mouse macrophage cell line RAW264.7 were used to assess the in vivo and in vitro anti-inflammatory activities, respectively, while ciliate, Tetrahymena pyriformis was used to determine antiprotozoal effects. Ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF-MS) was employed to analyze the chemical profiles of the extracts.*
Results: *All the extracts manifested anti-inflammatory effects in vitro and in vivo. The HDE exhibited significantly higher in vitro anti-inflammatory activity than SBE ($p < 0.05$). Furthermore, in vivo anti-inflammatory activity was higher in SBE compared to HDE. Both extracts exhibited antiprotozoal effects. The 96 % ethanol extract of both plants were more active than the 30 % ethanol extracts. Analysis by UHPLC-Q-TOF-MS revealed the presence of chrysin, apigenin and apigenin derivatives, naringenin, wogonin, quercetin and quercetin derivatives, as well as scutellarin.*
Conclusion: *The HDE and SBE extracts from Vietnam exhibit significant in vivo and in vitro anti-inflammatory activities and in addition, antiprotozoal activity against Tetrahymena pyriformis. These plants, therefore, are potential sources of antiprotozoal agents.*

Keywords: *Anti-inflammatory activity, Antiprotozoal activity, Ethanol extract, Hedyotis diffusa, Scutellaria barbata*

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INTRODUCTION

Inflammation is a normal host defense response to tissue damage caused by multiple stimuli such as physical trauma, chemicals, and infectious agents. Non-steroidal anti-inflammatory, steroidal, and immunosuppressant drugs have been used to treat various forms of inflammation. However, these medicines may cause many side effects such as vasculitis, headache and

problems related to immunodeficiency. The acute inflammatory response to infection produced by different microorganisms including viruses, bacteria and protozoa is an essential component of the host defense reaction. In particular, protozoa cause chronic and persistent infections because they have evolved many mechanisms to evade and resist specific immunity, and natural immunity against protozoa is fairly weak [1]. Quite a number of infectious diseases are caused by protozoa.

Treatment of protozoa infections is limited in effectiveness and often causes additional serious side effects. In addition, therapeutic efficacy is inhibited by the development of drug resistance [2]. Toxicity of the drugs, difficulties in administration, duration of treatment, and low effectiveness have promoted the study of new antiprotozoal agents.

In Vietnam, *Hedyotis diffusa* (family Rubiaceae) (HD) and *Scutellaria barbata* (Lamiaceae family) (SB) are used to treat multiple diseases including hepatitis, liver cancer, tonsillitis, acute pharyngitis, trauma, venomous snakebite, and tumors [3]. However, evaluation of the phytochemical profile and anti-inflammatory as well as antiprotozoal effects of these two medicinal herbs have not been studied. However, secondary metabolites of both HD and SB from different regions have been studied and several chemicals characterized [4,5]. Also, differences in chemical constituents at different locations have been reported [6,7]. Recently ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF-MS) became the method of choice for the determination of chemical components in biological materials, due to its great speed, efficiency, and resolution [8]. Therefore, UHPLC-Q-TOF-MS was used for the analysis of the phytochemicals from HD and SB extracts. This study therefore investigates the anti-inflammatory and antiprotozoal effects along with a phytochemical profile based on liquid chromatography with tandem mass spectrometry (LC/MS/MS) of HDE and SBE.

EXPERIMENTAL

Plant materials

Whole HD and SB plants were collected from Can Tho and Thanh Hoa provinces in Vietnam, in October 2020, and authenticated by Dr Luu Hong Truong. A voucher specimen (no. 015842) was deposited in the herbarium of Institute of Applied Materials Science (VAST).

Preparation of the extract

After harvesting, wilted leaves and waterlogged parts were removed. Then, the whole plants were dried in the oven at 60 ºC, ground into powder and extracted at room temperature with 30 and 96 % ethanol for 4 weeks as earlier described by Tin *et al* [9]. The solvent was removed on a rotary evaporator after filtration, and the resulting extracts were stored at 4 °C until use.

Anti-inflammatory activity

Cell culture

The RAW264.7 murine macrophage cell line (American Type Culture Collection) were cultivated in a humidified incubator with 5 % CO_2 at 37 °C in Roswell Park Memorial Institute (RPMI) medium (Sigma-Aldrich Co, USA) with 10 % fetal bovine serum (FBS), Sigma-Aldrich Co, USA.

Cell viability

Cell viability was determined using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) test.

Nitric oxide (NO) production

The cells were seeded into 96-well plates at a density of 2 x 10^4 cells/well/100 μL. After incubation for 24 h, they were treated with extracts or 15 μM dexamethasone (Sigma-Aldrich Co., USA) for 1 h and activated by lipopolysaccharide (LPS) (Sigma-Aldrich Co., USA) at 1 μg/mL. The level of NO in the culture supernatant was measured using Griess kit (Sigma-Aldrich Co., USA) according to manufacturer's instructions.

Mouse model of inflammation

Male Swiss albino mice were housed under standard conditions (23 ± 2 ºC and 12 h light-dark cycle) in plastic containers for two days to acclimatize in the laboratory. During this period, the animals were fed with normal feeds and water.

All animal procedures were approved by the Institutional Animal Care and Use Committee of the Institute of Applied Materials Science VAST (protocol no. 01/HDKH), and were cared for in compliance with the internationally accepted guide for the care and use of laboratory animals, published by the US National Institutes of Health [10].

Groups and treatments

Mice were preliminarily grouped (8 – 10 mice/group) according to body weight and paw volume (Table 1). In all groups, extracts and drug solutions were administered orally (0.1 mL/10 g) at the same time once daily for 6 consecutive days. Distilled water was used as negative control and diclofenac (5 mg/kg) as positive control. Study groups received HBE and SBE at 250 and 500 mg/kg (Table 1). The initial volume (V_o) of the mouse paw was measured. Thereafter, 1 % carrageenan suspension (0.025 mL) was administered into the left hind 1 h and 24 h after extract/drug administration. Paw edema was recorded 1, 3, 5, and 24 h after carrageenan injection (V_s). The change in paw volume (X) expressed as a percentage (%) was calculated using Eq 1.

$$X\ (\%) = ((V_s – V_o)/V_o)100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ (1)$$

Table 1: Groups of mice used in this study

Group	Drug received
SBE96/500	96% ethanol extract of *S. barbata* at dose of 500 mg/kg
SBE96/250	96% ethanol extract of *S. barbata* at dose of 250 mg/kg
SBE30/500	30% ethanol extract of *S. barbata* at dose of 500 mg/kg
SBE30/250	30% ethanol extract of *S. barbata* at dose of 250 mg/kg
HDE96/500	96% ethanol extract of *H, diffusa* at dose of 500 mg/kg
HDE96/250	96% ethanol extract of *H. diffusa* at dose of 250 mg/kg
HDE30/500	30% ethanol extract of *H. diffusa* at dose of 500 mg/kg
HDE30/250	30% ethanol extract of *H. diffusa* at dose of 250 mg/kg

Antiprotozoal activity

Protozoa culture

Tetrahymena pyriformis (*T. pyriformis*) (strain WH14) was obtained from the collection of the Research Institute of Veterinary Hygiene and Ecology (Moscow, Russia). Cells were grown in a sterile medium containing 0.5 % glucose, 0.5 % casein, 0.1 % Springer yeast extract 0251 and 0.1 % NaCl. Protozoa were inoculated into the medium and incubated for 4 - 5 days at 25 °C. The obtained protozoal culture was used for toxicity studies.

Analysis of antiprotozoal activity

Stock solutions for analysis of antiprotozoal activity were prepared by dissolution of extracts in either 30 or 96 % ethanol at 1 g/mL. For biological activity, the samples were cooled to 6 °C and centrifuged for 3 min at 3000 rpm. Toxicity of plant extracts to *T. pyriformis* was determined using a BioLaT-3.2 instrument [11]. Solution of extract (5 – 50 µL) were added to the protozoa in the instrument wells. Survived moving cells were counted each minute using the AutoCiliataXP program. Each extract concentration was tested in 3 wells (triplicate). The dependences of the survived cell number on time were measured. For a short-term experiment (about 1 h), 2000 to 4000 cells were taken. To carry out a long-term study, extract solutions (290 µL) were added to the wells of the BioLat-3.2 plate, the program AutoCiliataXP was started, and the plate with samples was installed in the device. The program evaluated the wells with samples without ciliates, then, a ciliate suspension (500 - 1000 cells) was added to wells of the total sample (300 µL). Each measurement was done in triplicate. After counting the cells ten times, the samples were installed in a thermostat and number of surviving ciliates was measured after 24 h. Survival rates (K) were calculated using Eq 2.

$$K = N_{24}/N_0 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ (2)$$

where N_{24} is the number of surviving ciliates in the sample after 24 h incubation, N_0 is the number of living ciliates in the sample at the start of study. Distilled water was used in control experiments, where the survival rate ranged from 2 to 2.5.

Chemical composition of extracts

The instrument used consisted of ExionLC™ UHPLC system and an X500R QTOF mass spectrometer. Chromatography was carried out on a Hypersil GOLD C18 column (150 x 2.1 mm, 3 µ). Mobile phase solvent A was water with 0.1 % formic acid, and solvent B was acetonitrile with 0.1 % formic acid. Flow rate was 0.4 mL/min. The following gradient was used: 0 – 1 min, 2 % B; 1 - 20 min, 2 - 98 % B; 20 - 25 min, 98 % B., 2 µL samples were applied for each analysis [8]. The negative electrospray ionization mode was applied for Q-TOF analysis with the following parameters: heater gas (45 psi), nebulizer gas (45 psi), curtain gas (30 psi), ion source temperature (500 °C). The mass range for TOF MS scan was m/z 70 – 2000. The mass range for TOF MS/MS scan was m/z 50 – 1500. For the negative mode, voltage of ion spray was −4.5 kV, the declustering potential was −70 V, collision energy was −20 eV, and the spread of collision energies was 10 eV. SCIEX OS software version

1.2.0.4122 was used to analyze the data obtained.

Statistical analysis

Data was analyzed using GraphPad Prism software version 7.00 (San Diego, USA). Values are presented as mean ± standard error of the mean (SEM) and one-way analysis of variance (ANOVA) was used for comparison. $P < 0.05$ was considered statistically significant.

RESULTS

In vitro anti-inflammatory activity

At concentrations higher than 150 µg/mL, the extracts (90 % ethanol) caused cell death, therefore, the lower extract concentrations were chosen for determination of the anti-inflammatory activity (25, 50 and 100 µg/mL, Figure 1).

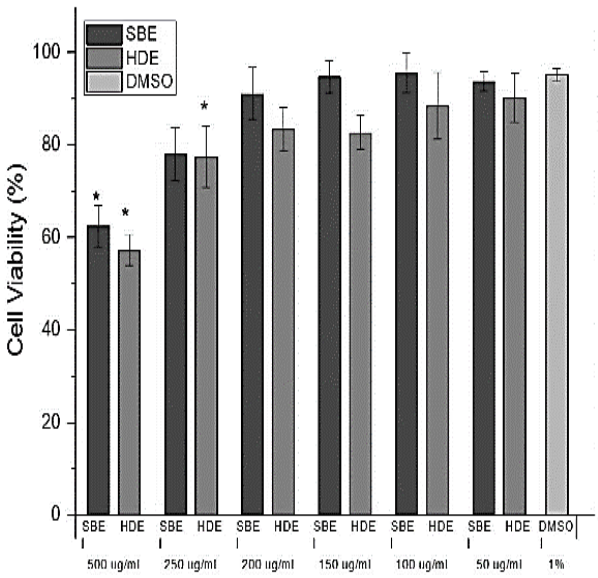

Figure 1: Toxicity of 96 % ethanol extracts. Values indicate mean ± SEM, n = 3. *$P < 0.05$ vs DMSO group

Nitric oxide production

The pro-inflammatory mediator, nitric oxide (NO) was used as a biomarker of inflammation and suppression of NO production indicated anti-inflammatory activity. It was found that after stimulation of the cells with LPS, concentration of NO increased (22.37 µM) by 87.63 % compared to unstimulated cells (11.9 µM). Dimethyl sulphoxide (DMSO) used as extract solvent had practically no effect on NO (11.6 µM). Application of both HD and SB extracts significantly inhibited NO production ($p < 0.05$) compared to LPS group, the best inhibition being observed at 100 µg/mL (Figure 2).

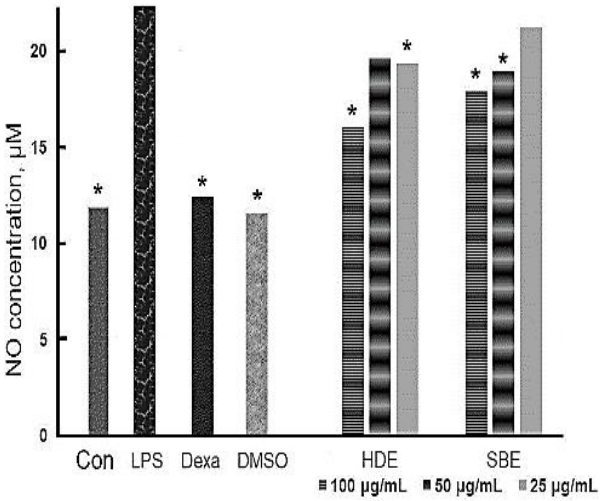

Figure 2: Effects of 96 % ethanol extracts on NO production by LPS-stimulated RAW 264.7 cells. SBE – cells treated with 96 % ethanol extract of *S. barbata*, HDE – cells treated with 96 % ethanol extract of *H. diffusa*; Con – untreated cells, LPS – cells treated with LPS only, Dexa – cells treated with 15 µM dexamethasone, DMSO – cells treated with DMSO. Values indicate mean ± SEM (n = 3). *$P < 0.05$ vs LPS group

In vivo anti-inflammatory activity

The extract effects on carrageenan-induced paw edema in mice were studied (Table 2). In this model, both 30 and 96 % ethanol extracts were investigated. Diclofenac (5 mg/kg) significantly reduced paw edema at 1, 3, 5, and 24 h. Similarly, both HDE and SBE also significantly reduced paw edema resulting from carrageenan stimulation compared to control ($p < 0.05$). However, HDE96/250 at 3 and 5 h as well as HDE30/250 at 3 h showed no significant reduction compared to control. The highest anti-inflammatory activity was demonstrated by SBE96/500 at 24 h after carrageenan application. In general, SB extracts were slightly more active than those of HD.

Antiprotozoal activity

This study showed that 30 % HDE at 1 h incubation showed practically no activity (Figure 3 A), while all cells were killed within 30 mins by 30 % SBE at 10 mg/mL (Figure 3 B). However, 96 % ethanol extracts of both plants were more active since all the infusoria were killed within 5 - 10 min at 1.25 mg/mL. After 24 h, the 96 % ethanol extract of *H. diffusa* was the most active; it killed all the cells at concentration of 2.5 mg/mL (Figure 4).

Table 2: The effect of extracts on paw edema

Group	Mouse paw edema (%)			
	1 h	3 h	5 h	24 h
Control (none)	52.88±3.48	69.64±3.43	72.54±4.72	63.88±3.48
Diclofenac (5mg/kg)	35.81±3.72*	46.07±2.33*	50.35±2.61*	19.22 ±2.16*&
SBE96/500	31.04±4.69*	48.24±5.61*	46.09±5.63*	18.45±4.48*&
SBE96/250	36.47±3.05*	52.17±2.78*	48.49±2.2*	25.85 ± 1.97*
SBE30/500	29.60±1.99*	53.69±4.97*	45.55±3.87*	21.79 ±1.85*&
SBE30/250	33.48±2.47*	56.38±2.85*	47.80±2.14*	24.95 ±2.67*&
HDE96/500	30.27±4.52*	56.27±4.73*	46.56±2.8*	20.03 ±2.19*&
HDE96/250	37.70±4.05**	69.73±6.03[a]	63.82±6.54	42.23±5.22*[a]
HDE30/500	29.74±3.93*	55.96±4.37*	42.83±3.88*#	25.68 ± 6.64*
HDE30/250	34.56±1.9*	60.25±4.38	50.00±4.59*	23.31±3.16*&

Note: *$P < 0.05$ vs control at the corresponding time, [a]$p < 0.05$ vs diclofenac at the corresponding time, [#]$p < 0.05$ vs HDE96/250 at 5 h, [&]$p < 0.05$ vs HDE96/250 at 24 h

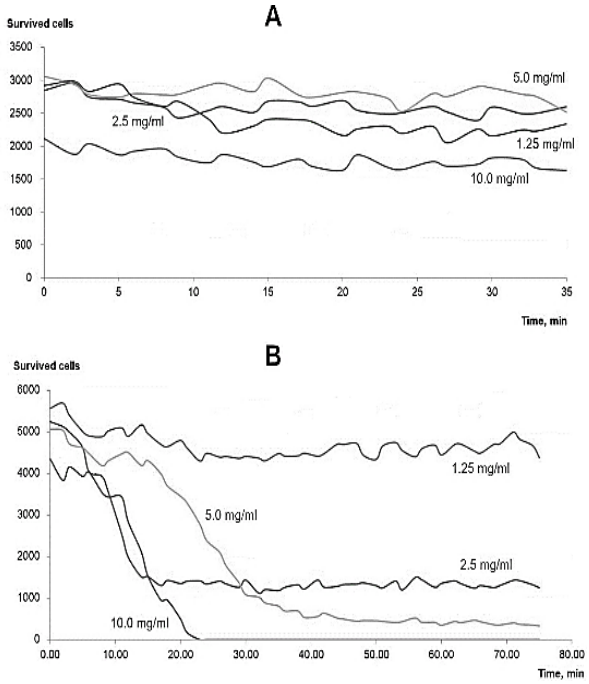

Figure 3: Toxicity of 30 % ethanol extracts on Tetrahymena pyriformis. A: H. diffusa, B: S. barbata. The 96 % ethanol extract of *H. diffusa* was the most active, killing all the cells at 2.5 mg/mL in 24 h. Thus, these experiments demonstrated significant antiprotozoal activity of both plant extracts

Chemical profile of extracts

Chemical profiling revealed the presence of chrysin, apigenin and apigenin derivatives, naringenin, wogonin, quercetin and quercetin derivatives as well as scutellarin (Table 3).

DISCUSSION

Plants are the most abundant natural source for search and development of new drugs with varying effects, and the search for newer molecules is unending. This study therefore investigated the anti-inflammatory and antiprotozoal activities for ethanol extracts of *H. diffusa* and *S. barbata* from Vietnam. Recently, the antiprotozoal activity for several plants from Vietnam had been studied [11]. Results of the present study are consistent with previous publication on *in vitro* anti-inflammatory effects of *S. barbata* samples from Taiwan [12] and mainland China [13] as well as *H. diffusa* samples from Chinese Fujian [14] and Anhui [15] provinces. The data of the present work showed that these plants growing in Vietnam also possess such activity. Furthermore, Vietnamese *H. diffusa* and *S. barbata* possess anti-inflammatory activity *in vivo* earlier identified for these plants from China [14] in paw edema model. This result suggests that *H. diffusa* and *S. barbata* from Vietnam exhibit anti-inflammatory activity in same way as plants growing in areas very remote from Vietnam.

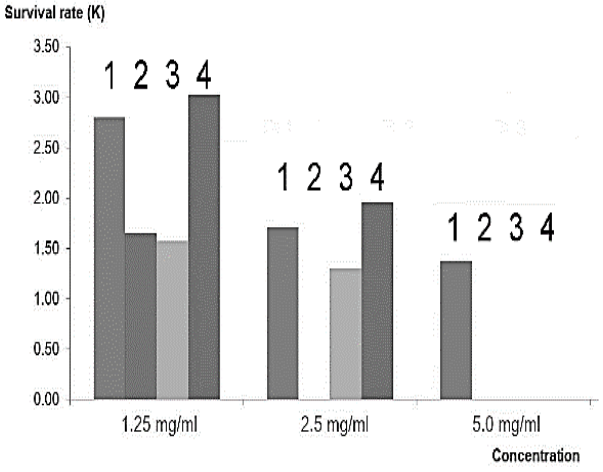

Figure 4: Activity of plant extracts at 24 h. (1) – 30% ethanol extract of *H. diffusa*; (2) – 96% ethanol extract of *H. diffusa*; (3) – 30% ethanol extract of *S. barbata* and (4) – 96% ethanol extract of *S. barbata*. Survival rate (K) is the ratio of the number of surviving ciliates after incubation for 24 h to the number of ciliates at the beginning of the experiment. Determinations were done in triplicate and coefficient of variation (CV) was not greater than 0.07, or 7 % of the mean for all extracts

Table 3: Chemical characterization of extracts by UHPLC-Q-TOF-MS

Compound	Pseudo-molecular ion, $(M-H)^-$	MS/MS fragments	96% ethanol extract	30% ethanol extract
Scutellaria barbata				
Chrysin	253.0521	143.0495; 63.0234	+	
Apigenin	269.0460	225.0546; 151.0026; 117,0333	+	
Naringenin	271.0607	151.0075; 119.0529; 107.0161; 177.0237	+	+
Wogonin	283.0627	268.0367; 163.0028	+	
Quercetin	301.0349	255.0286	+	+
Apigenin-7-O-β-D-glucopyranoside	431.0980	269.0439; 225.0554; 117.0325	+	
Quercetin-3-O-β-D-glucopyranoside	463.1029	301.0427; 174.9609		+
Scutellarin	461.0724	285.0389; 113.0238	+	
Hedyotis diffusa				
Scopoletin	191.0209	111.0079; 87.0076		+
Azelaic acid	187.0989	125,0961; 97.0654		+
1,3-Dihydroxy-2-methylanthraquinone	253.0598	238.0335; 210.0374	+	
5-Dehydroxykaempferol	269.0555	149,0288; 117.0366	+	
Isodonsesquitin A	285.2174	267.2034; 223.2129	+	
Fakalinediol	305.1768	121.0289		+
Scandoside	389.1098	165.0542; 121.0282; 209.0447		+
Geniposidic acid	419.1193	357.1173; 343.1015; 257.0659		+
Asperulosidic acid	431.1199	165.0566		+
Hesperidin	609.1823	193.0503;		+
Quercetin−3-sophoroside	625.1407	301.0344; 255.0287		+

This study further showed that *H. diffusa* and *S. barbata* possess antiprotozoal activity. Chemical compositions of the two medicinal herbs are similar to those previously reported [16,17]. The components of *H diffusa* extracts are flavonoids, triterpenoids, and iridoids. The chemical composition of *S. barbata* extracts consisted of mainly flavonoids. All extracts contain a set of compounds exhibiting anti-inflammatory and antiprotozoal activity. For example, scutellarin has earlier shown to exhibit anti-inflammatory, anti-neurotoxic, antioxidant, and apoptosis-inducing effects [18], while asperulosidic acid exhibited anti-inflammatory effects through inhibition of inflammatory mediators such as nitric oxide (NO), interleukin-6 (IL-6) and tumor necrotic factor-α (TNF-α) [19]. The compounds with antiprotozoal activity include naringenin, quercetin and other flavonoids as well as scopoletin and are present in HDE and SBE from Vietnam.

CONCLUSION

Hedyotis diffusa and *Scutellaria barbata* ethanol extracts exhibit *in vitro*, *in vivo* anti-inflammatory activity as well as antiprotozoal activity in the same way as the same samples from other geographic regions. Thus, these plants are potential sources of antiprotozoal drugs.

DECLARATIONS

Acknowledgement

None.

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Ethical approval

Institute of Applied Materials Science VAST Animal Care and Use Committee granted approval for this study (01/HDKH).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. PTNT, NTTT, AO, EC performed the pharmacological experiments and phytochemical characterization. YU, HNA, LTD, NCK provided biological material and prepared the manuscript. All authors read and approved the manuscript for publication.

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