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RESEARCH ARTICLE

Phytochemical analysis, antioxidant and cytotoxicity activities of *Clitoria ternatea* L. flower aqueous extract against HaCaT cell line

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Abstract

Clitoria ternatea L., traditionally used in Ayurveda is recognized for its antiparasitic and therapeutic properties. However, there is still limited evidence describing its bioactive components and biological activity using aqueous flower extracts relevant to topical and dermatological applications. This study aimed to determine the phytochemical composition, antioxidant potential and cytotoxic effects of *Clitoria ternatea* L. flower aqueous extract, highlighting its pharmacological relevance and safe application in human skin models. The extract was analysed for total phenolic content (TPC), total flavonoid content (TFC), total anthocyanin content (TAC) and screened for bioactive using liquid chromatography-mass spectrometry (LC-MS). Antioxidant activity was evaluated using DPPH and FRAP assays, while cytotoxicity against human keratinocyte (HaCaT) cells was assessed through 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. The extract demonstrated high levels of phenolics and flavonoids, recording 114.9 ± 0.47 mg GAE/g and 95.5 ± 0.59 mg QE/g respectively at 1000 µg/mL. TAC was 17.67 ± 5.42 mg/L at 10000 µg/mL. LC-MS identified key antioxidant and antiparasitic compounds including rutin, kaempferol and cyanidin-3-rutinoside. Antioxidant assays revealed moderate activity with IC_{50} values of 832 ± 10.22 µg/mL (DPPH) and 662 ± 8.32 µg/mL (FRAP), lower than ascorbic acid or gallic acid. The extract showed no cytotoxicity on HaCaT cells at concentrations below 2 mg/mL, supporting its safety for topical use. These findings advance our understanding of *Clitoria ternatea* L. phytoconstituent profile and support its development for natural skincare or therapeutic applications. Furthermore, the findings also support biodiversity conservation by promoting the sustainable use of medicinal plants.

Keywords: aqueous extraction; butterfly pea; keratinocytes; MTT assay; phytocompounds

Introduction

Clitoria ternatea L. is a perennial climber from the Fabaceae family and is also known as the Asian pigeonwings' pea plant or "*bunga telang*" in Malay. Its flowers are widely used in Southeast Asian cuisine as a natural coloring agent and have been utilized for centuries in Ayurvedic and folk medicine. The plant is rich in secondary metabolites such as flavonoids, anthocyanins, saponins, tannins and phenolic compounds, which contribute to its wide range of pharmacological properties, including antioxidant, anti-inflammatory, antimicrobial, anticancer and wound-healing activities (1). It was reported the antimicrobial and antibiofilm properties of *C. ternatea* L. flower and leaf extracts (2). The "SULAK" drug formulation and ointment for treating leprosy in the Indian Ayurvedic medicinal system incorporates *C. ternatea* L. powdered root water mixes as a primary ingredient (3). The Irulas of Kodiakkarai, India and several communities in Indonesia are among the various global tribal and nomadic ethnic groups that have also been applying *C. ternatea* L. floral paste as treatment for several health issues, such as eye infections, headaches, boils and skin conditions (4). Furthermore, other parts of *C. ternatea* L. have been employed in treating certain health conditions, including skin, indigestion, arthritis, constipation, liver and intestinal diseases (5).

Given these bioactivities, *C. ternatea* L. has emerged as a promising candidate in the search for novel plant-based therapies, particularly against pathogens that affect the skin. One such emerging concern is *Acanthamoeba* commonly found in nature, particularly in soil and water (6). The potential role of *Acanthamoeba* in ecosystems as a host for microbial pathogens and a model organism for mortality has garnered substantial attention. Concerns regarding the ability of *Acanthamoeba* to cause severe infections in humans are also rising from the

growing number of immunocompromised individuals. *Acanthamoeba* is an opportunistic protozoan parasite that typically exists in two forms, trophozoites and cysts (7). During the cyst stage, the amoeba remains dormant, which can lead to human infections. Failure to provide effective monotherapy following initial *Acanthamoeba* infection frequently results in chronic consequences. The amoeba can infect various tissues including the skin, cornea, respiratory tract and central nervous system by breaching epithelial barriers. Clinical manifestations include cutaneous acanthamoebiasis (CA), *Acanthamoeba* keratitis (AK) and granulomatous amoebic encephalitis (GAE) (6). The challenge in treating *Acanthamoeba* infections lies in the pathogen's ability to form double-walled cysts, which are highly resistant to conventional antimicrobial agents, resulting in prolonged infections and increased disease burden (7).

Currently, therapeutic options for *Acanthamoeba* infections are limited, often involving cytotoxic drugs with poor selectivity and side effects on human cells. In this context, there is an urgent need to identify plant-based compounds that can inhibit both the trophozoite and cyst stages and exhibit minimal cytotoxicity to human cells. However, to date, there is limited scientific evidence assessing the effect of *C. ternatea* L. extracts specifically on *Acanthamoeba*-associated infections or on keratinocyte models representing the human skin barrier. Thus, the present study aimed to evaluate the phytochemical composition, antioxidant activity and *in vitro* cytotoxicity of *C. ternatea* L. flower aqueous extract against HaCaT cells, a well-established model for human keratinocytes. By assessing the safety and bioactivity of the extract, this study seeks to support the development of safer, nature-based therapies for *Acanthamoeba*-related skin infections and to fill the existing gap in the literature regarding the skin-protective potential of *C. ternatea* L.

Materials and methods

Sample Collection and Morphological Identification

The blue flowers of Asian pigeon wings (*Clitoria ternatea* L.), or locally known as *bunga telang*, utilized in this study were collected from Jenderam Hulu Village, Dengkil, Selangor, Malaysia at GPS coordinates 2°51'28.6"N101°44'09.0" E. The flowers were sampled in November and December 2022. Standard procedure was followed during the preparation of the herbarium specimen. Identified as *C. ternatea* L., this study deposited the plant in the Forest Research Institute Malaysia (FRIM), Kuala Lumpur, Malaysia, with voucher number SBID: 023/22.

Sample Preparation

One kilogram of *C. ternatea* L. flowers was collected and prepared during the extraction process performed in this study. Following the collection, the flowers were cleaned. Only disease-free, undamaged blue pea flowers were selected for freezing at -80 °C. Subsequently, the flowers were freeze-dried in a Scanvac Coolsafe system in the biopharmaceutic laboratory, Atta-Ur-Rahman Institute for Natural Product Discovery (AuRIns) at -100 ° C for three days (8). The dried flowers were then weighed, establishing their water content before and after drying. Finally, the samples were ground with a blender (8011G Wiring, HGB2NTGA), yielding 122.56 g of flower powder.

Solvent Extraction for Phytoconstituents

This study implemented the maceration method during extraction. Water was employed as the solvent in a 1:30 (v/v) ratio for three days with a magnetic stirrer at room temperature. The extract obtained was then filtered through Whatman™ filter paper (110 mm). A rotary evaporator was utilized to concentrate the flower extract samples, yielding 10 g of the extract, which was 8.16 %. Finally, the solvent-free extract samples were stored in universal bottles and were keep at 4 °C in refrigerator.

Total Phenolic Content (TPC) Quantification

The total phenolic content (TPC) of the crude *C. ternatea* L. flower extract was quantified according to a slightly modified Folin-Ciocalteu method (9). Firstly, 6.52 µL of the sample solution (1 mg of the dry extract dissolved in 1 mL of an aqueous solvent) was mixed with 163 µL of 10 % (v/v) Folin-Ciocalteu's phenol reagent. The solution was then incubated at room temperature in the dark for 5 to 8 min after being thoroughly mixed to ensure homogeneity. The reaction mixture was briefly vortexed after adding 130.43 µL of a 7.5 % (w/v) sodium carbonate (Na_2CO_3) solution. The mixture was kept in the dark for 30 min to ensure colorimetric reaction completion. 96-well plates were employed for each reaction.

The absorbances of the *C. ternatea* L. flower extract reaction mixtures were determined at 765 nm with an ultraviolet-visible (UV-Vis) spectrophotometer (BMG LABTECH SPECTROstar® Nano) with a standard calibration curve constructed with gallic acid solutions provided by Sigma-Aldrich (Saint Louis, MO, USA) with purity more than 90 % between 0 and 1000 µg/mL in an aqueous solvent. The TPC values were expressed in per gram of dry extract in mg of gallic acid equivalents (GAE), ensuring reproducibility and accuracy in every phenolic compound quantification. The approach also offers efficient and reliable plant extract phenolic content estimations, which can be compared directly with the data reported in previous studies. This study provides a foundation for future *C. ternatea* L. flower extract bioactivity studies.

$$\text{TPC (mg GAE/g)} = \frac{(CxV)}{M} \qquad \text{(Eqn. 1)}$$

Where C is the concentration of gallic acid obtained from the calibration curve (mg /mL), V is the volume of the extract (mL) and M is the weight of the extract (g).

Total Flavonoid Content (TFC) Quantification

A stock solution of 1 mg of dried *C. ternatea* L. flower extract accurately measured and dissolved in 1 mL of methanol was employed in establishing the crude total flavonoid content (TFC) of the *C. ternatea* L. flower extract in this study (10). A reaction mixture of 50 µL of the extract solution, 2.15 mL of ethanol, 0.1 mL of aluminum chloride ($AlCl_3$, 10 % w/v), 0.1 mL of potassium acetate (1 M) and 2.8 mL of distilled water was prepared and vortexed to ensure homogeneity. A blank solution served as the control, which was an equal volume of distilled water to substitute for the $AlCl_3$ under similar conditions. All reaction mixtures and blank samples were then incubated at room temperature for 30 min to allow for a complete reaction. This study determined the absorbance of each sample with a UV-Vis spectrophotometer (BMG LABTECH SPECTROstar® Nano) at 415 nm.

The TFC values of the *C. ternatea* L. flower extract was expressed as mg of quercetin equivalents (QE) per gram of dry flower extract based on a calibration curve constructed with quercetin solutions by Sigma-Aldrich with purity more than 90 % (Saint Louis, MO, USA) at 0 to 1000 µg/mL. The reliable and reproducible method was vital for establishing the antioxidant potential of the *C. ternatea* L. flower extract. The protocol also ensured precise determinations of flavonoid levels. The bioactive profile of the extract was procured and compared with available data.

$$\text{TFC QE (mg/L}^{-1}) = \frac{(CxV)}{DE} \qquad \text{(Eqn. 2)}$$

Total Anthocyanin Content (TAC) Quantification

The pH differential method reported (11) has been formally acknowledged as an official analytical procedure by the Association of Official Analytical Chemists (AOAC) International. This study implemented the technique to assess the total anthocyanin content (TAC) of the *C. ternatea* L. flower extract. Based on the guideline, two buffer solutions with distinct pH values (1.0 and 4.5) were required to evaluate the structural changes in anthocyanins under different acidities.

This study dissolved 0.025 M potassium chloride and 0.4 M sodium acetate to procure pH 1.0 and 4.5 buffers respectively (Sigma Aldrich, United States of America). The *C. ternatea* L. flower samples were then subjected to pH differential analyses after being appropriately diluted. The step was performed to ensure that absorbances at 520 nm were within the linear 0.2 to 1.4 range. Accuracy across measurements was also maintained with a consistent dilution factor. Each sample was diluted separately with two buffer solutions at a 1:4 (10 mL:40 mL) dilution ratio. Equilibration was reached by incubating the mixtures at room temperature for 40 min.

The absorbance of each flower extract sample was measured at 520 and 700 nm with a UV-Vis spectro photometer (BMG LABTECH SPECTROstar® Nano). The maximum absorption of anthocyanins in their coloured form was represented by the absorbance at 520 nm, whereas the absorbance at 700 nm was corrected for potential turbidity and light scattering effects. The TAC was calculated using Equation (1) as follows:

$$\text{Total Anthocyanin } \left(\frac{\text{mg}}{\text{L}},\text{ME}\right) = \frac{A*MW*DF*10^3}{\varepsilon*1} \qquad \text{(Eqn.3)}$$

Where A = (A520 nm - A700 nm) pH 1.0 - (A520 nm - A700 nm) pH 4.5, denoting the differences in absorbance at 520 nm and 700 nm within a specific pH range, DF refers to the dilution factor, the molecular weight (MW) for cyanidin-3-glucoside (cyd-3-glu) and malvidin-3-glucoside are 493.43 g/mol and 449.2 g/mol respectively, the pathlength (l) is measured in cm, the molar extinction coefficient, ε, is 26900 L. mol^{-1}. cm^{-1} for cyanidin-3-glucoside and 28000 L. $mol^{-1}.cm^{-1}$ for malvidin-3-glucoside and the factor 103 is employed to convert from g to mg.

Liquid Chromatography-Mass Spectrophotometry (LC-MS) Optimization

This study performed chromatographic separation with a Thermo Scientific C18 column (Acclaim Polar Advantage II, 3 × 150 mm, 3 µm particle size) on an UltiMate 3000 UHPLC system (Dionex). The gradient elusion was conducted at a 0.4 mL/min flow rate and the column temperature was maintained at 40 °C. Water with 0.1 % formic acid (solvent A) and acetonitrile (MeCN) (solvent B) were the mobile phases. A total run time of 22 min and a 3 µL sample injection volume was also employed. The gradient program implemented was 5 % B from minutes 0 to 3, increased linearly to 80 % B for 3 to 10 min, maintained at 80 % B for 10 to 15 min before being reverted to 5 % B for 15 to 22 min.

The mass spectrometry analysis in this study was conducted in the positive ionization mode. A time-of-flight (TOF) analyzer was utilized to obtain accurate mass measurements of the molecular ions, which were then processed with the Compass Data Analysis software (Bruker Daltonik GmbH). The spectrum data obtained from the LC-MS analysis were also processed utilizing several available platforms, including GNPS.

Global Natural Social Molecular Networking (GNPS)

After processing LC-MS/MS data with MZmine, the outputs were exported as a quantitative feature table (in TXT or CSV format) containing ion intensities and an MS/MS spectral summary file in MGF format, which listed the MS/MS spectra linked to the detected ion features. The files were uploaded to the SuperQuick FBMN tool via GNPS with user credentials and an associated email address. The "Feature Generation Tool" was selected for submitting the feature quantification table and the spectral files and the FBMN analysis was executed by selecting the “analyze uploaded files with GNPS Molecular Networking” tab. Direct links to the results were provided via email. Nevertheless, the processing period depends upon the sample size and the analytical instrument employed, which can require between several minutes to a maximum of 10 hr (12).

2,2-Diphenyl-1-picrylhydazyl (DPPH) Radical Scavenging Method

Ascorbic acid served as the standard during the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method performed in this study (13). The procedure assessed the free radical scavenging potential of the *C. ternatea* L. flower extracts. The DPPH (5 mg) was dissolved to yield a 1 mM DPPH solution in 100 mL of methanol, while 50 µL of dimethyl sulfoxide (DMSO) and 200 µL of 1 mM DPPH solution were employed as the control samples. Subsequently, 25 µL of 1000, 500, 250, 150, 125, 62.5, 31.25, 15.625 and 7.8125 µg/mL standard and sample solutions were placed in a 96-well plate. The 1 mM DPPH solution was then added to each well. Under light-restricted conditions, the plate was incubated at room temperature for 30 min. The absorbance of all samples was determined at 517 nm with a UV-Vis spectrophotometer (BMG LABTECH SPECTROstar® Nano).

Scavenging activity and absorbance are inversely linked. An increased activity reflects a diminished absorbance, which can be observed by a purple-to-yellow colour change. Equation (2) was applied to calculate the scavenging capacity of the *C. ternatea* L. flower extract and positive controls towards DPPH free radicals. Radical scavenging activity comparisons were also performed based on the IC_{50} values of the extract samples. IC_{50} values denote the concentration at which 50 % of the DPPH free radical scavenging reactions are inhibited.

$$\frac{\text{A Blank - A Sample}}{\text{A Blank}} \times 100 \quad \text{(Eqn.4)}$$

Ferric Reducing Antioxidant Power (FRAP) Assay

This study slightly modified the FRAP assay reported (14). A fresh FRAP reagent, consisting of acetate buffer (300 mM), 2,4,6-Tris (2-pyridl)-s-triazine (TPTZ) solution (10 mM in 40 mM HCl) and $FeCl_3$ solution (20 mM in distilled water) at a 10:1:1 (v/v/v) volumetric ratio was the first step of the assessment. After incubation at 37 ° C for 10 min, 1.5 mL of the fresh FRAP reagent was mixed with 50 µL of 1.0 mg/mL of the *C. ternatea* L. flower extract in a 90:10 methanol solution (sample solution). The mixture was then incubated at 37 °C for 10 min. The absorbance of each sample was determined at 593 nm with a UV-Vis spectrophotometer (BMG LABTECH SPECTROstar® Nano).

Gallic acid standards at concentrations ranging from 0.0039 to 0.5 mg/mL were employed for constructing the calibration curve in this study. Consequently, the C_{TE} values were directly determined from the absorbance of the *C. ternatea* L. sample solution based on the calibration curve. The values obtained were calculated using Equation (3) as follows:

$$C_{TE}(\text{FRAP})[\mu g/mL] = \frac{A_{\text{Clitoria ternatea}} - B_{Cal}}{M_{Cal}} \quad \text{(Eqn.5)}$$

Culturing of HaCaT Cell Line

The HaCaT human keratinocyte skin cells (CVCL-0038, CLS, 300493) employed in this study were procured from Cell Line Service (CLS), Germany. A Dulbecco's Modified Eagle Medium (DMEM) medium enriched with 10 % fetal bovine serum (FBS) and 1 % penicillin (100 units/mL) and streptomycin (100 µg/mL) was applied for routine maintenance (15). Upon reaching confluence with a uniform monolayer (24 hr), the spent medium was discarded. Trypsin (2 mL) was added to detach the cells, which were subjected to centrifugation at 3500 × g for 5 min. Resuspension of the cell pellets was then achieved with 25 mL of a fresh growth medium. Each well in a 96-well plate was dispensed with 200 µL of the suspension. The filled plate was then incubated in a humidified and 5 % carbon dioxide (CO_2) environment at 37 °C for 24 hr, producing a uniform monolayer when observed under a light microscope.

Cell Viability Assay

The cell viability assay performed in this study was per the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay outlined (16) with minor modifications. The cytotoxic effects of the *C. ternatea* L. flower extract on HaCaT cells were evaluated. Each well of a 96-well plate was first seeded with 1 × 10^4 HaCaT cells and left for 24 hr for cell attachment. *C. ternatea* L. aqueous flower extracts at 500 to 30000 µg/mL were then added and incubated for another 24 hr. After discarding the spent media, phosphate-buffered saline (PBS) was employed to gently rinse the cells.

A complete and freshly prepared medium of 0.5 % MTT was incorporated into each well before incubating them for 3 hr, allowing formazan crystal formation by metabolically active cells. Finally, the MTT-containing medium was removed, while the formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The absorbance of the resulting purple solution, indicative of cell viability was determined spectrophotometrically at 570 nm. All experiments in this study were conducted in triplicate.

$$\text{Percentage (\%)} = \left(\frac{\text{Average}}{\text{Negative Control}}\right) \times 100 \quad \text{(Eqn.6)}$$

Statistical Analysis

A one-sample t-test with a two-tailed distribution in Microsoft Excel was performed to compare the means between two independent groups evaluated in this study. A $p<0.05$ significance level was also adopted for all assessments. The error bars on the y-axis of the graphical data representations indicate the mean ± standard error (SE) derived from experiments conducted in triplicate.

Results

Total Phenolic Content (TPC), Total Flavonoid Content (TFC), Total Anthocyanin Content (TAC) and Extract Yield Quantification

Table 1 summarizes the TPC values of the flower samples. At 7.81 µg/mL, a 5.25 ± 0.94 mg GAE/g extract TPC was recorded, which increased to 19.9 ± 0.67 mg GAE/g extract at 500 µg/mL and 114.9 ± 0.47 mg GAE/g extract at 1000 µg/mL. Based on the results in Table 1, 7.81 µg/mL of the *C. ternatea* L. flower extract recorded a 10.2 ± 0.40 mg QE/g extract TFC value. The value increased to 42.2 ± 0.17 mg QE/g extract at 500 µg/mL of the sample and 95.5 ± 0.59 mg QE/g extract at 1000 µg/mL.

In this study, the TAC of the *C. ternatea* L. aqueous flower extracts assessed was expressed as cyanidin-3-glucoside equivalent mg L^{-1} per gram of extract. According to the data in Table 1, the flower extract documented a 17.67 ± 5.42 mg/L extract at 10000 µg/mL.

Table 1. Total phenolic, flavonoid and anthocyanin contents of aqueous *C. ternatea* L. flower extract at different concentrations

Concentration of aqueous extract (µg/mL)	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QE/g)	Total anthocyanin content (mg/L)
7.81	5.25 ± 0.94	10.2 ± 0.40	
500	19.9 ± 0.67	42.2 ± 0.17	17.67 ± 5.42
1000	114.9 ± 0.47	95.5 ± 0.59	

Each points represents the mean of the results of triplicate experiments. **GAE**: Gallic Acid Equivalent: **QE**: Quercetin Equivalent: mg/L: cyanidin-3-glucoside equivalent. The data expressed as (mean ± SD, n = 3).

Liquid Chromatography Mass Spectrometer (LC-MS) Analysis

The LC-MS analysis of the flower extract in this study revealed key bioactive compounds (Table 2). Rutin, kaempferol and cyanidin were among the predominant compounds detected, which are naturally present in the floral components of *C. ternatea* L.

DPPH Free Radical Scavenging Assay

The antioxidant activities of samples are commonly determined with the DPPH assay. The DPPH absorbs UV-Vis light at 517 nm, yielding a stable free radical. Consequently, the absorbance of a sample decreases in the presence of an antioxidant as it lowers

Table 2. Chemical composition of active compounds in aqueous extract of *C. ternatea* L. flower using LC-MS analysis at positive ESI

No	RT (min)	Compound Name	Formula	Mass	Mz Error PPM
1	6.15	DL-Indole-3-lactic acid	$C_{11}H_{11}NO_3$	188.07	10
2	6.68	Abrine	$C_{12}H_{14}N_2O_2$	188.07	21
3	8.52	3-[4,5-*D*ihydroxy-3-[(2*R*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxy-6-[(2*R*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl]oxy-5-hydroxy-2-(4-hydroxyphenyl)-7-methoxychromen-4-one	$C_{34}H_{42}O_{20}$	755.24	26
4	8.58	Rutin	$C_{27}H_{30}O_{16}$	611.16	7
5	8.76	7-[4,5-*D*ihydroxy-6-(hydroxymethyl)-3-[(2*S*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyloxan-2-yl] oxyoxan-2-yl] oxy-2-(3,4-dihydroxyphenyl)-5-hydroxychromen-4-one	$C_{27}H_{32}O_{14}$	595.16	3
6	8.79	7-O-beta-glucopyranosyl-4'-hydroxy-5-methoxyisoflavone	$C_{22}H_{22}O_{10}$	447.13	19
7	8.94	Kaempferol-3-O-galactoside-6''-rhamnoside-3'''	$C_{33}H_{40}O_{19}$	741.22	11
8	9.05	Kaempferol|3,5,7-trihydroxy-2-(4-hydroxyphenyl) chromen-4-one	$C_{15}H_{12}O$	287.06	3
9	9.08	Cyanidin 3-rutinoside	$C_{27}H_{30}O_{15}$	595.17	6
10	9.11	Kaempferol-3-O-glucoside	$C_{21}H_{20}O_{11}$	449.11	3
11	9.15	Kaempferol-O3-alpha-rhamnopyranoside	$C_{21}H_{20}O_{10}$	617.15	1
12	9.23	Acacetin-7-O-neohesperidoside	$C_{28}H_{32}O_{14}$	593.19	32
13	10.27	Luteolin 7-(6''-malonylglucoside)	$C_{24}H_{22}O_{14}$	535.11	6
14	13.67	Methyl (4*R*)-4-(5*R*,8*S*,9*S*,10*R*,13*R*,17*R*)-10,13-dimethyl-3,6-dioxohexadecahydro-1*H*-cyclopenta[a]phenanthren-17-yl) pentanoate	$C_{24}H_{40}O_4$	827.54	11

the DPPH radical levels. The extent of inhibition reflects the antioxidant capacity of a sample, where IC_{50} represents the concentration required for 50 % inhibition.

The percentage inhibition of DPPH by the *C. ternatea* L. flower aqueous extract is demonstrated in Fig. 1. Based on the findings, the *C. ternatea* L. aqueous flower extract exhibited significant activity with $p<0.001$, achieving an 832 ± 10.22 µg/mL IC_{50}, whereas the standard antioxidant, ascorbic acid, documented 92 ± 5.17 µg/mL of a sample decrease in the presence of an antioxidant as it lowers the DPPH radical levels. Gallic acid (positive control) has a much lower IC_{50} compared to the flower extract, it shows the gallic acid is much stronger antioxidant activity.

Ferric Reducing Antioxidant Power (FRAP) Assay

According to FRAP assay results illustrated in Fig. 2, the *C. ternatea* L. aqueous flower extract exhibited a statistically substantial activity, recording a 662 ± 8.32 µg/mL IC_{50} ($p<0.001$). Meanwhile, IC_{50} of the standard antioxidant ascorbic acid had 68 ± 6.22 µg/mL.

HaCaT Cell Treated with *C. ternatea* L. Aqueous Inhibit the Cell Viability of the Target Cells

Fig. 3 demonstrates the effects of 0.039 to 30 mg/mL of *C. ternatea* L. aqueous flower extract on the viability of HaCaT cells after 24 hr of treatment. The flower extract did not exhibit cytotoxic effects at lower concentrations (0.039 to 1.25 mg/mL). Cell viability also remained above 80 %, with certain concentrations resulting in a slight improvement compared to the control. Conversely, a significant dose-dependent reduction in cell viability was observed at concentrations ≥2.5 mg/mL, dropping below 50 %, indicating the onset of cytotoxicity. The IC_{50} value was estimated at 2.5 mg/mL. The results suggested that the extract was safe at concentrations up to 1.25 mg/mL. Higher doses may compromise cell viability, requiring caution in therapeutic applications. The images of cells treated with 0, 2.5 and 25 mg/mL of the *C. ternatea* L. flower extract are demonstrated in Fig. 4.

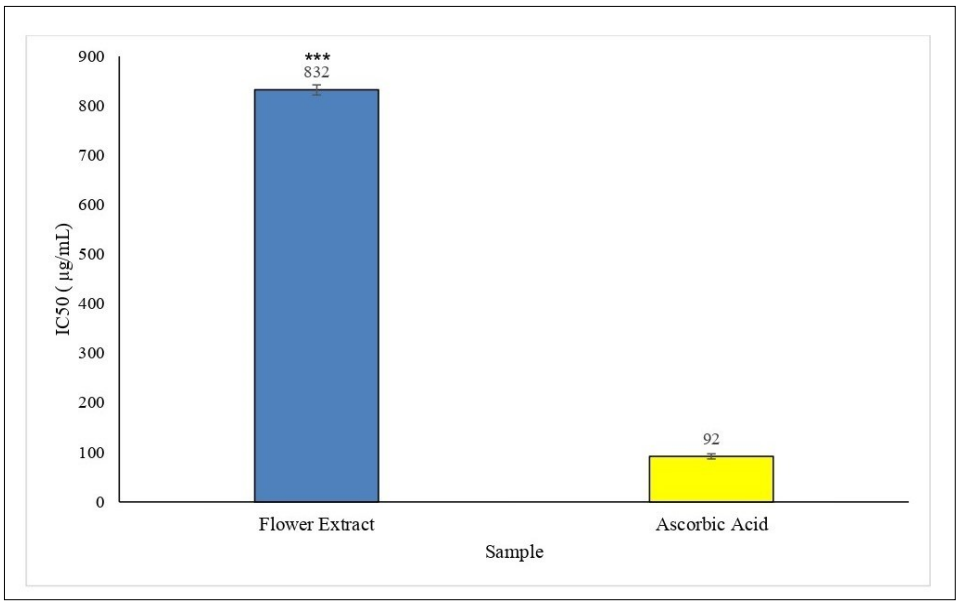

Fig. 1. IC_{50} of *C. ternatea* L. aqueous flower extract and positive control (ascorbic acid) in antioxidant assay. Each value is expressed as mean ± SD (n=3). *** represent a significant different at the level of $p<0.001$.

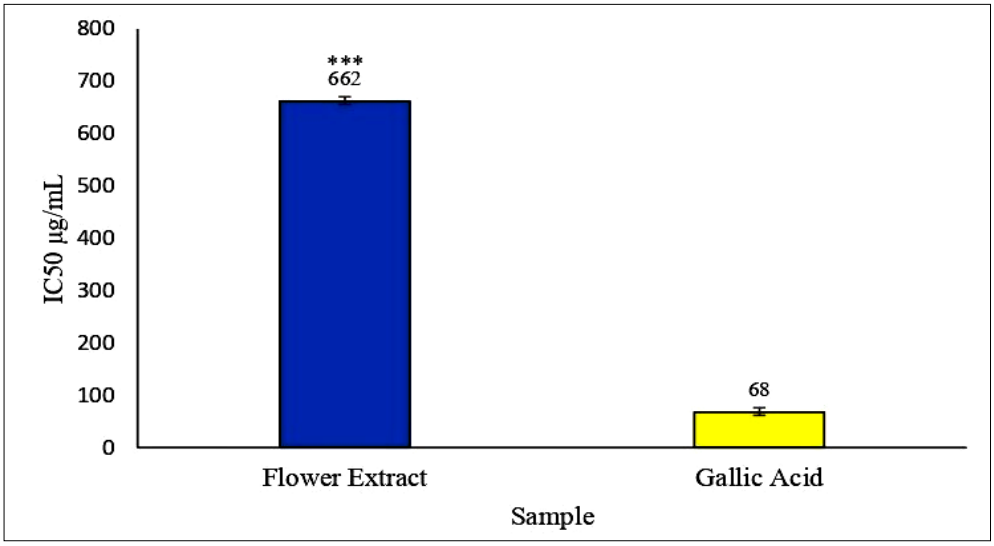

Fig. 2. IC_{50} of *C. ternatea* L. aqueous flower extract and positive control (gallic acid) in antioxidant assay. Each value is expressed as mean ± SD (n=3). *** represent a significant different at the level of p<0.001.

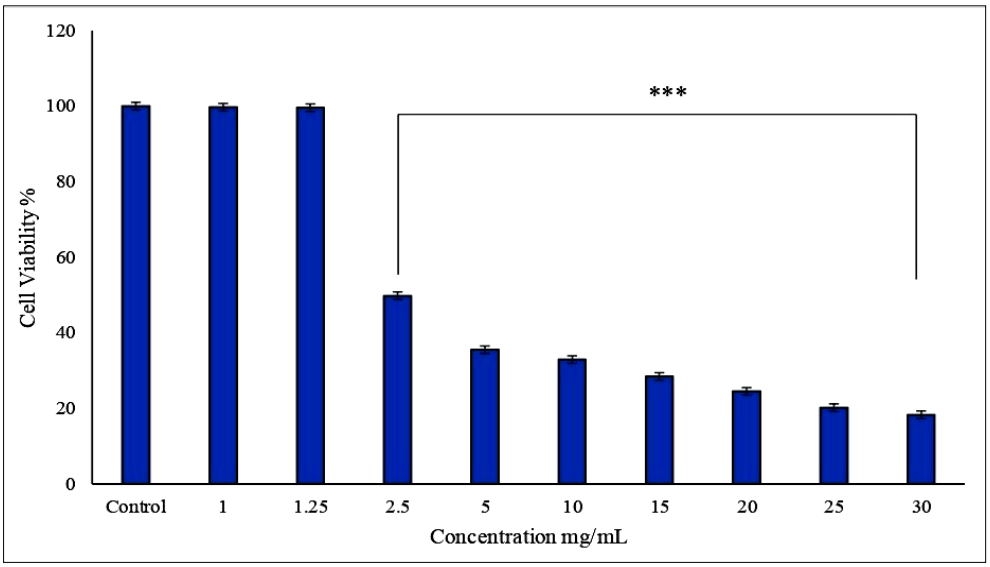

Fig. 3. Effect of *C. ternatea* L. aqueous flower extract on HaCaT cell viability assessed by MTT assay. Concentrations ranged from 1 to 30 mg/mL. Data are expressed as mean ± SD (n=3). Statistical analysis was conducted using an independent t-test; *** indicates p<0.001 compared to control.

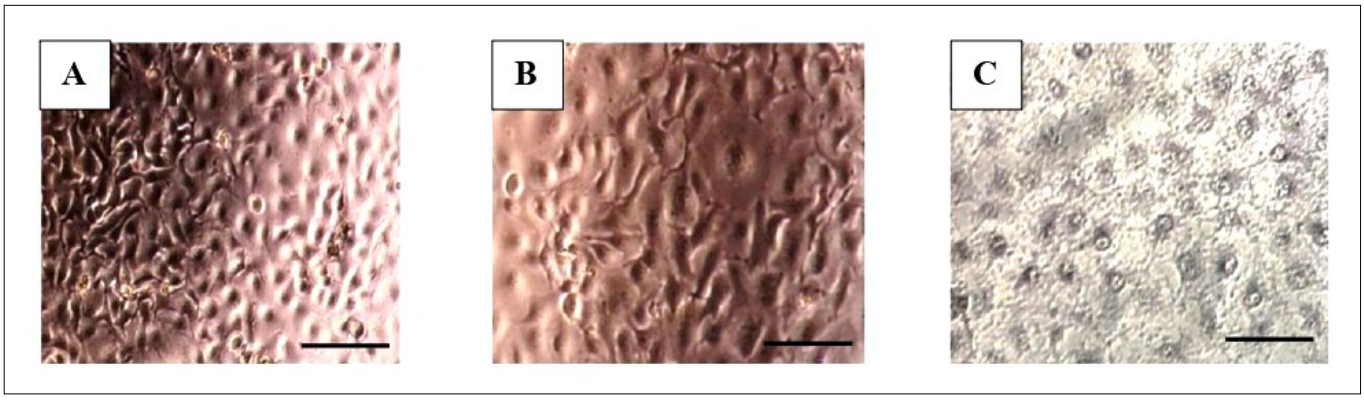

Fig. 4. The image of human skin HaCaT keratinocytes in culture upon *C. ternatea* L. aqueous flower extract compared with control under inverted microscope (10x). (**A**) Control, (**B**) The cell viability of 2.5 mg/mL of *C. ternatea* L. flower aqueous extract and (**C**) The cell viability of 25 mg/mL of *C. ternatea* L. flower aqueous extract. The image shows reduced clarity due to high background interference from the intense coloration of the extract.

Discussions

The wide range of pharmacological properties exhibited by *C. ternatea* L., or commonly referred to as butterfly pea, has gained considerable scientific interest and positioned it as a promising candidate for therapeutic applications (17). However, the present study did not directly assess the antiparasitic effects of *C. ternatea* against *Acanthamoeba in vitro*. These compounds have been previously associated with antiparasitic and cytoprotective mechanisms, including oxidative stress modulation, membrane disruption and inhibition of parasite growth pathways (18). Therefore, we hypothesize that *C. ternatea* L. aqueous extract could be

explored as a supportive anti-*Acanthamoeba* agent. Such potential is especially relevant in developing countries where *Acanthamoeba* poses risks to both agricultural productivity and public health. Furthermore, the sustainable cultivation and use of *C. ternatea* L. aligns with biodiversity conservation strategies, offering a low-cost, eco-friendly source of phytochemicals with therapeutic promise The findings also corroborated with previous report validating the antibacterial, antiparasitic and antifungal properties of traditional skin disease remedies, even against microorganisms resistant to drugs (19). Previous study has characterized anthocyanin-rich or solvent-based extracts of *C. ternatea* using general cell lines such as human embryonic kidney (HEK-293) and mouse macrophage (RAW 264.7) (20). However, none have explored its full aqueous phytochemical profile nor its effect on HaCaT skin keratinocytes. This study fills that gap by quantifying the extract's TPC, TFC and TAC across concentrations. The identifying key flavonoids via LC-MS and evaluating cytotoxicity in HaCaT cells, a model relevant to dermatological and topical applications.

The *C. ternatea* L. aqueous extract exhibited a concentration-dependent increase in TPC. At the lowest concentration assessed, the TPC was 5.25 mg GAE/g extract, which notably rose to 19.9 mg GAE/g before reaching a maximum of 114.9 mg GAE/g extract at 1000 µg/mL. The TPC values recorded in this study were higher than those recorded by Jeyaraj et al. (20) and Azahar et al. (21), which documented 50.4 ± 3.2 mg GAE/g and 106.79 ± 7.14 mg GAE/g respectively. Different extraction and quantification and sample preparation (might be associated with the employment of whole *C. ternatea* L. flowers) might have led to the variation in the results reported.

A study suggested that methanol can improve polyphenol extraction by enhancing penetration into cell structures (22). Nevertheless, the study did not record a TPC value (61.70 ± 0.12 mg GAE/g) higher than the figure documented in this study, indicating that the solubility of bioactive compounds in plant extracts depends on the polarity of the solvents employed. Phenolic compounds contain an aromatic ring with one or more hydroxyl substituents, making them more soluble in water than ethanol. Phenolic compounds are typically located in the vacuoles of plant cells and may combine with sugars, producing glycosides. Plants also contain polyphenols that are either bound to cell wall fibers or floating freely within the liquid medium in the cells. The wide range of polyphenols contributes to the higher TPC levels in water extractions than the methanol counterparts (23).

Flavonoids are secondary metabolites commonly found in plants. The substances are present in almost all parts of a plant, including flowers, roots, seeds and leaves. In this study, rutin and kaempferol were the predominant flavonoids identified in the flower extract. Flavonoids are phenolic compounds and they are crucial antioxidants that donate electrons to prevent the oxidation of free radicals and reactive oxygen species (24). The *C. ternatea* L. aqueous extract evaluated in this study recorded a 10.2 mg QE/g extract TFC, which increased to 42.2 mg QE/g and reached a maximum of 95.5 mg QE/g extract (slightly lower than the TPC at 1000 µg/mL). Previous studies also reported TPC values higher than flavonoids in *C. ternatea* L. flower extract (25). Although TPC levels should theoretically be higher than TFC, different species have varying phenolic compound and flavonoid structures. Furthermore, different TPC assessment methods might result in output variations, considering that TPC is not exclusively ascertained through the Folin-Ciocalteu technique (26).

The TFC levels of aqueous *C. ternatea* L. extracts vary. For instance, Azahar et al. (21) noted a TFC of 85.05 ± 5.01 mg QE/g, while Lakshan et al. (27) documented 42.75 ± 1.74 mg QE/g. The differences might be due to the extraction methodology, as distinct techniques yield varying flavonoid amounts. The TAC of the *C. ternatea* L. aqueous extract procured in this study via the maceration method was slightly lower (17.67 mg/L) than the figures recorded by Destiana et al. (28) (54.55 ± 0.45 mg/L). Similarly, Handayani et al. (29) reported TAC values between 125.24 and 275.53 mg/L utilizing the differential method.

Another study reported that maceration extract yielded a higher level of TFC (53.127 mg QE/g) than reflux (averaging 24.527 mg QE/g) and Soxhlet (recording a mean of 21.060 mg QE/g) (30). Maceration does not require heating, producing the highest flavonoid content, whereas reflux and Soxhlet necessitate heating. Heating during extraction can reduce flavonoid levels by 15 % to 78 %. Increasing temperature can also affect the phenolic contents of samples. Nevertheless, phenol levels can rise to a specific temperature but diminish with higher temperatures. Maceration is a conventional extraction method. Relying on passive diffusion, plants subjected to maceration are soaked in a solvent for an extended period to extract bioactive compounds. Nevertheless, prolonged extraction time can lead to degradation of anthocyanins due to extended exposure to oxygen and light (31). Meanwhile, microwave-assisted extraction (MAE) techniques offer decreased extraction time and solvent and exhibit excellent reproducibility. Despite limitations, maceration remains widely implemented, considering its simplicity and suitability for food and pharmaceutical applications.

Solvent choice can influence anthocyanin extraction. Acidified solvents, such as ethanol or water consisting of a slight acid percentage can improve anthocyanin stability and yield (31). A study noted that the interactions between acidified solvents promote the release of pigments, such as anthocyanins from cell wall membranes, even though the effect depends on the type of acid employed (32). In the study, a 5771.13 ± 54.19 mg/L anthocyanin yield was obtained in an acidified solvent compared to a solvent (3772.28 ± 116.74 mg/L). The variations in the efficacy of a solvent and acid combination may be attributed to the processing of pigment extraction procedures. The effects are particularly significant when a concentration step is necessary, as anthocyanin concentration in a solution may increase with the removal of the extraction solvent. Acid concentration may also rise from the occurrence or prolonged anthocyanins hydrolysis, affecting the final pigment recovery (33).

The antiparasitic potency of *C. ternatea* L. and its antimicrobial, memory-enhancing and anticonvulsant benefits are due to its phytochemical compounds. *C. ternatea* L. flowers possess phytochemical substances, including alkaloids, tannins, glycosides, resin and flavonoids (1). Nonetheless, *C. ternatea* L. roots reportedly have the most phytochemical substances, while the flowers do not. Manjula et al. (34) documented that *C. ternatea* L. flowers contained the second highest levels of flavonoids after the roots at 42 ± 1 mg/mL.

The LC-MS data obtained in this study confirmed the presence of several flavonoid compounds, including rutin, kaempferol-3-O-galactoside-6''-rhamnoside-3'''-rhamnoside, kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl) chromen- 4-one), cyanidin-3-rutinoside and kaempferol-3-O-glucoside in the flower extracts assessed. The antioxidant, anti-inflammatory, anticancer and cardioprotective properties of rutin have been extensively studied (35). Kaempferol is an essential flavonoid in *C. ternatea* L. flower extract. Demonstrating significant anti-inflammatory properties by modulating cytokine expression and suppressing pro-inflammatory signaling pathways, kaempferol is a promising therapeutic application candidate (36). Kaempferol and rutin also improved acetylcholine and cholinergic activities in animal models and can affect helminth activities (34).

C. ternatea L. flowers are blue primarily due to an anthocyanin pigment, cyanidin-3-rutinoside, which also reportedly exhibits protective anti-inflammatory properties. Kongthitilerd et al. (37) also noted the anti-diabetic properties of anthocyanins, including cyanidin-3-rutinoside. In this study, key flavonoids identified via LC-MS namely rutin and kaempferol are well-established antioxidants. Rutin exerts cytoprotective effects through free radical scavenging, enhancement of endogenous antioxidant enzymes such as SOD, CAT, GPx and inhibition of pro-inflammatory cytokines (38). Kaempferol has been shown to suppress oxidative stress-induced apoptosis via modulation of MAPK and PI3K/Akt pathways and to inhibit lipid peroxidation, contributing to cellular membrane protection (39). Both flavonoids are reported to disrupt parasite membrane integrity and interfere with protozoan metabolic pathways, indicating their potential anti-*Acanthamoeba* roles (18). The substance enhances glucose metabolism and modulates insulin sensitivity. Nevertheless, further studies are required to validate the medical potential of cyanidin-3-rutinoside (40). Meanwhile, carbohydrates, alkaloids, tannins, saponins and flavonoids are predominantly responsible for antiparasitic activities of *C. ternatea* L. (41). The data underscored *C. ternatea* L. as a potential source of bioactive compounds with diverse pharmacological benefits, including an antiparasitic agent against *Acanthamoeba*.

Antioxidants in *C. ternatea* L. flowers can prevent several diseases by counteracting reactive and unstable free radicals in cells (42). In this study, the *C. ternatea* L. flower extract demonstrated concentration-dependent increases in radical scavenging capacity. An improved IC_{50} antioxidant activity concerning DPPH (860 ± 0.10 µg/mL) and FRAP (chemical-based) (662 ± 0.10 µg/mL) was observed when water was employed as the solvent. Nevertheless, the inhibition capacity of the flower extract was lower than ascorbic acid and gallic acid, as it is a as pure reference compounds such as ascorbic acid and gallic acid are structurally simple, possess high electron-donating capacity and directly scavenge free radicals with greater efficiency. Jumina et al. (43) described that a lower IC_{50} value means a smaller amount of the substance was needed to inhibit 50 % of the DPPH radicals signifying higher antioxidant activity. The findings were consistent with the TPC and TFC results, revealing a direct correlation between enhanced phenolic and flavonoid contents and the antioxidant capacity of the *C. ternatea* L. flower extract. Additionally, the aqueous nature of the extract limits the solubility and extraction of certain non-polar or moderately polar antioxidant constituents, such as some flavonoid aglycones or phenolic acids, compared to organic solvents like methanol or ethanol (44). The antioxidant mechanisms of flavonoids such as rutin and kaempferol, identified in the LC-MS analysis of this study, may also be slower or involve more complex pathways such as metal ion chelation or modulation of redox-sensitive signaling, which are not fully captured in rapid *in vitro* assays like DPPH (45). Therefore, the comparatively higher IC_{50} value should not be interpreted as a lack of bioactivity but rather as a reflection of the extract's complexity, the moderate efficacy of water as a solvent and the limitations of the assay system.

Although *C. ternatea* L. flower extracts demonstrated potential protective effects against oxidative damage activities, dissimilar antioxidant values have been recorded (25), including IC_{50} values ranging from 0.08 to 4 mg/mL in DPPH assays of solvent or water extracts (46). Lopez-Prado et al. (47) also reported that the anthocyanin-rich *C. ternatea* L. flower water extract demonstrated comparable potency and superior antioxidant activity to its 100 % and 50 % methanol extract counterparts. Meanwhile, no significant differences were observed in the antioxidant activity of butterfly pea flower anthocyanin extracts procured through water and 50 % ethanol extraction in DPPH and FRAP assays (20). The outcomes suggested that distilled water can be effectively utilized for anthocyanin extraction, which permits its application as a natural blue-colored agent with potent antioxidant properties (31).

Human keratinized skin cell (HaCaT) was chosen to represent the outer skin barrier, which was often the first line of defense against environmental pathogens like *Acanthamoeba* (7). Their barrier-forming capability and physiological relevance make HaCaT cells an appropriate *in vitro* model for assessing potential cytotoxic effects of therapeutic agents, particularly those with possible dermal or transdermal exposure routes (48). The cytotoxicity of *C. ternatea* L. extract improved with prolonged incubation and increased extract concentrations, indicating a dose-dependent pattern of diminishing cell viability corresponding to rising cytotoxicity. This study documented the most significant growth inhibition of the HaCaT cell line at 30 mg/mL of the *C. ternatea* L. flower extract, while 2.5 to 30 mg/mL of the extract resulted in the lowest IC_{50} values. The findings indicated the substantial cytotoxic effects of *C. ternatea* L. flower extract on HaCaT cells.

No cytotoxic studies are available on the HaCaT cell line. Previous reports primarily focused on the cytotoxic activities of water and solvent *C. ternatea* L. flower extracts on various cancer cell lines, such as MCF-7 (hormone-dependent breast cancer cell line), K562 (human leukemia cells) and SKBR (human breast carcinoma) cell lines, recording IC_{50} values between 27.2 and 68.2 µg/mL (49). In another study, the solven *C. ternatea* L. flower extracts had IC_{50} values between 36 µg/mL and 57 µg/mL against Dalton's lymphoma ascites (DLA) cells (50). The water and solvent extracts of *C. ternatea* L. flowers were also reportedly cytotoxic to different cancer cell lines below 100 µg/mL and were not toxic up to 100 µg/mL on the normal human foreskin fibroblast (Hs27) cell line. Nonetheless, the cytotoxic concentration on the normal cell line was not determined, considering that the highest concentration evaluated was 100 µg/mL. Conclusively, *C. ternatea* L. flower water extracts were more toxic to cancer cell lines than normal cell lines (22). The observations also contributed to the

cytotoxic activity of *C. ternatea* flower extracts to flavonoids. Nevertheless, further *in vivo* studies are necessary to determine the toxic effects of *C. ternatea* flower extract and stability and bioavailability of other compounds.

Conclusion

Clitoria ternatea L. flower aqueous extract contains multiple bioactive compounds exhibiting antioxidant properties and low cytotoxicity toward HaCaT cells. Its traditional application in treating skin-related conditions is supported by these findings, though further *in vitro* and *in vivo* studies are recommended to validate anti-*Acanthamoeba* activity and safety. The aqueous extract also demonstrated low toxicity and interacted well with human keratinized skin cells. Furthermore, this study aimed to broaden the utilization of traditional medicinal plants, directing more attention to Malaysian plants and acknowledging their usefulness. The data can indirectly contribute to the conservation of biodiversity, as strategies to sustainably employ medicinal plants will be implemented to ensure their future availability.

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Authors' contributions

MAHR collected and performed the analysis of the plant material, statistical analyses and wrote the manuscript. TSA reviewed the manuscript and supervised all activities, participated in field research, data analysis and manuscript preparation. FS and HH supervised all activities and provided technical support for manuscript preparation. All authors have read and agreed to the publication of the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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