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Original Article

Antioxidant, anti-inflammatory, and antibacterial activities against acne-causing bacteria of *Miliusa velutina* (A.DC.) Hook.f. & Thomson extracts

[Actividad antioxidante, antiinflamatoria y antibacteriana contra las bacterias causantes del acné de los extractos de *Miliusa velutina* (A.DC.) Hook.f. & Thomson]

Phornphan Phrompanya[1,2], Wararut Buncharoen[1], Yingmanee Tragoolpua[1], Kanokporn Saenphet[1*]

[1]Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand
[2]Ph.D.'s Degree Program in Biology (International Program), Faculty of Science, Chiang Mai University, Chiang Mai, Thailand.
*E-mail: kanokporn.saenphet@cmu.ac.th, stit.lilo123@gmail.com

Abstract

Context: Acne is often associated with bacterial infection, inflammation, and oxidative stress. In Thailand, *Miliusa velutina* has traditionally been used to relieve pain and to heal wounds. Given its biological properties, *M. velutina* extracts may represent an alternative approach to treat acne.

Aims: To characterise *M. velutina* extracts regarding their antioxidant and anti-inflammatory properties and antibacterial activity against acne-causing bacteria.

Methods: We prepared crude methanolic and aqueous extracts of *M. velutina* leaves and stem bark by maceration. We evaluated the antioxidant activity of the extracts by using four *in vitro* methods and determined the total phenolic and flavonoid contents. We tested the anti-inflammatory activity of the extracts by examining their ability to inhibit nitric oxide production in RAW 264.7 macrophages. We tested the antibacterial activity against acne-causing bacteria by using the agar diffusion method and by determining the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC).

Results: The methanolic bark extract showed potent antioxidant properties, denoted by its ability to scavenge the ABTS and DPPH radicals and its iron-reducing power and total antioxidant activity. The methanolic leaf extract presented the best ability to inhibit nitric oxide production, with a half maximal inhibitory concentration (IC_{50}) of 7.20 ± 1.92 µg/mL. Additionally, *M. velutina* extracts exhibited antibacterial activity against *Cutibacterium acnes*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, methicillin-resistant *S. aureus* and *Pseudomonas aeruginosa*, with the MIC and MBC ranging from 0.49 to 62.50 mg/mL.

Conclusions: These findings indicate that *M. velutina* extract can be used as a novel therapeutic agent for acne treatment owing to its antioxidant, anti-inflammatory and antibacterial properties.

Keywords: antioxidant; *Cutibacterium acnes*; nitric oxide; plant extract; RAW 264.7 cell.

Resumen

Contexto: El acné se asocia a menudo con infección bacteriana, inflamación y estrés oxidativo. En Tailandia, la *Miliusa velutina* se ha utilizado tradicionalmente para aliviar el dolor y curar heridas. Dadas sus propiedades biológicas, los extractos de *M. velutina* pueden representar un enfoque alternativo para tratar el acné.

Objetivos: Caracterizar los extractos de *M. velutina* en cuanto a sus propiedades antioxidantes y antiinflamatorias y su actividad antibacteriana contra las bacterias causantes del acné.

Métodos: Se prepararon extractos acuosos y metanólicos crudos de hojas y corteza del tallo de *M. velutina* por maceración. Evaluamos la actividad antioxidante de los extractos mediante cuatro métodos *in vitro* y determinamos el contenido total de fenoles y flavonoides. Probamos la actividad antiinflamatoria de los extractos examinando su capacidad para inhibir la producción de óxido nítrico en macrófagos RAW 264.7. Probamos la actividad antibacteriana contra las bacterias causantes del acné utilizando el método de difusión en agar y determinando la concentración inhibitoria mínima (CIM) y la concentración bactericida mínima (CBM).

Resultados: El extracto metanólico de corteza mostró potentes propiedades antioxidantes, denotadas por su capacidad para eliminar los radicales ABTS y DPPH y su poder reductor del hierro y actividad antioxidante total. El extracto metanólico de hoja presentó la mejor capacidad para inhibir la producción de óxido nítrico, con una concentración inhibitoria media máxima (IC_{50}) de 7,20 ± 1,92 µg/mL. Además, los extractos de *M. velutina* mostraron actividad antibacteriana contra *Cutibacterium acnes*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *S. aureus* resistente a la meticilina y *Pseudomonas aeruginosa*, con una CMI y una CBM que oscilaron entre 0,49 y 62,50 mg/mL.

Conclusiones: Estos resultados indican que el extracto de *M. velutina* puede utilizarse como un nuevo agente terapéutico para el tratamiento del acné debido a sus propiedades antioxidantes, anti-inflamatorias y antibacterianas.

Palabras Clave: antioxidante; célula RAW 264.7; *Cutibacterium acnes*; extracto vegetal; óxido nítrico.

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AUTHOR INFO
ORCID: 0000-0003-2369-5098 (WB)
0000-0002-0754-0638 (YT)
0000-0003-3844-4267 (KS)

INTRODUCTION

Acne vulgaris is one of the most prevalent skin problems: it affects over 90% of adolescents and persists into adulthood in approximately 14% of cases (Fabbrocini et al., 2010). It is a chronic inflammatory disease that affects the pilosebaceous glands. It is influenced by various factors, such as androgen-mediated stimulation of sebaceous gland activity, hormonal imbalance, inflammation, and bacterial infection (Thiboutot et al., 2009). Moreover, inflammatory acne lesions often result in permanent scars, leading to aesthetic, social, and psychological consequences. Although acne is not life-threatening, its impact on quality of life can be significant. Successful acne treatment typically involves a multifactorial approach that requires a trial period of various regimens. The objectives of acne treatment are to prevent the formation of new lesions, reduce the risk of scarring, and minimise the negative psychological effects. Therefore, standard acne treatment strategies often incorporate anti-inflammatory and antibacterial processes to achieve these goals (Jalian et al., 2007).

The major skin pathogens linked to the formation of acne are *Cutibacterium acnes* and *Staphylococcus epidermidis*. Colonisation of a normal follicle by these bacteria promotes proinflammatory mediators, resulting in inflammation. These mediators stimulate a localised immune response causing the formation of pustules; more severe inflammation is accompanied by the development of comedonal acne (Bhambri et al., 2009; Harper, 2004). Additionally, enzymes released by *C. acnes* can contribute to the rupture of the comedonal wall, leading to the development of larger inflammatory lesions (Jalian et al., 2007). Apart from *C. acnes* and *S. epidermidis*, other resident bacteria such as *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), and *Pseudomonas aeruginosa* can also cause skin diseases via skin wounds; thus, they can play a pathogenic role in acne.

During the inflammatory process of acne, many types of reactive oxygen species (ROS), such as superoxide, the hydroxyl radical and nitric oxide (NO), are produced by neutrophils and are involved in the irritation and destruction of the follicular wall (Briganti and Picardo, 2003). Excessive ROS production can lead to oxidative stress, which plays a role in the pathogenesis of acne. Therefore, antioxidant agents may be an effective approach to reduce cell damage during acne-related inflammation.

In recent years, there has been a growing interest in using natural ingredients with multiple properties to treat skin problems (Nasri et al., 2015; Zagórska-Dziok et al., 2021). Moreover, the global cosmeceutical industry has been shifting towards natural bioactive ingredients; this shift has been driven by an increasing awareness of the limitations of synthetic cosmetics in modern life (Smit et al., 2009). *Miliusa* plants (*Annonaceae*) are well known for their traditional medicinal uses in Southeast Asia and have been reported to have various biological properties, including acetylcholinesterase inhibition, anticancer, antibacterial and anti-inflammatory activities (Promchai et al., 2018; Promgool et al., 2019; Thao et al., 2015; Xu et al., 2019). Among *Miliusa* species, *Miliusa velutina* is renowned for treating ulcers and relieving pain through topical application of its stem bark, as recommended by Thai folk physicians (Hermhuk et al., 2018). Such potential wound healing and pain-relieving properties may be attributed to its anti-inflammatory properties. Although this plant is widely found in Asia (Son, 2019) and has been utilised in traditional herbal remedies, it remains largely unknown to most people and is not often used for beneficial purposes. Thus, pharmacological activities underlying the wound-healing and pain-relieving properties of this plant should be thoroughly studied. Previous studies have reported that alcoholic extracts of *M. velutina* leaves demonstrated potent antibacterial activity, while aqueous extracts derived from its stem bark exhibited activity against pathogenic bacteria and antioxidant activity, as indicated by the ability to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals (Fahad et al., 2020; Wongsa et al., 2017). These reports have shown that extracts prepared using different parts of the plants and solvents show distinct biological activities. Furthermore, the key secondary phenolic and flavonoid metabolites in plant extracts are influenced by these factors (Dirar et al., 2019; Shabir et al., 2011). Thus, it is necessary to find the proper extraction solvent and plant part to produce an extract with the desired pharmacological activities.

Overall, there is a lack of evidence regarding the phytochemical (phenolic and flavonoid) contents of *M. velutina*, and it has not been demonstrated whether it has anti-inflammatory and antibacterial effects against acne-causing bacteria. This situation increased our interest in evaluating the biological activities of this plant. Therefore, we examined the antioxidant and anti-inflammatory properties and the antibacterial activity against acne-causing bacteria of *M. velutina* extracts. We compared the efficacy of extracts prepared with different solvents and plant parts. Studying the properties of *M. velutina* provides scientific evidence to enhance the credibility of traditional medicine and could lead to the development of new herbal formulations of this plant to treat acne.

MATERIAL AND METHODS

Plant material and extraction

Leaf and stem bark parts of *M. velutina* were collected from Chiang Mai University, Chiang Mai, Thailand (18°48'19.1"N 98°57'18.1"E) and identified by Assoc. Prof. Dr Tanawat Chaowasku at the Herbarium of the Department of Biology, Faculty of Science, Chiang Mai University (voucher no. 39964). The leaves and bark were dried and ground into a fine powder. The dried powder (50 g) was macerated in 500 mL of two different solvents, namely water and 70% methanol, for 24 h. The extracts were filtered through Whatman No. 1 filter paper and then concentrated using a vacuum rotary evaporator (Labtech, EV400) at 40°C. Four crude extracts – methanolic leaf (Leaf-MeOH), methanolic bark (Bark-MeOH), aqueous leaf (Leaf-Aq) and aqueous bark (Bark-Aq) were stored at -20°C until further analysis.

Total phenolic content

The total phenolic content of the extracts was estimated with the Folin–Ciocalteu assay (Wolfe et al., 2003). Briefly, 100 µL of extract (1 mg/mL) was added to 2 mL of Folin–Ciocalteu reagent (10% v/v) and 2 mL of sodium carbonate (7.5% w/v). After incubation for 20 min at room temperature, the absorbance was measured at 760 nm. The calibration curve was prepared using gallic acid as a standard, and the results are expressed as milligrams gallic acid equivalents per gram of extract (mg GAE/g extract). The experiment was repeated three times.

Total flavonoid content

The total flavonoid content was determined by using the aluminium chloride method, according to Ordoñez et al. (2006). Briefly, 100 µL of extract (1 mg/mL) was mixed with 0.5 mL of aluminium chloride solution (2% w/v). The absorbance was measured at 420 nm after incubation for 60 min at room temperature. Quercetin was used as a standard agent, and the results are expressed as milligrams of quercetin equivalents per gram of extract (mg QE/g extract). The experiment was repeated three times.

Determination of antioxidant activity

Radical scavenging assays

The antioxidant activity of the extracts was tested with the 2,2-azino-bis-[3-ethylbenzothiozoline-6-sulphonic acid] (ABTS) and DPPH radical scavenging assays. The ABTS and DPPH radical scavenging activities of each extract are expressed as the half maximal inhibitory concentration (IC_{50}).

The ABTS assay was performed using the method described by Re et al. (1999). The radical ABTS solution was prepared through oxidation of ABTS (7.46 mM) by potassium persulphate (4.9 mM); the measured absorbance at 734 nm was 0.70 ± 0.02. Two hundred microlitres of each extract at different concentrations were added to 2 mL of the ABTS solution. The absorbance was measured at 734 nm after 1 min to determine the degree of decolourisation of the ABTS radical. Gallic acid was used as a reference compound (20–100 µg/mL). The percentage of inhibition was calculated according to the formula [1].

$$\% \text{ inhibition} = [(A_{0min} - A_{1min})/ A_{0min}] \times 100 \quad [1]$$

In the formula [1], A_{0min} is the absorbance of the ABTS solution, and A_{1min} is the absorbance in the presence of the sample (extract or gallic acid) at 1 min. The data are presented as the mean of triplicate analyses.

The ability of each extract to scavenge DPPH radicals was determined following the method of Susanti et al. (2007). Briefly, 2 mL of DPPH in methanol (0.13 mM) was mixed with 100 µL of each extract at various concentrations. The mixture was incubated for 30 min in the dark, and then the absorbance was measured at 517 nm. Gallic acid (5–25 µg/mL) was used as a reference compound. The absorbance of the control mixture was used to calculate the initial absorbance. The percentage of inhibition was calculated according to the formula [2].

$$\% \text{ inhibition} = [(A_{0min} - A_{30min})/ A_{0min}] \times 100 \quad [2]$$

In the formula [2], A_{0min} is the absorbance of the DPPH solution, and A_{30min} is the absorbance of the sample (extract or gallic acid) at 30 min. The data are presented as the mean of triplicate analyses.

Iron-reducing power assay

The reducing capacity of the extracts was determined using the method of Oyanaizu (1986). One hundred microlitres of each extract at various concentrations were added to 2 mL of phosphate buffer (200 mM, pH 6.6) and 2 mL of potassium ferricyanide (1% w/v). After incubation at 50°C for 20 min, 2 mL of trichloroacetic acid (10% w/v) were added. Two millilitres of the mixture were then added to 2 mL of distilled water and 0.4 mL of ferric chloride (0.1% w/v). The absorbance was measured at 700 nm after incubation for 10 min. The percentage increase in reducing power was calculated according to formula [3].

$$\% \text{ effective} = [(A_{test} - A_{blank})/ A_{blank}] \times 100 \quad [3]$$

In the formula [3], A_{test} is the sample (extract or ascorbic acid) absorbance, and A_{blank} is the control

absorbance. Ascorbic acid was used as standard. The reducing capacity is presented as the half maximal effective concentration (EC_{50}). The data are presented as the mean of triplicate analyses.

Total antioxidant activity

The total antioxidant capacity of the extracts was evaluated by using the method of Umamaheswari and Chatterjee (2007). The reaction mixture containing 0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate was mixed with 100 µL of each extract. The mixture was incubated at 95°C for 90 min, and then the absorbance was measured at 695 nm. The total antioxidant activity is expressed as milligrams of ascorbic acid equivalents per gram of extract (mg AAE/g extract). The data are presented as the mean of triplicate analyses.

Determination of anti-inflammatory activity

NO inhibitory assay

The inhibitory effect of the extracts on NO production was performed in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells, a murine macrophage cell line. The RAW 264.7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and incubated at 37°C in 5% CO_2. The cell viability was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Briefly, the cells were seeded in a 96-well plate (1×10^5 cells/well). After overnight incubation, the cells were treated with various concentrations of the extracts. Then, the medium was replaced with 30 µL of MTT solution (2 mg/mL), and the cells were incubated for 4 h. The purple formazan crystals were dissolved in 200 µL of dimethyl sulphoxide (DMSO), and the absorbance was measured at 540 nm.

The NO inhibitory activity of extracts was evaluated with the Griess reaction. RAW 264.7 cells (1×10^5 cells/well) were activated by incubation in a medium containing 1 µg/mL LPS and treated with different concentrations of the extracts. Gallic acid was used as a standard at 12.5, 25, 50, 100 and 200 µg/mL. After incubation for 24 h, an equal amount of the culture medium was mixed with the Griess reagent and incubated for 15 min; then, the absorbance was measured at 550 nm. The percentage of NO inhibition was calculated based on the capacity of each extract to inhibit NO production compared with the control (the cells treated with LPS). The experiment was repeated three times.

Determination of antibacterial activity

Bacterial strains

Four gram-positive skin pathogenic bacteria (*C. acnes*, *S. aureus*, MRSA and *Staphylococcus epidermidis*) and one gram-negative bacterium (*Pseudomonas aeruginosa*) were obtained from the Division of Microbiology, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand. Mueller Hinton broth and agar (MHB and MHA, respectively) were used for bacterial growth and the bacterial assay under aerobic conditions at 37°C for 24 h, except for *C. acnes*, which was grown in Brain Heart Infusion (BHI) medium under anaerobic conditions at 37°C for 72 h.

Agar well diffusion assay

The agar well diffusion method is commonly used to screen the antimicrobial ability of extracts. The agar plate surface was spread with the bacterial inoculum. A well with a diameter of 6–8 mm was made, and 100 µL of each extract was added to the well. Then, the agar plates were incubated under suitable conditions depending on the tested bacterium before measuring the diameter of the inhibition zone around each well (Collins et al., 1995). The experiment was repeated three times.

Determination of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC)

The MIC was evaluated with the broth dilution method. Two-fold serial dilutions of each extract were prepared in sterile MHB. The bacterial culture was adjusted to 0.5 McFarland standard (1×10^8 colony-forming units [CFU]/mL), and the dilutions of extracts were added to the culture and incubated at 37°C for 72 h (for *C. acnes*) or 24 h (for the other bacteria). Gentamicin was used as a standard antibiotic for all bacterial strains, except for MRSA, for which vancomycin was used. The MIC was recorded as the lowest concentration of the extract at which the bacterial growth was inhibited. For the MBC determination, the cultures with no bacterial growth were streaked onto agar plates and incubated under suitable conditions for each bacteria. The MBC was recorded as the lowest concentration at which there was no visible bacterial growth (Collins et al., 1995). The experiment was repeated three times.

Statistical analysis

The data are expressed as the mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons in SPSS Statistics

Version 22 for Windows. A p-value < 0.05 was considered statistically significant.

RESULTS

Total phenolic and flavonoid contents

The phenolic and flavonoid contents are important contributors to the antioxidant capacity (Nur et al., 2019). The Bark-MeOH extract exhibited the highest total phenolic content (28.94 ± 1.56 mg GAE/g extract), while the Bark-Aq extract demonstrated the highest total flavonoid content (48.65 ± 2.37 mg QE/g extract) (Table 1). However, there was no significant difference ($p>0.05$) in the total phenolic and flavonoid contents between the Bark-MeOH, Bark-Aq, and Leaf-MeOH extracts. On the other hand, the Leaf-Aq extract showed the lowest total phenolic and total flavonoid contents ($p<0.05$), suggesting variations in phenolic and flavonoid compound levels among the different extraction methods and *M. velutina* parts.

Antioxidant activity

The antioxidant activity of *M. velutina* leaf and bark extracts is summarised in Table 2. The IC_{50} for the DPPH and ABTS radical scavenging assays ranged from 0.37 to 4.25 mg/mL and 3.44 to 64.00 mg/mL, respectively. Remarkably, the Bark-MeOH extract displayed the highest free radical scavenging ability, with the lowest IC_{50} for DPPH (0.37 ± 0.02 mg/mL) and ABTS (3.44 ± 0.07 mg/mL) radical scavenging. Moreover, the Bark-MeOH extract exhibited the strongest iron-reducing power, with an EC_{50} of 0.52 ± 0.01 mg/mL, and the highest total antioxidant activity (401.23 ± 12.54 mg AAE/g extract). In contrast, the Leaf-Aq extract showed a significantly lower antioxidant capacity compared with the other extracts ($p<0.05$). Notably, the methanolic extract displayed greater antioxidant activity than the aqueous extract from the same plant part, and the bark extract demonstrated a greater antioxidant capacity than the leaf extract with the same solvent. These findings demonstrate that the extraction solvent and plant part influence the antioxidant activity.

Anti-inflammatory activity

Cytotoxic effects of M. velutina extracts on RAW 264.7 cells

We used the MTT assay to examine the cytotoxic effect of the extracts at different concentrations (0–625 µg/mL) on RAW 264.7 cells (Fig. 1). The extracts had a concentration-dependent effect on RAW 264.7 cell viability.

Table 1. Total phenolic and flavonoid contents of *Miliusa velutina* extracts.

Extract	Total phenolic content (mg gallic acid equivalents/ g extract)	Total flavonoid content (mg quercetin equivalents/ g extract)
Leaf-MeOH	24.30 ± 2.66^{b}	43.65 ± 3.73^{a}
Bark-MeOH	28.94 ± 1.56^{a}	39.90 ± 4.05^{a}
Leaf-Aq	18.17 ± 0.89^{b}	22.68 ± 5.84^{b}
Bark-Aq	25.93 ± 2.70^{a}	48.65 ± 2.37^{a}

Methanolic leaf extract (Leaf-MeOH), methanolic bark extract (Bark-MeOH), aqueous leaf extract (Leaf-Aq), and aqueous bark extract (Bark-Aq). The data represent the mean ± standard deviation (n = 3). Different letters (a and b) for each column indicate significant differences by analysis of variance followed by a Tukey's test ($p<0.05$).

Table 2. Determination of antioxidant activity of *Miliusa velutina* extracts from DPPH, ABTS, iron reducing power and total antioxidant activity assays.

Extracts	DPPH IC_{50} (mg/mL)	ABTS IC_{50} (mg/mL)	Iron reducing power EC_{50} (mg/mL)	Total antioxidant (mg ascorbic acid equivalents/g extract)
Leaf-MeOH	1.79 ± 0.10^{d}	47.67 ± 0.74^{d}	0.82 ± 0.01^{d}	268.46 ± 11.94^{b}
Bark-MeOH	0.37 ± 0.02^{b}	3.44 ± 0.07^{b}	0.52 ± 0.01^{b}	401.23 ± 12.54^{a}
Leaf-Aq	4.25 ± 0.06^{e}	64.00 ± 1.16^{e}	7.49 ± 0.04^{e}	98.60 ± 14.59^{c}
Bark-Aq	1.14 ± 0.02^{c}	6.06 ± 0.02^{c}	0.63 ± 0.03^{c}	283.14 ± 10.44^{b}
Gallic acid	0.012 ± 0.0004^{a}	0.060 ± 0.002^{a}	-	-
Ascorbic acid	-	-	0.29 ± 0.003^{a}	-

Methanolic leaf extract (Leaf-MeOH), methanolic bark extract (Bark-MeOH), aqueous leaf extract (Leaf-Aq) and aqueous bark extract (Bark-Aq). The data are represented as the mean ± standard deviation (n = 3). Different letters (a–e) for each column indicate significant differences by analysis of variance followed by Tukey's test ($p < 0.05$).

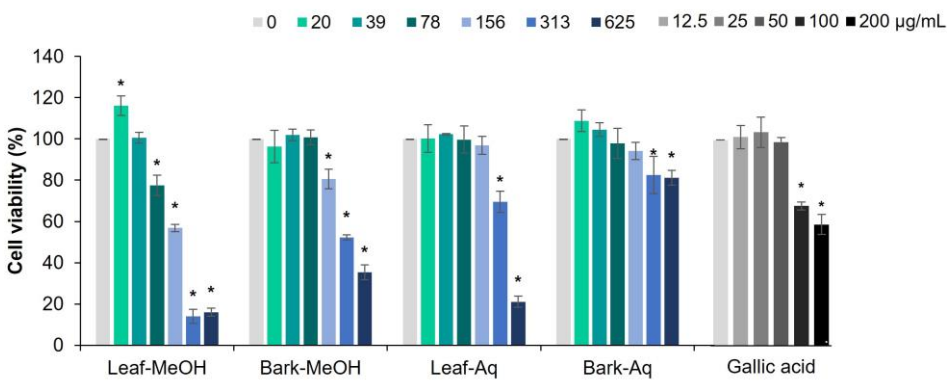
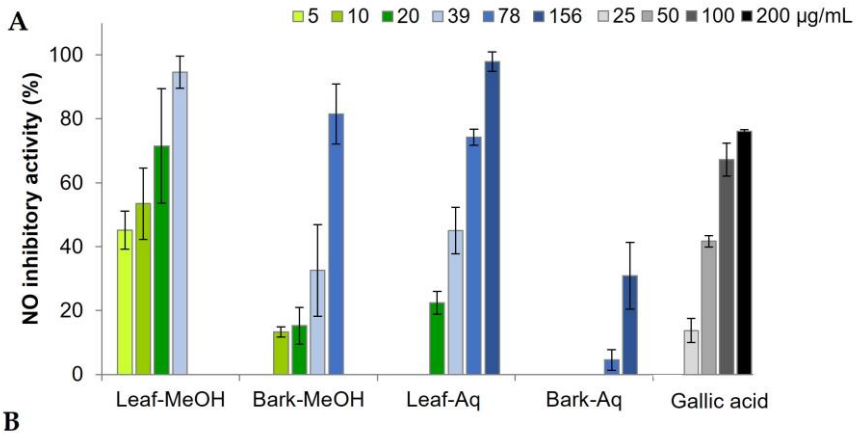


Figure 1. The effect of *Miliusa velutina* methanolic leaf (Leaf-MeOH), methanolic bark (Bark-MeOH), aqueous leaf (Leaf-Aq) and aqueous bark (Bark-Aq) extracts and gallic acid on RAW 264.7 cell viability.

The data are expressed as the mean ± standard deviation (n = 3). *Significantly different value from control (0 µg/mL) by analysis of variance followed by Tukey's test ($p<0.05$).



B

Inhibition of NO Production	Extracts				
	Leaf-MeOH	Bark-MeOH	Leaf-Aq	Bark-Aq	Gallic acid
IC_{50} (µg/mL)	7.20 ± 1.92[a]	49.67 ± 4.66[b]	54.07 ± 5.29[b]	> 156	74.00 ± 0.00[c]

Figure 2. The effect of *Miliusa velutina* methanolic leaf (Leaf-MeOH), methanolic bark (Bark-MeOH), aqueous leaf (Leaf-Aq) and aqueous bark (Bark-Aq) extracts and gallic acid on inhibition of nitric oxide (NO) production in lipopolysaccharide-stimulated RAW 264.7 cells (A) and the half-maximal inhibitory concentration (IC_{50}) for each extract and gallic acid (B).

The data are expressed as the mean ± standard deviation (n = 3). Different letters (a–c) indicate significant differences by analysis of variance followed by Tukey's test ($p<0.05$).

The Leaf-MeOH extract was cytotoxic at >39 µg/mL, and the Bark-MeOH extract was cytotoxic at >78 µg/mL. On the other hand, the aqueous extracts only affected cell viability at concentrations above 156 µg/mL. Gallic acid at a concentration of up to 50 µg/mL had no effect on RAW 264.7 cell viability. Hence, the Bark-MeOH, Leaf-Aq, and Bark-Aq extracts exhibited lower cytotoxicity than the standard compound (gallic acid). Based on these results, we assessed the inhibitory effect of the extracts on NO production by using concentrations up to 156 µg/mL.

Ability of M. velutina extracts to inhibit NO production

We evaluated the effect of the *M. velutina* extracts on NO release in LPS-stimulated RAW 264.7 cells by using the Griess reaction. The extracts inhibited NO production in a concentration-dependent manner (Fig. 2A). The Leaf-MeOH extract at a concentration of 39 µg/mL inhibited almost all NO production (94.65%). Moreover, the Leaf-MeOH extract showed the most potent inhibitory effect on NO release, with an IC_{50} of 7.20 ± 1.92 µg/mL. The Bark-MeOH and Leaf-Aq extracts exhibited an IC_{50} of 49.67 ± 4.66 and

54.07 ± 5.29 µg/mL, respectively (Fig. 2B). Moreover, the Leaf-MeOH, Bark-MeOH, and Leaf-Aq extracts exhibited a significantly higher capacity to reduce NO production compared with gallic acid, which had an IC_{50} of 74.00 µg/mL.

Antibacterial activity

We examined the ability of *M. velutina* extracts at a concentration of 250 mg/mL to inhibit the growth of acne-causing bacteria. We observed varying degrees of inhibition (Fig. 3). The inhibition zones ranged from 14.33 ± 0.58 to 58.33 ± 0.58 mm (Table 3). Notably, the Leaf-MeOH and Leaf-Aq extracts exhibited the largest inhibition zones against *C. acnes* at 58.33 ± 0.58 and 57.33 ± 0.58 mm, respectively. They were more potent than gentamicin at a concentration of 1 mg/mL.

The MIC and MBC for the *M. velutina* extracts are summarised in Table 4. The MIC ranged from 0.49 to 31.25 mg/mL, while the MBC ranged from 0.98 to 62.50 mg/mL. All extracts showed the lowest MIC and MBC against MRSA. Furthermore, the antibacterial activity of the *M. velutina* extracts varied depending on the plant part. The leaf extracts exhibited more potent inhibitory and bactericidal effects against *S. epidermidis*, MRSA, and *C. acnes*, while the bark extracts showed more potent inhibitory activity against *S. aureus*. Overall, the *M. velutina* leaf and bark extracts demonstrated antibacterial activity against acne-causing bacteria.

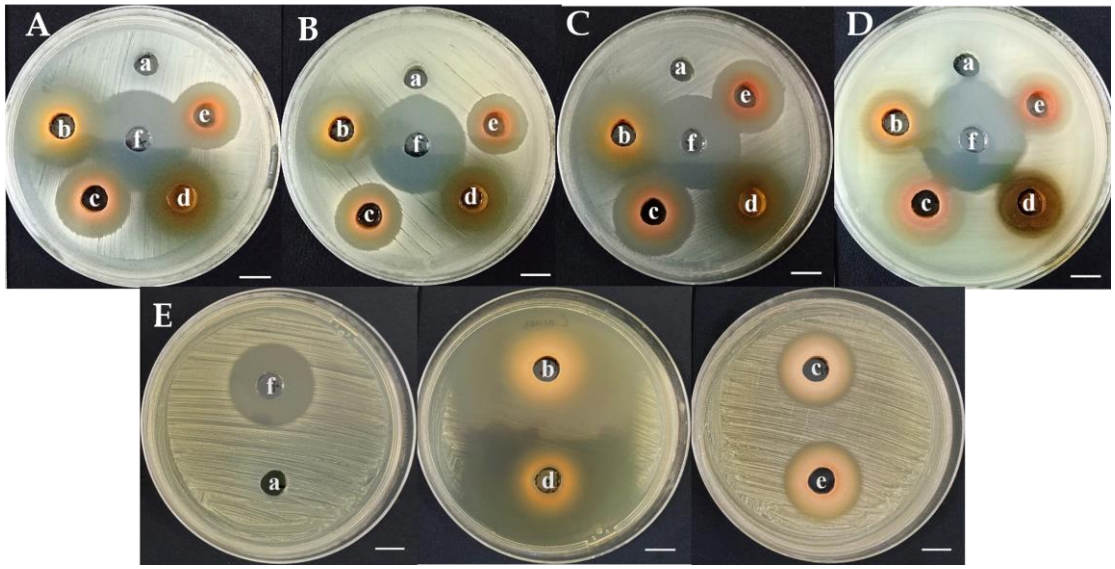


Figure 3. Screening the antibacterial activity of *Miliusa velutina* extract against *Streptococcus epidermidis* **(A)**, *Staphylococcus aureus* **(B)**, methicillin-resistant *S. aureus* (MRSA) **(C)**, *Pseudomonas aeruginosa* **(D)** and *Cutibacterium acnes* **(E)** with the agar well diffusion method.

Negative control (a), methanolic leaf extract (Leaf-MeOH) (b), methanolic bark extract (Bark-MeOH) (c), aqueous leaf extract (Leaf-Aq) (d), aqueous bark extract (Bark-Aq) (e) and positive control (gentamicin or vancomycin) (f). The extract concentration was 250 mg/mL, and the positive control concentration was 1 mg/mL. Scale bar = 10 mm.

Table 3. Antibacterial activity of *Miliusa velutina* extracts against acne-causing bacteria using agar well diffusion assay.

Extract	Zone of inhibition (mm in diameter)				
	S. epidermidis	***S. aureus***	**MRSA**	***C. acnes***	***P. aeruginosa***
Leaf-MeOH	26.67 ± 0.58^{b}	24.33 ± 1.15^{b}	28.33 ± 0.58^{b}	58.33 ± 0.58^{a}	16.33 ± 0.58^{cd}
Bark-MeOH	24.00 ± 0.00^{c}	23.00 ± 0.00^{b}	27.00 ± 0.00^{c}	27.33 ± 0.58^{b}	19.00 ± 0.00^{b}
Leaf-Aq	26.00 ± 1.00^{b}	24.33 ± 1.15^{b}	27.67 ± 0.58^{bc}	57.33 ± 0.58^{a}	18.00 ± 1.73^{bc}
Bark-Aq	23.67 ± 0.58^{c}	22.33 ± 1.15^{b}	25.67 ± 0.58^{d}	28.33 ± 2.36^{b}	14.33 ± 0.58^{d}
Gentamicin	34.33 ± 0.58^{a}	34.67 ± 0.29^{a}	ND	28.33 ± 1.15^{b}	34.83 ± 0.29^{a}
Vancomycin	ND	ND	32.00 ± 0.00^{a}	ND	ND

Methanolic leaf extract (Leaf-MeOH), methanolic bark extract (Bark-MeOH), aqueous leaf extract (Leaf-Aq), and aqueous bark extract (Bark-Aq). The results represent the mean ± standard (n = 3) using 250 mg/mL for each extract and 1 mg/mL for gentamicin and vancomycin. Different letters (a–d) indicate significant differences by analysis of variance followed by a Tukey's test ($p<0.05$). ND = not determined.

Table 4. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Miliusa velutina* extracts against acne-causing bacteria.

Extracts	*S. epidermidis*	*S. aureus*	MRSA	*C. acnes*	*P. aeruginosa*
MIC (mg/mL)					
Leaf-MeOH	3.91	0.98	0.49	3.91	3.91
Bark-MeOH	15.63	0.98	0.98	7.81	31.25
Leaf-Aq	3.91	15.63	15.63	1.95	31.25
Bark-Aq	31.25	0.49	31.25	3.91	3.91
Gentamicin (µg/mL)	5	0.04	ND	12.5	12.5
Vancomycin (µg/mL)	ND	ND	7.81	ND	ND
MBC (mg/mL)					
Leaf-MeOH	7.81	7.81	3.91	3.91	31.25
Bark-MeOH	62.50	1.95	0.98	7.81	31.25
Leaf-Aq	15.63	31.25	31.25	1.95	31.25
Bark-Aq	31.25	1.95	31.25	31.25	31.25
Gentamicin (µg/mL)	5	0.04	ND	12.5	12.5
Vancomycin (µg/mL)	ND	ND	15.63	ND	ND

Methanolic leaf extract (Leaf-MeOH), methanolic bark extract (Bark-MeOH), aqueous leaf extract (Leaf-Aq), and aqueous bark extract (Bark-Aq). ND = not determined.

DISCUSSION

Natural substances derived from plant extracts are a good source of bioactive compounds and have become a prominent group of cosmeceutical ingredients. These compounds have been shown to exhibit efficacy for various skin conditions because of the diverse properties of secondary metabolites found in plants (Aburjai and Natsheh, 2003; Hussein and El-Anssary, 2018). Among these, polyphenols, particularly phenolic and flavonoid compounds, are known for their biological activities, including anti-inflammatory, antioxidant, antiviral, and antitumour (Ghasemzadeh and Jaafar, 2011; Lee et al., 1993; Paliwal et al., 2005). We found that *M. velutina* leaf and bark extracts are rich in important bioactive phenolic and flavonoid compounds. However, the content of these bioactive compounds in the extracts depends on the solvent used for extraction and the plant part. Several studies have reported variations in the total phenolic and flavonoid contents in extracts based on solvent polarity (Dessalegn et al., 2020; Ismael et al., 2021; Shahinuzzaman et al., 2020). Polar solvents are more effective than non-polar solvents for extracting phenolic and flavonoid compounds due to the presence of a hydroxyl group (Panche et al., 2016; Wang and Weller, 2006). The results from this study revealed that the *M. velutina* bark extracts exhibited a significantly higher total phenolic content than the leaf extracts. However, there were no significant differences in the total flavonoid content between the Leaf-MeOH, Bark-MeOH and Bark-Aq extracts. We found that both water and 70% methanol are suitable solvents for *M. velutina*.

The results from the ABTS and DPPH radical scavenging, iron-reducing power, and total antioxidant assays revealed that the *M. velutina* extracts have a potent antioxidant capacity. The ABTS and DPPH radical scavenging activities indicate the ability of extracts to eliminate free radicals by offering their hydrogen atom and an electron. We also performed a reducing power assay to estimate the ability to chelate ferrous ions, a pro-oxidant. The reducing capacity is typically attributed to the presence of various reductants. The antioxidant action of reductants is based on their ability to break the free radical chain by donating a hydrogen atom. Additionally, reductants can react with peroxide precursors and thus effectively prevent peroxide formation (Loganayaki et al., 2013). The antioxidant capacity of different extracts may be attributed to their variable reducing power, which contributes to their overall antioxidant activity. Furthermore, the total antioxidant capacity is based on the reduction of Mo (VI) to Mo (V) by the water-soluble and fat-soluble antioxidants in extracts (Aliyu et al., 2013). In this study, the bark extracts showed more potent antioxidant activity than the leaf extracts. The strongest antioxidant activities in the Bark-MeOH extract may attributed to the phenolic and flavonoid compounds in this extract. There have been reports of the correlation between the antioxidant activities and total phenolic and flavonoid contents in plant extracts (Anh et al., 2021; Aryal et al., 2019). This result is also

related to another report in which the ethanolic extract of *M. velutina* stem bark exhibited good antioxidant activity in terms of the DPPH radical scavenging, iron reducing power and total antioxidant capacity assays (Trang et al., 2020a).

Accumulation of reactive nitrogen species (RNS) has been associated with oxidative stress and inflammatory conditions (Di Meo et al., 2016; Pizzorno and Murray, 2020). During the inflammatory process of acne, proinflammatory mediators are released. Among these mediators, NO is associated with the immune response; indeed, NO production is very important to defend the body. Nevertheless, overproduction of NO could induce cell damage and chronic inflammation (Abaffy et al., 2019; Williams et al., 2007). We determined the effect of the *M. velutina* extracts on the inhibition of NO production in LPS-stimulated RAW 264.7 cells. This murine macrophage cell line has been used to screen anti-inflammatory agents. The Leaf-MeOH extract presented the lowest IC_{50} (7.20 ± 1.92 µg/mL) and, thus, the strongest capacity to inhibit NO production. A previous study reported high anti-inflammatory activity of *M. velutina* aqueous leaf extract in the anti-albumin denaturation assay, with an IC_{50} of 21.13 ± 0.95 µg/mL (Trang et al., 2020b). The total phenolic content of plant extracts is highly related to free radical scavenging activities (Choi et al., 2007). The ability of the *M. velutina* extracts to potently inhibit NO production may be due to the phenolic compounds contained in the extracts. Moreover, previous studies have demonstrated that medicinal plants are a good source of antioxidant molecules, which effectively inhibit the inflammatory process by reducing NO production (García Díaz et al., 2022; Mfotie Njoya et al., 2017; Ravipati et al., 2012). Furthermore, the concentrations of *M. velutina* extracts in this assay presented >90% cell viability. Therefore, the decrease in NO production in the treated cells was due to the inhibitory effect of the extracts and not cell death.

Besides antioxidant and anti-inflammatory activities, we evaluated the antibacterial activity of the *M. velutina* extracts. Plant-derived antibiotics have been used as an alternative strategy to treat acne because pathogenic bacteria have developed antibiotic resistance and have become a major threat to public health. Stem bark extracts from *M. velutina* presented the antibacterial effect on pathogenic bacteria such as *Escherichia coli*, *P. aeruginosa*, *Sarcina lutea*, *Shigella boydii*, *Shigella dysenteriae* and *S. aureus* (Fahad et al., 2020). In this study, the *M. velutina* extracts exhibited notable antibacterial activity against gram-positive and gram-negative bacteria that are known to be involved in the development of acne. Nevertheless, each extract's antibacterial activity differed depending on the extraction solvent, plant part and bacterial strain. The *M. velutina* Leaf-MeOH and Leaf-Aq extracts showed the highest inhibitory effect against *C. acnes*, a major bacteria associated with acne. Meanwhile, the Bark-MeOH and Leaf-MeOH extracts demonstrated the highest inhibitory effect against MRSA. Nevertheless, all extracts in this study showed no appreciable effect in killing *P. aeruginosa*, a gram-negative bacteria, with an MBC of 31.25 mg/mL. Gram-negative bacteria are more resistant than gram-positive bacteria because their outer membrane contains LPS, which can limit membrane permeability (Amenu and Andualem, 2014; Debalke et al., 2018). As a result, *P. aeruginosa* could not be easily damaged by the *M. velutina* extracts.

The *M. velutina* extracts have potent bioactive substances, which we showed have antibacterial activity against a wide range of acne-causing bacteria. The effective antibacterial activity of the *M. velutina* extracts may be correlated to the various phytochemicals in each extract. A study on phytochemicals of *Miliusa* species showed several bioactive compounds such as flavonoids, alkaloids, terpenoids, phenylpropanoids, styrylpyrones, and homogentisic acid derivatives (Sawasdee et al., 2010). Several studies have reported that the high phenolic and flavonoid contents of plant extracts are correlated with effective antibacterial activity (Shan et al., 2007; Sivapriya et al., 2011). The antibacterial activity of these compounds is probably associated with their incorporation into bacterial cell membranes, leading to changes in membrane permeability and integrity, and causing the loss of vitally important cellular material (Nourbakhsh et al., 2022; Sivapriya et al., 2011).

CONCLUSION

Antioxidants can indirectly contribute to anti-inflammatory and antibacterial effects by reducing oxidative stress, while anti-inflammatory agents can manage inflammation associated with infections. The results of this study revealed that *M. velutina* extracts possess all three properties. However, the extraction solvent and plant part influence the biological properties. The methanolic extracts exhibited better biological properties than the aqueous extracts. The stem bark extract had the most effective antioxidant activity, while the leaf extract had the most effective anti-inflammatory activity and ability to inhibit acne-causing bacteria. Consequently, the *M. velutina* leaf-MeOH extract is suitable for the development of anti-acne formulations. These findings provide scientific support for the traditional uses of this plant. Additional studies should focus on isolating the potential compounds from this plant and delving into other biological properties in more detail.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Phrompanya P	Buncharoen W	Tragoolpua Y	Saenphet K
Concepts or ideas		x	x	x
Design		x	x	x
Definition of intellectual content			x	x
Literature search	x			x
Experimental studies	x			
Data acquisition	x			x
Data analysis	x	x		x
Statistical analysis	x			x
Manuscript preparation	x			x
Manuscript editing				x
Manuscript review	x	x	x	x

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