Unlocking the Healing Power: Antioxidant, Anti-inflammatory, and Antibacterial Potentials of *Pavetta siamica* Bremek Leaves

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Summary

The aim of the present study was to evaluate the antioxidant, anti-inflammatory and antibacterial activities of *Pavetta siamica* Bremek leaves from the Rubiaceae family. The study also aimed to identify the phytochemical components responsible for these bioactivities. The result revealed that *n*-hexane extract showed variable activity against all tested bacteria which are *Staphylococcus aureus* (14.7 ± 0.4 mm), *Enterobacter aerogenes* (9.0 ± 1.0 mm), *Enterococcus faecalis* (10.0 ± 1.0 mm) and *Pseudomonas aeruginosa* (8.3 ± 0.4 mm). The antioxidant activity of all extracts showed significant differences between less polar and more polar extracts with total phenolic content calculated as gallic acid equivalents ($mg \cdot L^{-1}$), ranged from 704 ± 38.0 to 12480 ± 75.3 mg, whereas the radical scavenging activity was evaluated at 517 nm with a value ranging between 3.71% ± 0.13 to 90.11% ± 0.05. On the other hand, the anti-inflammatory activity of less polar extracts showed 100% inhibition at a constant dosage of 500 $\mu g' mL^{-1}$ in both anti-denaturation and anti-hyaluronidase assays. Phytochemical screening of extracts revealed the presence of flavonoids, phenols and tannins in both methanol and aqueous extracts while glycosides, terpenoids and steroids were found in less polar extracts.

Key words

Pavetta siamica Bremek, Rubiaceae; antioxidant, anti-inflammatory, antibacterial

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Introduction

The Rubiaceae, which consist mostly of flowering plants, contain at least 611 genera with more than 13,000 species of trees, shrubs and herbs. They are widely found in tropical regions around the world. *Pavetta* is one of the genera of flowering plants in the Rubiaceae family that includes about 360 species of both shrubs and evergreen shrubs. Typically, plants from this genus are found in scrub, grassland and sometimes in forests in sub-tropical and tropical areas of Asia and Africa. While many species have historically been employed for medical purposes, there much remains to be discovered about their prospective applications in the pharmaceutical business. Some *Pavetta* species are used in traditional medicine for their pharmacological qualities, which include anti-inflammatory, antioxidant, antibacterial and antidiabetic effects. Extracts from the roots and leaves of various *Pavetta* species have been utilised in African traditional medicine. The primary origins of these plants are in tropical and subtropical regions of Africa, but some of the species can also be found in Asia and Pacific Islands.

P. siamica is a flowering plant species found in Southeast Asia, specifically in Malaysia, Thailand, and Indonesia. In Thailand, it is known to be used in traditional medicine to cure a variety of diseases. Other names include Siam *Pavetta*, which is also known as *'Kaca Piring'* in Malaysia (Salleh et al., 2022). *P. siamica* is a threatened species due to habitat loss, overharvesting and due to human activities (Chantaranothai, 2021). This study is the latest in a series of studies from past findings on the volatile components of *P. siamica* leaves (Salleh et al., 2022).

The primary aim of this study is to assess the potential health benefits of *P. siamica* through bioactivity screening, focusing on its antioxidant, anti-inflammatory, and antibacterial properties. As demonstrated in several studies, these bioactive properties play a significant role in promoting healthy aging (Kumar et al., 2020), while also combating oxidative stress (Kumar et al., 2020), inflammation (Peng et al., 2017), and bacterial infections (Ding et al., 2018). Additionally, the study aims to identify the phytochemical components responsible for these bioactivities, which could provide further insights into its therapeutic potential. Several species from the *Pavetta* genus have proven to exhibit all three of these bioactivities. For example, *P. owariensis* (Baldé et al., 2015), *P. indica* (Thayyil and Muthu, 2018), and *P. crassicaulis* (Rao and Naika, 2017) possess remarkable antioxidant properties, comparable to those of standard ascorbic acid. Additionally, the anti-inflammatory properties of *P. owariensis* (Tahiri et al., 2022) have been found to inhibit enzymes responsible for inflammatory processes, further supporting its therapeutic potential.

Material and Methods

Plant Materials

The leaves of *P. siamica* were collected from Behrang, Perak and identified by Shamsul Khamis from Universiti Kebangsaan Malaysia (UKM), Selangor, Malaysia. The voucher specimen (SK146/19) was deposited at the Herbarium of Faculty of Science and Technology, UKM.

Plant Extraction

The dried powdered leaves (10.0g) of *P. siamica* underwent a cold extraction process using various polarities of solvents which are *n*-hexane, dichloromethane (CH_2Cl_2), methanol (CH_3OH) and distilled water (dis. H_2O) sequentially. *P. siamica* leaves were selected due to their availability, accessibility and well-known traditional medicinal uses. Following extraction, the obtained extracts were filtered, and the solvent was removed under pressure. These extracts were then preserved by freezing until they were ready to be used in the experiments. The percentage yields (w/w) of the leaves *P. siamica* were 0.60 g, 0.31 g, 0.80 g and 0.93 g, respectively.

Solvents and Chemicals

Analytical grade *n*-hexane, CH_2Cl_2 and CH_3OH used for extraction were purchased from QReC (Asia). Antioxidants: gallic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), bovine serum albumin (BSA), and tris-buffer saline (TBS) were obtained from Sigma-Aldrich (Merck, Malaysia). Antibacterial materil: nutrient agar (NA), nutrient broth (NB) and ciprofloxacin were purchased from Thermo Fisher Scientific. The bacteria used in this study were *S. aureus*, *E. faecalis*, *E. aerogenes* and *P. aeruginosa* refrigerated between 2 °C to 8 °C. All standard strains were obtained from the American Type Culture Collection (ATCC).

Antioxidant Activities

Total phenolic content (TPC) of all extracts was tested using established method (Singleton and Rossi, 1965) with modifications throughput microplate systems. Briefly, distilled water (0.1 mL) and 10% Folin-Ciocalteu reagent (0.1 mL) were added to each tested sample (extract and standard reference). The sample mixture was left for 3 to 5 min before 7.5% Na_2CO_3 (w/v) (0.1 mL) was added. A spectrophotometer with a wavelength of 765 nm was used to measure the absorbance of extract after 2h. The activity capacity of the extract was estimated using a gallic acid (GA) calibration curve. TPC in the sample was reported in terms of gallic acid equivalents (GAE) per unit weight, based on the concentration of gallic acid obtained from the calibration curve. The absorbance value of the sample was matched to the corresponding concentration of GA.

On the other hand, DPPH radical scavenging activities of extracts were determined according to the method (Blois, 1958) with minor modifications. Briefly, extracts with different concentrations: 200, 100, 50, 25 and 12.5 $mg{\cdot}mL^{-1}$ (50 mL) and green tea extract (with the same concentration) were mixed with different concentration of DPPH solution (50 mL) and ethanol (150 mL) in a 96-well microplate. The mixture was homogenized at 300 rpm for 15 s and left in the dark at room temperature for 30 min. A spectrophotometer with a wavelength of 520 nm was used to measure the absorbance of the mixed solution. Three independent experiments were performed for each experiment.

Anti-Inflammatory Activities

The anti-inflammatory activity of all extracts was evaluated using two *in vitro*-based assays which were protein denaturation inhibitory and hyaluronidase inhibitory. Protein denaturation

inhibitory assay was performed according to (Williams et al., 2008) with minor modifications. Briefly, a test solution (0.55 mL) consisting of plant extract (0.05 mL) and 1% w/v BSA (0.5 mL) was used in this assay. Prior to use, BSA was prepared in TBS and adjusted to pH 6.74 using glacial acetic acid. Diclofenac sodium was used as a standard drug. All tested extracts were dissolved in methanol to obtain stock solution (1.1 mg·mL^{-1}). These solutions were used to produce a final concentration of 100 µg·mL^{-1}. Reagent mixtures were prepared as follows: control solution consisting of BSA and methanol, product control solution of extract, test sample consisting of plant extract solution, standard solution consisting of diclofenac sodium (2000 µM) and blank solution. Each test tube was heated at a temperature of 72 °C for about 5 min and then cooled for up to 20 min. The UV-Vis Microplate Spectrophotometer (EPOCH 2) at a wavelength of 416 nm was used to determine the absorbance of the mixture.

Meanwhile, the hyaluronidase inhibitory effect was evaluated as the method described by Sigma protocol (Ling et al., 2003) with few modifications. A 1500-2000 U hyaluronidase in 20 mM sodium phosphate buffer (100 µL) was preincubated with the test sample (25 µL) in DMSO at 37 °C for 10 min. Later, the assay was started by adding hyaluronic acid (100 µL) and was incubated at 37 °C for another 45 min. Any undigested hyaluronic acid was collected using acid albumin solution (1 mL) as a precipitate. The absorbance of the reaction mixture was then measured at 600 nm after 10 min standing at room temperature, where the reference value for maximum inhibition was obtained from the absorbance in the absence of enzyme. The inhibitory activity of the test sample was determined by calculating the percentage of absorbance in the presence of the sample compared to that without the enzyme. Apigenin was used as a reference to validate the assay under identical experimental conditions. Compounds were tested at a maximum concentration of 5 x 10^2 µM in the final reaction mixture. The results were reported as the mean percentage inhibition ± the standard error of the mean (SEM), based on three independent experiments, each performed in triplicate.

Antibacterial Activities

The disc diffusion assay was used to investigate the antibacterial activities of four extracts. In this method petri plates containing 15 mL of nutrient agar medium were inoculated with 108 CFU·mL of each test bacterium: *Staphylococcus aureus*, *Enterobacter aerogenes*, *Enterococcus faecalis* and *Pseudomonas aeruginosa*. Whatman No.1 filter paper disc with the diameter of 6mm was impregnated with concentration of 250 mg·mL^{-1}. The test was conducted in three replicates and left 30 min at room temperature for compound diffusion then incubated 24h at 37 °C. A standard disc containing ciprofloxacin antibiotic drug (30 µg·disc^{-1}) was used as a positive control for antibacterial activity. Then, the antibacterial activity was observed through the presence of the clear inhibition zone by the bacteria around the disc.

Phytochemical Screening Methods

In this study, eight phytoconstituents which are flavonoids, alkaloids, saponins, phenols, tannins, glycosides, steroids and terpenoids were screened utilizing four leaves extracts.

The presence of alkaloids was tested using Mayer's test where 0.5 mL of leaves extract was treated with Mayer's reagent (mercuric-potassium iodide) to give a cream-coloured precipitate (Suresh et al., 2015). Meanwhile, for saponins, 1 mL of alcohol was diluted in 0.5 mL of extract. Then, the mixture was re-diluted to 20 mL of distilled water and shaken well for 15 min. Foam formation was observed (Suresh et al., 2015). For phenols and tannins, ferric chloride test was used, where 0.5 mL of extract, 0.5 mL of distilled water and 1 mL of $FeCl_3$ 5% or 10% solution were mixed. The formation of bluish black solution indicates the presence of phenol while the formation of a dark blue or greenish-black solution indicates the presence of tannin (Suresh et al., 2015). The Lieberman–Burchard reagent was used to detect glycosides. The extract (0.5 mL) was dissolved in chloroform (2 mL). Later, the resulting solution was treated with acetic acid, acetic anhydride and concentrated H_2SO_4 to give a deep green coloration (Suresh et al., 2015). The presence of flavonoids was screened using alkaline reagent test where 1 mL of extract was added with NaOH solution (2 mL) and a few drops of diluted HCl. A colour change from intense yellow to colourless when dilute acid is added indicates the presence of flavonoids (Junaid and Patil, 2020). Meanwhile, the Salkowski test was used to detect steroids where the extract (0.5 mL) was dissolved in chloroform (1 mL). When the resulting solution is treated with few drops of concentrated H_2SO_4, it turns red (Suresh et al., 2015). The Salkowski test can also be used to detect terpenoids, but after the addition of concentrated H_2SO_4, the mixture needs to be heated for at least 2 min to give a grey mixture (Yadav and Agarwala, 2011).

Results and Discussion

The sequential extraction yielded *n*-hexane extract (0.60 g, 6%), CH_2Cl_2 extract (0.31 g, 3%), CH_3OH extract (0.80 g, 8%) and aqueous extract (0.93 g, 9%). The plant material may include a considerable number of water-soluble polar compounds and comprise less non-polar or moderately polar chemical constituents. Table 1 summarizes the qualitative phytochemical contents for all four crude extracts. Methanol crude extract possesses the most promising constituents as it shows strong positive for flavonoids, moderate for phenols and tannins, as well as weak positive for saponins and terpenoids, while the least promising extract, *n*-hexane only shows positive results for glycosides and steroids.

Table 2 summarizes TPC of four tested extracts. The values, expressed as TPC mg GAE·100 g^{-1}, represent the mean of triplicate wells from duplicate experiments, with SEM < 15 % included. Methanol crude extract has the greatest TPC value at 12480 in mg GAE·100 g^{-1} ± 75.3 with SEM% of 0.35% followed by the aqueous crude extract at 7239 in mg GAE·100 g^{-1} ± 21.7 with SEM% of 0.17. Meanwhile, TPC value for the CH_2Cl_2 crude extract is equally high, coming in at 5010 GAE·100 g^{-1} ± 47.7 with SEM% of 0.55. The *n*-hexane crude extract, with a TPC value of 704 GAE·100 g^{-1} ± 38.0 with SEM% of 3.13 records the least activity.

To validate TPC assay, DPPH free radical scavenging activity for all four crude extracts was evaluated. Based on the results summarized in Table 3, the methanol crude extract again showed the highest antioxidant activity at 90.11% ± 0.05, followed by the aqueous crude extract at 52.46% ± 0.58.

Table 1. Phytochemical contents of *P. siamica* leaves extracts

Test	Method	Phytoconstituent	Crude Extract			
			n-Hex	CH_2Cl_2	CH_3OH	Aq
Mayer's	[14]	Alkaloids	-	-	-	-
Foam	[14]	Saponins	-	-	+1	-
Ferric Chloride	[14]	Phenols	-	-	+ 2	+ 3
	[14]	Tannins	-	-	+ 2	+ 1
Libermann's Burchard	[14]	Glycosides	+ 2	+ 3	-	-
Ammonia	[15]	Flavonoids	-	-	+ 3	+ 2
Salkowski	[14]	Steroids	+ 2	-	-	-
	[16]	Terpenoids	-	+ 3	+ 1	-

Note: +1 = Weak, +2 = Moderate, +3 = High

Table 2. Antioxidant assay of *P. siamica* leaves extracts using TPC assay

Extract	Total Phenolic Content (TPC) (mg GAE·100 g^{-1})
n-Hex	704 ± 38.0
CH_2Cl_2	5010 ± 47.7
CH_3OH	12480 ± 75.3
Aq	7239 ± 21.7

Table 3. DPPH Free Radical Scavenging Activity of extracts in percentage (%)

Extract	Concentration	DPPH Free Radical Scavenging Activity (%)
n-Hex	100 μg·mL^{-1}	3.71 ± 0.13
CH_2Cl_2	100 μg·mL^{-1}	21.46 ± 0.32
CH_3OH	100 μg·mL^{-1}	90.11 ± 0.05
Aq	100 μg·mL^{-1}	52.46± 0.58

Meanwhile, the CH_2Cl_2 crude extract showed 21.46% ± 0.32 inhibition, and the *n*-hexane crude extract had the lowest antioxidant activity at 3.71% ± 0.13.

The findings suggest that the methanol and aqueous crude extracts contain high levels of secondary metabolites and phytoconstituents with antioxidant properties. Flavonoids, phenolic acids and other polyphenolic substances may be among them. To identify individual chemicals and assess their bioactivity, further investigation and purification of these extracts are required.

Table 4 relates to the protein denaturation inhibitory assay of four crude extracts at a constant dosage of 500 μg·mL^{-1}. The mean inhibition (%) with SEM was calculated from triplicate measurements across three independent experiments. The study's positive control, diclofenac sodium is a well-known anti-inflammatory medication, which at a dosage of 636.26 μg/mL inhibited protein denaturation by 99.22%. The data showed that both *n*-hexane and CH_2Cl_2 crude extracts had 100% inhibition indicating a complete protein denaturation inhibition. On the other hand, the methanol crude extract showed absent inhibition, while the aqueous crude extract had a moderate anti-inflammatory action as evidenced by the 83.73% reduction of protein denaturation.

Table 4. Protein denaturation inhibition of samples in percentage (%)

Extract	Final Concentration (μg·mL^{-1})	Protein denaturation inhibition (% SEM)
n-Hex	500	100
CH_2Cl_2	500	100
CH_3OH	500	NA
Aq	500	83.73 ± 1.22
Diclofenac sodium*	636.26	99.22 ± 1.07

Note: NA (non-applicable); *: positive control

The amount of hyaluronidase enzyme inhibition taken by each crude extract at a concentration of 500 μg·mL^{-1} is shown in Table 5. Based on the results, it has been proven that hyaluronidase enzyme is completely inhibited by *n*-hexane, CH_2Cl_2, and aqueous crude extracts, showing substantial anti-inflammatory efficacy. On the other hand, the methanol crude extract demonstrates 89.78% inhibition of the hyaluronidase enzyme, indicating modest anti-inflammatory action. Meanwhile, the positive control apigenin, demonstrates 84.91% inhibition of hyaluronidase with a SEM of 2.24 at a dosage of 40.51 μg·mL^{-1}.

Table 5. Hyaluronidase Inhibition (%SEM) Results

Extract	Final Concentration (μg·mL^{-1})	Hyaluronidase inhibition (% SEM)
n-Hex	500	100
CH_2Cl_2	500	100
CH_3OH	500	89.78 9.00
Aq	500	100
Apigenin*	40.51	84.91 2.24

Note: *: positive control

Overall, the evidence shows strong anti-inflammatory activity of *n*-hexane and CH_2Cl_2 crude extract, which should be further studied as a prospective source of natural anti-inflammatory drugs. In addition, the inhibitory activity shown by the aqueous and methanolic extracts also needs to be refined because they may have anti-inflammatory bioactive substances.

Among the four extracts, only *n*-hexane extract showed inhibitory activity against all tested bacteria *S. aureus*, *E. faecalis*, *P. aeruginosa* and *E. aerogenes* (Table 6). The higher solubility of lipophilic chemicals in *n*-hexane compared to the aqueous extract may cause the *n*-hexane extract to have higher antibacterial activity. Bacterial cell membranes may be easier for lipophilic chemicals to reach and destroy more successfully.

Table 6. Antibacterial activities of four crude extracts using disc diffusion method (Mean ± SD)

Extract	Zone of inhibition (mm)			
	Gram-positive bacteria		Gram-negative bacteria	
	S. aureus	*E. faecalis*	*E. aerogenes*	*P. aeruginosa*
n-Hex	14.7 ± 0.4	10.0 ± 1.0	9.0 ± 1.0	8.3 ± 0.4
CH_2Cl_2	-	-	-	-
CH_3OH	-	-	-	-
Aq	8.7 ± 0.6	-	-	-
Ciprofloxacin	28.0 ± 0.2	28.0 ± 0.2	28.0 ± 0.2	28.0 ± 0.2

Note: *S. aureus* (ATCC 25923), *E. faecalis* (ATCC 29212), *E. aerogenes* (ATCC 13048), *P. aeruginosa* (ATCC 27853).

(-): No inhibition observed; SD: standard deviation

On the other hand, the decreased solubility of hydrophilic chemicals in aqueous extract may result in less access to the bacterial cell membrane and therefore less efficiency in disrupting it. Meanwhile, *S. aureus* is somewhat inhibited by the aqueous extract, while *E. aerogenes*, *P. aeruginosa*, and *E. faecalis* are unaffected. None of the four microorganisms examined were inhibited by the CH_2Cl_2 and methanol extracts.

Conclusion

In conclusion, the study has demonstrated that *P. siamica* Bremek leaves exhibit significant antioxidant, anti-inflammatory and antibacterial properties. The *n*-hexane extract showed notable antibacterial activity against a range of bacteria, including *S. aureus*, *E. faecalis*, *E. aerogenes*, and *P. aeruginosa*, with varying inhibition zones. The antioxidant activity varied significantly between less polar and more polar extracts, with higher total phenolic content and radical scavenging activity observed in the more polar extracts. The anti-inflammatory potential was particularly evident in the less polar extracts, which exhibited complete inhibition in both anti-denaturation and anti-hyaluronidase assays at a concentration of 500 μg/mL. Phytochemical screening identified the presence of key bioactive compounds, including flavonoids, phenols and tannins in methanol and aqueous extracts, while glycosides, terpenoids and steroids in less polar extracts. These findings suggest that *P. siamica* leaves possess promising bioactive properties that may contribute to their therapeutic potential, with the identified phytochemicals potentially playing a role in these activities. Further research is needed to isolate and identify specific compounds responsible for these bioactivities.

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CRediT Authorship Contribution Statement

Shamuntheswary Arumugam: Conceptualization, investigation and performed the experiments. **Nurul Syafiqah Rezali:** Original draft preparation, manuscript revision, funding acquisition and supervision of the study. **Zunoliza Abdullah:** Co-supervision. **Mazura Md Pisar:** Providing research and analysis facilities for antioxidant and anti-inflammatory assays. **Wan Mohd Nuzul Hakimi Wan Salleh:** Providing plant sample and manuscript editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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