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RESEARCH ARTICLE OPEN ACCESS

Effect of Extraction Temperature on the Phytochemical Profile, Antioxidant Activity and Antibacterial Activity of Aqueous *Boehmeria nivea* Leaf Extracts

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Abstract

***Boehmeria nivea* (L.) is one of functional plants, but its phytochemical composition and bioactivities are not yet well standardized. The objective was to study the phytochemical profile, antioxidant and antibacterial property of the aqueous extracts from leaves prepared at 25 °C (G1) and at 60 °C (G2). The study included qualitative phytochemical screening, quantitative analysis with thin layer chromatography and biological properties such as antimicrobial and antioxidant activities. G1 exhibited higher TPC (2.7340 ± 0.049 mg GAE/g) than G2 (2.2421 ± 0.010 mg GAE/g), whereas G2 showed greater TFC (0.581 ± 0.053 mg NE/g) than G1 (0.267 ± 0.049 mg NE/g). In the DPPH assay, G1 demonstrated stronger antioxidant activity (EC_{50} = 256.8 ± 0.09 µg/mL) compared to G2 (271.2 ± 0.26 µg/mL). Antibacterial testing revealed MIC values of 80 mg/mL for G1 against *Staphylococcus epidermidis* ATCC 12228 and *Vibrio parahaemolyticus* ATCC 17802, while G2 showed enhanced activity against *Staphylococcus epidermidis* (40 mg/mL). These findings indicate that extraction temperature influences phytochemical yield and biological performance, supporting the potential application of *Boehmeria nivea* leaf extracts.**

Keywords: Ramie, Aqueous Extraction, Phytochemicals, DPPH Radical Scavenging, Minimum Inhibitory Concentration, LC-MS

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INTRODUCTION

Many plants can long be used as traditional medicine, including infusions, decoctions, and powders.[1] The development of modern phytopharmaceuticals from these natural sources has been received increasing attention due to a low cost and safe.[2] However, a substantial gap remains between traditional use and evidence based clinical application because many herbal preparations have not been scientifically evaluated for their efficacy and safety. Therefore, studies are needed to optimize extraction conditions and ensure the quality, and biological effectiveness of plant derived products.

Boehmeria nivea (L.) Gaudich. (*B. nivea*) is well known for its use in remedies. Previous studies have reported that its leaves contain abundant phenolic compounds, flavonoids, chlorogenic acid, vitamin C, and minerals, contributing to anti-inflammatory, antimicrobial, antioxidant activities.[3,4] However, previous studies have focused on identifying phytochemicals or evaluating biological activities, whereas the influence of water and temperature are important for extracting bioactive compounds. The resulting biological activities have received limited attention. Since extraction temperature is a critical factor affecting the phytochemicals, optimizing this parameter is important for obtaining extracts with biological efficacy. Therefore, the research focused on the profile including phytochemicals, antibacterial and antioxidant capacity of the aqueous extracts prepared from the *B. nivea* leaves prepared at 25 °C and 60 °C, providing evidence for selecting an appropriate extraction condition for future utilization of this plant.

MATERIALS AND METHODS

Strains and chemicals

HCl, H_2SO_4 (Sigma, Aldrich, USA), TCA, $FeCl_3$, NaOH, $CHCl_3$, $CuSO_4$, KI, Na_2CO_3, $NaNO_2$, $AlCl_3$ (Biobasic, Canada), CH_2O_2, CH_3CN, Folin-Ciocalteu reagent (Merck, USA), gallic acid, naringenin, ascorbic acid standard (Thermo Fisher Scientific, USA), DPPH (Merck, USA), silica gel 60 F_{254} aluminium-backed TLC plates (Merck, Germany).

Staphylococcus epidermidis (ATCC 12228) and *Vibrio parahaemolyticus* (ATCC 17802) were used in this study. Each strain was inoculated from a colony into agar broth and incubated until vigorous growth was achieved. The bacterial solution was prepared at 0.5 McFarland standard, which is approximately equal to 1×10^8 CFU/mL, and then diluted with sterile broth to provide the needed inoculum concentration for additional antibacterial experiments.

Sample preparation and extraction

Fresh *B. nivea* leaves were collected in May in Vietnam (10°462 323 N, 106°422 073 E). The plant material was identified based on morphology information presented in Figure 1. The washed leaves in distilled water (DW) were dried by drying cabinet (50 °C). The dried leaves were powdered by grinder. Then, DW was added into powder at a material-to-solvent ratio of 1:10 (mg/mL) and left for 90 minutes in a water bath at either 25 °C (G1) or 60 °C (G2).[5] Next, the clear supernatants were collected after combining both centrifugation at optimized condition (9,000 rpm, 30 minutes), and filtration through filter paper (Whatman No. 1) to remove insoluble residues. The filtrated samples were heat evaporated at 50 °C until complete removal of water. The samples were preserved at -20 °C for further tests.

Phytochemical screening

The samples were prepared in DW (10 mg/mL) and qualitative phytochemical was screened according to the previous methods with minor modifications.[7-10]

Polyphenol detection

Before determination of total polyphenol content, all the extracts were screened for the presence of polyphenols. Particularly, 5 drops of 5% $FeCl_3$ was added to the extracts (1 mL). The deep blue color represents the positive result.[7]

Tannin detection

A portion of 2-3 drops of 5% $FeCl_3$ was dropped into the extract. A blue- black precipitate was taken as the tannin presence.[8]

Saponin detection

1 mL of the sample was mixed with DW and vigorously shaken for around 5 minutes. The test tube was then kept standing for a further 5

minutes. The extent of stable froth was observed to confirm the presence of saponins.[9]

Alkaloid detection

The alkaloids presence was detected by adding concentrated HCl and 10 drops of Dragendorff reagent into 1 mL extract solution. Orange precipitate confirmed that alkaloids presented in the extract.[8]

Flavonoid detection

Flavonoids were confirmed whether present in the extract by adding 15 drops of concentrated H_2SO_4 to 1 mL of extract. An orange color appeared to illustrate flavonoid compound.[10]

Steroid detection

The Rosenheim reaction was used to detect steroids. The reaction was described by combining extracts with TCA solution. The positive reaction was that after 20 minutes, the solution turned into blue color.[9]

Protein detection

Biuret test was done to confirm the existence of proteins. Four drops of 3% $CuSO_4$ and four drops of 10% NaOH were mixed with extracts, the red color formation determining that proteins are present.[9]

Starch detection

One milliliter of iodine solution with KI was added into extracts, and the blue color indicated that starch exists in the extract.[9]

Glycoside detection

The glycoside presence was detected by adding 2 mL extract followed by adding $CHCl_3$ and concentrated H_2SO_4. The brown-red rings illustrate having glycoside.[10]

Thin-layer chromatography (TLC)

In the study, TLC was used to observe the preliminary phytochemical screening in the extracts, which mainly based on the contact between mobile phase and stationary phase.[11] The DW dissolved the extracts (1 mg/mL) were used for TLC application. A volume of each sample (5 µL) was applied onto the TLC plates. The plates were developed using n-hexane:acetone (11:9, v/v) as the mobile phase. Naringenin was used as reference flavonoids, betulinic acid was reference for triterpenoid. After development, plates were first observed under UV light (254 nm) to detect the clear separation and subsequently derivatized by spraying with $AlCl_3$ and vanillin H_2SO_4 reagent, followed by heating until colored spots were clearly visualized.

Screening by liquid chromatography-mass spectrometry (LC-MS)

The phytochemicals profile of two aqueous extracts was analyzed using an UHPLC (Ultimate 3000) with a mass spectrometer (MSQ Plus) and a diode-array detector (Thermo Fisher Scientific, USA) with A Phenomenex ODS (C18), 150 × 4.6 (mm) in length and 5 µm in diameter. The mobile phase included 0.1% formic acid as (A) with concentrated acetonitrile as (B). A flow rate was 1.2 mL/minute. The gradiented following the step that were 0-25 minutes, (B: 20%-50%); 25-27 minutes, (B: 50%-100%); 27-30 minutes, (B: 100%). The injection volume was 5 µL, and chromatograms were monitored at 280 nm. Mass spectra were acquired using electrospray ionization with both ionization modes (positive and negative). Chromeleon software (version 7.2.4.8179) was applied in data acquisition and process.

Total phenolic contents quantification (TPC)

TPC was calculated by the Folin-Ciocalteu's method with minor modifications.[12] 25 µL the samples, 25 µL diluted Folin-Ciocalteu reagent solution and 200 µL DW were pipetted into the 96-well plate and kept for 5 minutes. After that, 25 µL 10% Na_2CO_3 were added. After that, the incubation step was occurred in the dark condition with 25 °C for 1 hour. The results were observed at 765 nm. The gallic acid standard (concentration from 20-100 µg/mL) was prepared for the standard curve. The TPC were calculated as mg GAE/g. Each performance was triplicated. All data were presented in mean ± SD.

Total flavonoid content quantification (TFC)

TFC was measured following the previous method.[13] Adding DW (100 µL), 5% $NaNO_2$ (10 µL) and 25 µL sample extract into the 96 well plate. After 5 minutes, 15 µL of 10% $AlCl_3$ was mixed.

After 6 minutes, 1M NaOH (50 µL) and DW (50 µL) were added and measured at 510 nm. Naringenin was used (600 to 2200 µg/mL) for calibration. TFC determinations were calculated (mg NE/g). The results were triplicated and expressed as mean ± SD.

Antioxidant activity determination

DPPH free radical scavenging assay was applied in antioxidant activity measurement *B. nivea* leaves.[14] For the control, methanol was mixed with DPPH solution. An ascorbic acid stock solution was prepared with DW to produce series of references concentrations. Similarly, extract solutions were prepared at different concentrations (200-1000 µg/mL) using DW. 50 µL the standard solutions or samples were then mixed with 50 µL DPPH working solution (0.1 mM), avoiding light (30 minute incubation). The wavelength (517 nm) was applied for measurement. The scavenging activity (%) was calculated basing on percentage reduction in absorbance of the sample compared with the control. Higher percentage of scavenging activity indicated the higher radical scavenging capacity of sample.

Antibacterial property determination

Minimum inhibitory concentrations (MICs) were illustrated using a process of microdilution guided in the Clinical and Laboratory Standards Institute (CLSI).[15] *S. epidermidis* and *V. parahaemolyticus* cultures were obtained in Luria–Bertani (LB) medium with 0.5 McFarland criteria (1 × 10^8 CFU). Serial two-fold dilutions of the aqueous extracts (160-0.078 mg/mL) were added in 96-well microplates. Each well contained of the extract solution (100 µL) and of the bacterial suspension (100 µL, 5 × 10^4 CFU). The microplates were set up at 37 °C in 16 hours, and MICs were calculated based on bacterial turbidity and assessed by the OD at 600 nm. Chloramphenicol (100 µg/mL) was used as the positive control, while bacterial cultures in LB broth without extract and sterile LB broth served as the growth and sterility controls, respectively. The MICs were triplicated.

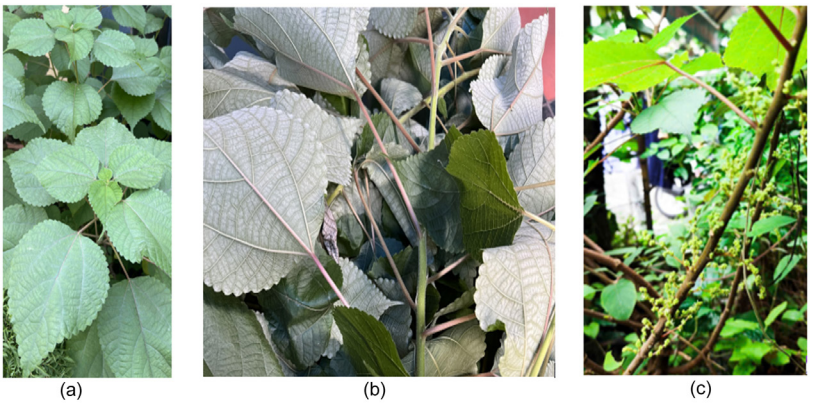
Data analysis

The mean ± standard deviation (SD) was expressed for the results that obtained after three replications. Statistical analyses were done via Microsoft Excel 2020. One-way analysis of variance (ANOVA) was applied to compare the mean values of the extraction conditions. The statistical significance at $P < 0.05$ showed the meaningful differences. The figures were drawn by GraphPad Prism.

RESULTS AND DISCUSSION

Phytochemical screening

Polyphenols, flavonoids, tannins, and alkaloids were identified in both extracts, whereas steroids, proteins, saponins, and starch were not detected. TLC analysis provided additional chromatographic evidence supporting the presence of several classes of phytochemicals



(a) (b) (c)

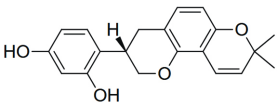
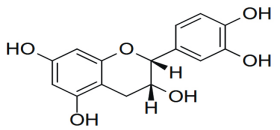
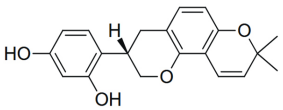
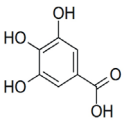
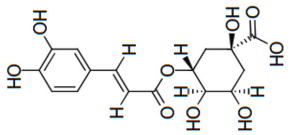
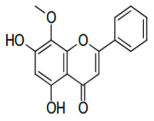
Figure 1. The morphology of leaves. Upper layer (a) and lower layer (b) and the distribution of fruits (c) of *B. nivea*. Leaves are long-petioled and hair; the top layer is dark green and the lower surface is covered with dense white hairs, giving the leaves a pale appearance, the clusters arranged in panicles shorter than the petioles; this appearance is consistent with the previous identifications.[6]

in the extracts (Figures 1-3). Under UV light at 254 nm, several UV-absorbing spots were observed on the TLC plates (Figure 2). Because UV absorption at this wavelength is not specific to a particular class of compounds, these spots were interpreted as UV-active constituents. Following derivatization with vanillin/H_2SO_4 reagent, distinct colored spots became visible. Comparison of the chromatographic profiles of the ethyl acetate fractions (G12 and G22) with those of the reference standards showed that several sample spots exhibited migration behavior and coloration comparable to naringenin and betulinic acid (Figures 3 and 4). These observations suggest the presence of flavonoid and triterpenoid constituents. Comparison of the chromatograms also revealed differences between G12 and G22, particularly in the flavonoid region, suggesting that extraction temperature may influence the composition of flavonoid constituents.

Liquid chromatography - mass spectrometry

The amount of phenolic, alkaloid, flavonoid, and steroid compounds was indicated in all the extracts. These findings expand upon the previous study conducted by Ikhlas et al. in 2021, as more phytoconstituents were analyzed and identified.[16]

Table 1. Tentatively annotated compounds in *B. nivea* aqueous extracts based on LC-MS analysis

Sample	Tentatively annotated compound	Molecular weight	Monoisotopic mass	Structure
G1	Glabridin	324.14	324.14	
	Epicatechin	290.27	290.08	
G2	Glabridin	324.14	324.14	
	Gallic acid	170.12	170.02	
	Chlorogenic acid	354.31	354.10	
	Wogonin	284.26	284.07	

Note: Compound annotations are tentative and were assigned based on comparison of theoretical molecular masses with published literature and publicly available databases

LC-MS analysis provided a preliminary phytochemical profile of the aqueous extracts by separating constituents according to their chromatographic retention times and mass spectral characteristics. The chromatographic profiles indicated that the G1 and G2 shared several common constituents but also exhibited differences in their chemical composition (Table 1). Based on comparison of the measured molecular masses with published literature and publicly available databases, several compounds were tentatively annotated. The G1 extract was tentatively annotated to contain glabridin, and epicatechin, whereas the G2 extract was tentatively annotated to contain glabridin, gallic acid, wogonin and chlorogenic acid (Table 1). Some of these tentatively annotated compounds, including gallic acid and glabridin, have not, to the best of our knowledge, been previously reported in *B. nivea* leaf extracts. These compound annotations were assigned based on molecular mass matching with published literature and publicly available databases and should therefore

Table 2. MICs results of extracts against *S. epidermidis* and *V. parahaemolyticus*

Test organisms	Plant extracts (mg/mL)	
	G1	G2
S. epidermidis	80	40
V. parahaemolyticus	80	80

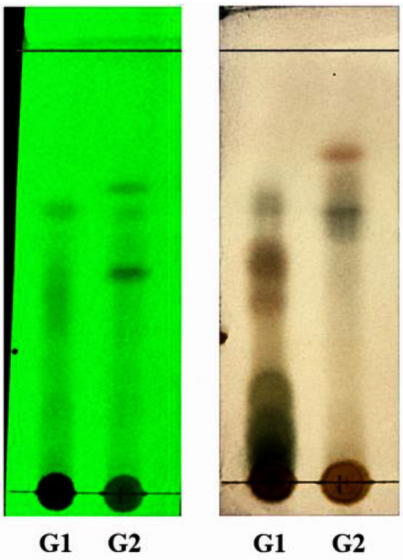


Figure 2. TLC of the G1 and G2. Chromatograms were visualized under UV light (254 nm) (left) and after derivatization with vanillin-H_2SO_4 (right)

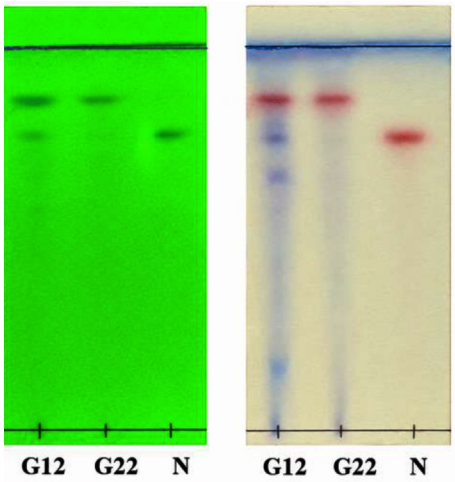


Figure 3. TLC of ethyl acetate fractions (G12, G22) and Narigenin standard (N). Chromatograms were visualized under UV light (254 nm) (left) and after derivatization with vanillin-H_2SO_4 (right)

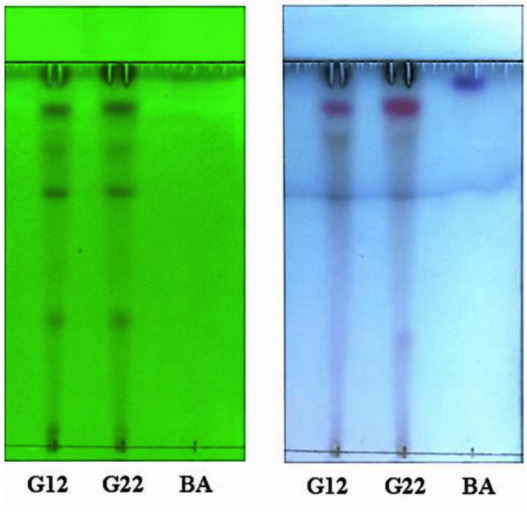


Figure 4. TLC of ethyl acetate fractions (G12, G22) and Betulinic acid standard (BA). Chromatograms were visualized under UV light (254 nm) (left) and after derivatization with vanillin-H_2SO_4 (right)

be regarded as preliminary. Further confirmation using authentic reference standards and MS/MS fragmentation analysis is required to verify their identities.

Total phenolic content quantification

The total phenolic content (TPC) was determined to evaluate the phenolic constituents of the aqueous extracts and their potential contribution to biological activities. Gallic acid was used as the standard, yielding the calibration equation y = 0.0058x + 0.0389 (R^2 = 0.9983), demonstrating the linearity of the analytical method.

The TPC values were 2.7340 ± 0.049 mg GAE/g for G1 and 2.2421 ± 0.010 mg GAE/g for G2 (Figure 5), indicating that extraction at 25 °C yielded a higher total phenolic content than extraction at 60 °C ($P < 0.05$). Although increasing extraction temperature generally enhances mass transfer by improving solute solubility, reducing solvent viscosity, and increasing diffusion rates, elevated temperatures may also accelerate the thermal degradation or oxidation of heat sensitive phenolic compounds.[17] Therefore, the lower TPC observed in G2 suggests that, under the extraction conditions employed in this study, the degradation or transformation of phenolic compounds outweighed the improvement in extraction efficiency associated with the higher extraction temperature.

Total flavonoid content quantification

The TFC of G1 and G2 was quantified by a standard curve generated from Naringenin as the standard equivalent (NE).

The TFC obtained was 0.267 ± 0.049 mg NE/g for G1 extract and 0.581 ± 0.053 mg

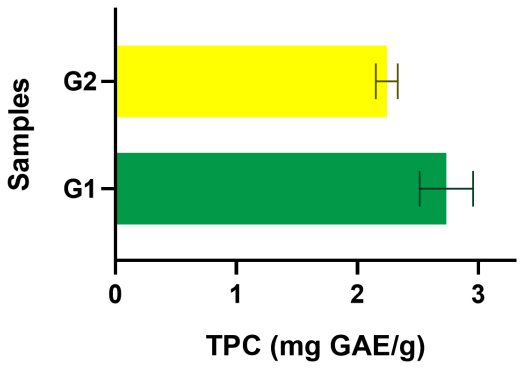


Figure 5. TPC results of G1 and G2 (mg GAE/g)

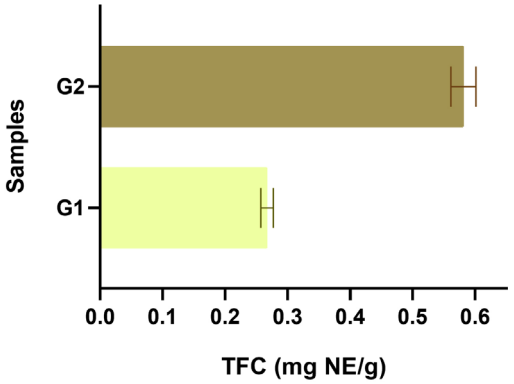


Figure 6. TFC results of G1 and G2 (mg NE/g)

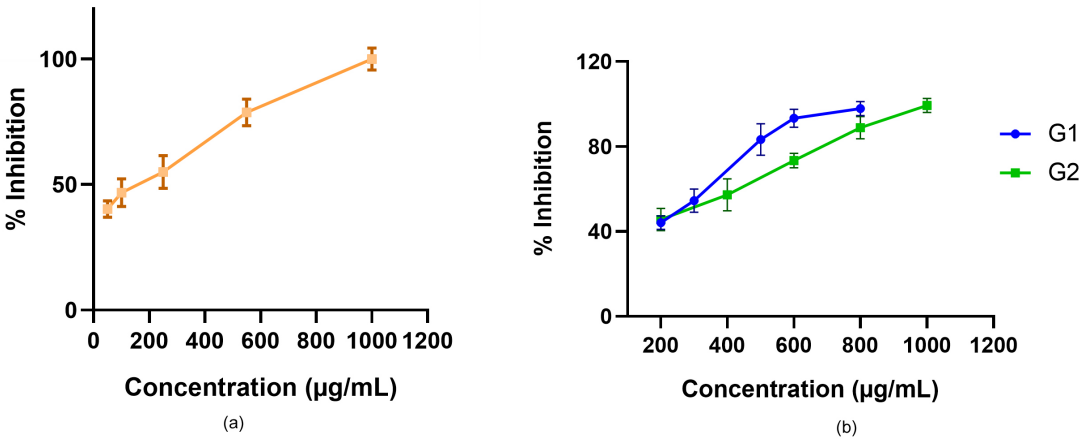


Figure 7. The results of antioxidant property of ascorbic acid (a) and G1, G2 (b)

NE/g for G2 (Figure 6). However, these values are significantly lower, compared to the published reports. Lee et al. reported a much higher TFC of 49.24 ± 0.38 mg/g for an ethanolic extract.[18] The difference was attributed to the different extraction solvents for TFC determination. In addition, biological and environmental factors are significant. Generally, the origin, season of harvest and location of cultivation of the plant samples lead to a difference in the secondary metabolites biosynthesis, which affects the TFC and TPC results.

Antioxidant property determination

The DPPH assay measures the reduction of the stable DPPH radical (deep violet, $\lambda_{max} \approx 517$ nm) to its non-radical yellow form after donation of a hydrogen atom or electron by antioxidants.[18]

The EC_{50} value of ascorbic acid was 99.5 ± 0.072 µg/mL, showing its strong radical scavenging activity compared to G1 and G2. The EC_{50} value of G1 (256.8 ± 0.09 µg/mL) was lower than that of G2 (271.2 ± 0.26 µg/mL) (Figure 7), indicating stronger radical scavenging activity of the G1. This result is consistent with the higher total phenolic content determined for G1, suggesting that phenolic compounds contributed substantially to the antioxidant activity of the extracts.[19] In addition, several tentatively annotated antioxidant related phytochemicals, including epicatechin, chlorogenic acid, and wogonin, may also contribute to the observed radical-scavenging activity.[20-23]

Antibacterial activity determination

The evaluation of the antimicrobial potential was shown in Table 2 using MIC testing to determine the lowest concentration that could inhibit bacterial growth.

Two bacterial strains were subjected to antimicrobial susceptibility testing, and the MIC values are summarized in Table 2. Both G1 and G2 inhibited the growth of *V. parahaemolyticus* at 80 mg/mL. Against *S. epidermidis*, the MIC values were 80 mg/mL (G1) and 40 mg/mL (G2). Results indicate that both samples showed antibacterial activity against the tested bacterial strains, with comparable inhibitory effects under the experimental conditions employed. The antibacterial activity may be caused by the bioactive phytochemicals in the extracts.[24,25] In particular, the higher total flavonoid content observed in G2 may have contributed to its lower MIC against *S. epidermidis*. In addition, tentatively annotated compound (glabridin), have previously been reported to possess antibacterial properties.[26] However, the mechanisms would be confirmed in further study.

CONCLUSION

In summary, the present study demonstrated that extraction temperature influenced the phytochemical composition and biological activities of aqueous *B. nivea* leaf extracts. Preliminary LC-MS based annotation suggested the presence of several phytochemical constituents. Moreover, the aqueous extracts exhibited antioxidant activity and antibacterial activity against the tested bacterial strains. These findings provide preliminary scientific evidence supporting the potential biological properties of *B. nivea* leaves and may be a foundation for further phytochemical characterization and biological investigations. Future studies employing comprehensive phytochemical identification and in vivo validation are required to identify the active constituents and understand their biological activities.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

KTHN performed experimental design, supplying chemicals, bacterial strains and supervised the study. MTUP and PATL performed analysis, data interpretation and wrote the manuscript. KTHN reviewed, revised and approved the final manuscript for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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