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Research Article

HPLC QUANTIFICATION, ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY OF ALKALOIDS FROM *Stephania glandulifera* MIERS

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ABSTRACT

Stephania glandulifera Miers has been extensively used for many years as a traditional medicine; mainly roots, caudex, stem and leaves are used in the treatment of various diseases in the eastern world. This study aimed to standardize the caudex of *S. glandulifera* through the development and validation of a high-performance liquid chromatography method for the quantification of its major compounds. Among the five previously isolated compounds, tetrahydropalmatine (1), palmatine (2), stepholidine (4), and stepharine (5) were successfully quantified for the first time, yielding concentrations of 20.6 mg, 20.4285 mg, 19.962 mg, and 62.262 mg per gram of plant respectively. Chromatographic separation was achieved using an Agilent, Eclipse plus C18 5 µm reversed-phase column with a methanol-water gradient system with a flow rate of 1mL/ min detecting tetrahydropalmatine at 290 nm and other compounds at 190 nm. The antibacterial activity of all the four compounds along with stepharanine (3) was evaluated, with palmatine (2) showing significant efficacy against Staphylococcus aureus ATCC 25925 and vancomycin-resistant Staphylococcus aureus (MIC=0.3225 mg/mL, MBC=0.625 mg/mL). Additionally, stepharanine (3) and stepharine (5) demonstrated notable antioxidant activity (IC_{50}=0.1384 mg/mL and 0.2944 mg/mL, respectively). These highlight stepharine, stepharanine, and palmatine as promising candidates for future drug development. Future research should explore their pharmacological potential and clinical applicability.

Keywords: Antibacterial activity, Antioxidant property, Bioactive compounds, High-performance liquid chromatography, *Stephania glandulifera* Miers

INTRODUCTION

Stephania falls under Menispermaceae family of angiosperm and is the largest among 70 genera. The plants are perennial herbs growing around 4 meters tall with large woody caudex; mostly found in tropical regions (Hao *et al.*, 2015; Wang *et al.*, 2019). These plants have been widely used in the treatment of various diseases such as tuberculosis, dysentery, hyperglycemia, cancer, fever, and inflammation (De Wet *et al.*, 2014). The medicinal importance is due to the presence of alkaloids which are the major phytochemicals in the genus *Stephania* (Semwal *et al.*, 2010a). *Stephania gladulifera* Miers is a glabrous woody climber with scattered lenticels (Forman, 1988). The genus *Stephania* contains eight types of alkaloids: quinolines, isoquinolines, proaporphine, aporphine, protoberberine, tetrahydroprotoberberine, benzylisoquinoline, and morphinans (Desgrouas *et al.*, 2014; Hao *et al.*, 2015). Several studies have reported the antibacterial and antioxidant activity of alkaloids from *Stephania* species. For instance, more than 30 alkaloids from *Stephania glabra*, including bisbenzylisoquinoline, hasubanalactams, berberine, and aporphine, were found to possess antipsychotic, antidiabetic, antimicrobial, and antihypertensive activities (Semwal & Semwal, 2015). Tetrandrine isolated from *Stephania tetrandra* was reported to have antioxidant, immunomodulatory, anti-fibrogenetic, and anti-allergic activities, along with approved clinical use in silicosis treatment (Qiang-min *et al.*, 2002).

In our previous work, we successfully identified and characterized the chemical structures of the major alkaloids (Supp 1.) from *Stephania glandulifera* (Dhungel *et al.*, 2023). Moreover, we demonstrated their promising anticancer potential through in vitro studies. However, the full spectrum of biological activities and the quantitative analysis of these alkaloids have yet to be explored comprehensively. In this study, we investigate the additional biological activities exhibited by these alkaloids including their antibacterial and antioxidant effects. Furthermore, we

employ High Performance Liquid Chromatography (HPLC) to quantify the alkaloid content in specific plant species. HPLC is widely applied analytical techniques used for pharmaceutical analysis commonly used in quantification of compounds (Bhujbal *et al*., 2024). As it is a very sensitive, accurate, precise and reproducible method, it is also being used in purity testing and stability assessment. HPLC is based on the principle of differential interaction of compounds between a mobile phase (liquid solvent) and a stationary phase (packed column material); the mobile phase carries a sample through a column generally used C18 column in reversed phase HPLC (Hussein, 2025). The compounds elute at different retention time detected by UV–Vis or diode array detectors. Quantification is done by comparing peak area with a standard sample (Siddiqui *et al.*, 2017).

Despite above mentioned reports, the antibacterial and antioxidant properties of alkaloids from *S. glandulifera* remain unexplored and their quantitative profile in the plant caudex has not been developed. The lack of comprehensive biochemical characterization impacts the development of standardized formulations and the exploration of these compounds as potential therapeutic agents. Therefore, the present study aims to combine HPLC-based quantification with evaluation of antibacterial and antioxidant properties of major alkaloids from *S. glandulifera* caudex, providing a more complete understanding of their potential applications.

MATERIALS AND METHODS

Materials

HPLC-grade solvents (Thermofisher Scientific, India) were used for quantification via HPLC. Deionized water was purified by the Ultra ClearTM system (Siemens Water Technologies Corp, Germany). HPLC was performed on an Ultrafast Liquid Chromatography (UFLC, Shimadzu High Prominence Liquid Chromatography, Japan) equipped with an LC-20AD Solvent Delivery unit, HPLC Degassing Units, SPD M20A Photodiode Array Detector (PDA), and CTO-20A Column Oven. The physical parameters set up on the HPLC instrument for analysis are as shown in Supp 2. The separation was done on an Agilent, Eclipse plus C18 5 µm reversed-phase column with a dimension of 4.6 ×150 mm (PN 959993-902, SN USUXB05354 and LN B12182). The mobile phases were (A) water and (B) methanol.

The gradient solvent system with methanol and water were used. Table 1 shows the information of the solvent system and flow rate maintained throughout the analysis. The flow rate was set at 1 mL/min with a controlled temperature of 40˚C. PDA detector was set at the wavelength of 290 nm for the detection of tetrahydropalmatine, and 190 nm for palmatine, stepharine, and stepholidine. The injection volume was 10 µL for every sample and standard.

Table 1. Gradient solvent system and flow rate in HPLC.

Time (minutes)	Concentration of methanol in percentage
0.01	10
0.01-10	30
10-20	50
20-30	70
30-35	90
40	Control stops

Quantitative Analysis

Preparation of Calibration Curve of Standard Compounds

Each of the working solutions of all compounds (1, 2, 4 and 5) were separately injected with the HPLC condition mentioned in the experimental section. Further calibration curve of each the compounds were prepared (concentration vs area). The linearity, limit of detection (LOD) and limit of quantification (LOQ) were analysed further for every compounds. Linearity of the method was analysed by injecting at least three of the concentration of compounds from 50- 1000 µg/mL while, signal to noise ratio was calculated under the HPLC condition, LOD was considered as 3:1 and LOQ as 10:1.

Quantification of Compounds in the Plant

Five gram each of powder caudex plant samples was taken in two separate beakers then 100 mL of methanol was added to both the beakers and were left for 24 hours. Sonication was done for 30 mins. The filtrates were taken after filtration and concentrated with rotatory evaporator to obtain crude extract with both samples. Further, 1 mg/mL concentration of each crude extracts (A and B) were prepared and filtered with a syringe filter before injection. Both the extracts were separately injected in the already set up HPLC

condition and further the peak of all compounds was analysed; taking their peak area, concentration of each of the compounds was calculated in both the cases. The analysis was done to duplicate and average concentration was resulted to calculate the average amount (g) of compounds present in the plant (Figure 1).

Biological Activity

All the chemicals were purchased from HiMedia (HiMedia Laboratories, India) unless otherwise specified.

Determination of Antioxidant Activity

Preparation of DPPH and Quercetin Solution

1, 1- Dimethyl-picrylhydrazyl (DPPH) with a concentration of 0.1 mM was prepared in methanol. The stock solution of quercetin (0.1 mg/mL), used as a positive control was prepared in methanol and serially diluted to obtain diluents with a concentration of 10, 20, 30, 40, and 50 µg/mL.

Procedure

Plant extracts/compounds (1 mg/mL) were diluted to a 10^{-7} dilution (10:100 ratio) with 50% DMSO/water. Each well of a microtiter plate contained 100 µL of the diluted solution. Then, 100 µL of DPPH solution was added. After incubation at 37ºC for 30 minutes, absorbance was measured at 517 nm using a multi-plate reader (EPOCH, Biotek). The percentage of DPPH free radical scavenging activity was calculated using a formula.

$$\text{Percentage scavenging} = \frac{\text{As} - \text{Ao}}{\text{Ao}} \times 100$$

Where,

Ao=absorbance of the DPPH solution

As= absorbance of the DPPH free radical solution containing the sample.

IC_{50} values for significant results were calculated using Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA)

Antibacterial Activity Assay

Antibacterial assay of purified compounds and solvent extracts were performed by agar well diffusion method in Mueller-Hinton Agar (MHA) (Figure 1). Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were also evaluated by agar dilution method recommended by Clinical and Laboratory Standards Institute (CLSI, 2020).

Preparation of Inoculums

Bacterial strains including VRSA (Vancomycin resistant *Staphylococcus aureus*), carbapenem-resistant *Klebsiella pneumoniae*, multi-drug resistant *Pseudomonas aeruginosa*, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25925, and *Klebsiella pneumoniae* ATCC 700603, were cultured in nutrient broth for 18 hours at 37ºC. The culture density was adjusted to 0.5 McFarland standards (approximately 10^8 CFU/mL) using Muller Hinton Agar Broth (MHB). The pathogenic strains, except ATCC strains, were obtained from the Institute of Medicine, TUTH, Maharajgunj, Kathmandu, while ATCC strains were collected from Sukraraj Tropical & Infectious Disease Hospital, Teku, Kathmandu.

Preparation of Compounds and Plant Extracts

Compounds and extracts with a concentration of 5 mg/mL and 50 mg/mL was prepared with 50% DMSO solution and stored in 4ºC.

Screening and Evaluation of Antimicrobial Activity

Mueller-Hinton Agar (MHA) media was prepared and dried. Organism inoculum was swabbed onto the media plates. Wells were made using a sterile cork borer (4 mm). Compound/extract solution (40 µL) was added to the wells, along with negative and positive controls. After settling for 30 minutes, the plates were incubated at 37ºC for 24 hours. The zone of inhibition, indicated by a clear area without growth, was analysed.

MIC values were analysed for pure compounds with strong antibacterial activity. A 5 mg/mL stock solution, prepared in 50% DMSO/water, was serially diluted (2.5 mg/mL to 0.0195 mg/mL) using MHB. After 3-4 hours of incubation at 37ºC, 0.02% resazurin was added to the microtiter plate to determine the MIC. Purple colour indicated bacterial death, while pink showed viability (Elshikh *et al.*, 2016). The concentrations showing MIC, along with others, were streaked on nutrient agar media to determine MBC after 24 hours of incubation.

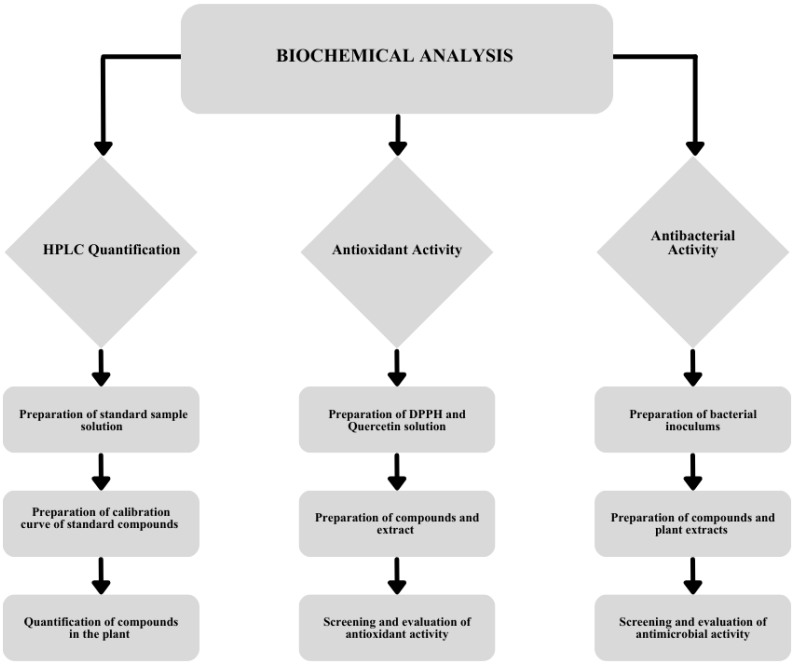

Figure 1. Flow chart showing entire process of Quantification and biological analysis of compounds from *Stephania glandulifera* Miers.

RESULTS AND DISCUSSION

Quantitative Analysis

Preparation of Calibration Curve of Standard Compounds

The chromatograms revealed consistent retention time for all concentrations of the compounds, with varying peak heights. Higher concentration of the compounds resulted in taller peak chromatograms and larger peak areas. Supp 3-6, depict the stacked chromatograms of compound 1, 2, 4, and 5 respectively. Calibration curves (Supp 7-10) were plotted to relate the area coverage to the concentration of compound dilutions. The data for corresponding concentration and area of compounds 1, 2, 4, and 5 can be found in Supp 11-14, respectively. Using Microsoft Excel, these calibration curves were utilized to determine the LOD and LOQ for compounds 1, 2, 4, and 5, as shown in Supp 15, Supp 16, Supp 17, and Supp 18, respectively. The calibration curve of each compound has provided the linearity of the method validation with good correlation coefficient (Supp 15, Supp 16, Supp 17, and Supp 18).

Quantification of Compounds in Extract

From 5 g each of plant powder, 0.731 g (A) and 0.6563 g (B) of methanol extract was obtained. The area of each of the compounds in the chromatogram of injected plant extracts (A and B) was taken for quantification. As shown in the Figure 2, the peak of the compounds 1, 2, 4 and 5 were obtained at retention time 32, 38, 25 and 23 minutes respectively. The average concentration of each of the compounds in both the extract A and B were calculated. This finally yielded the amount of each compound per gram of plant which is shown in the Supp 19-22.

The calibration curves generated for compounds 1, 2, 4, and 5 exhibited excellent linearity, confirming that the analytical method was strong and reliable for quantitative assessment. The strong correlation coefficients reflect good sensitivity of the HPLC method, consistent with findings from similar phytochemical studies conducted elsewhere. For example, recent HPLC analyses of alkaloids from *Stephania cambodica* and *Stephania venosa* reported comparable linearity and sensitivity (Dary *et al*., 2017; Kongkiatpaiboon *et al*., 2017), supporting the validity of the present methodology.

The quantification of compounds from methanol extracts exhibited consistent recoveries and stable retention times. Such reproducibility is essential for pharmacological evaluation and aligns with international reports on the reproducibility of alkaloid analysis in *Stephania* species. These results highlight the suitability of caudex extract of *Stephania glandulifera* as a reliable method for alkaloid profiling.

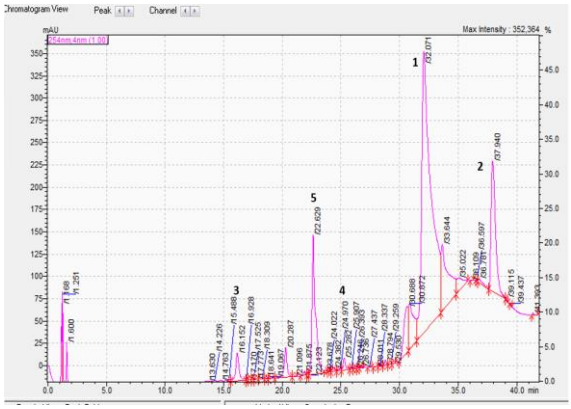

Figure 2. Chromatogram showing all five isolated compounds present in the caudex extract of *Stephania glandulifera*

Biological Activity

Antibacterial Assay

Antibacterial effects of pure compounds and extracts were tested. Neomycin (1mg/mL) served as a positive control, while Dimethyl Sulphoxide (DMSO) was the negative control. Compound 2 showed antibacterial activity against VRSA and *Staphylococcus aureus* ATCC (Supp 23). No other compounds exhibited antibacterial effects. Zone of Inhibition (ZOI) of control and compounds against respective organisms are presented in Supp 27. Extracts also showed antibacterial activity (Supp 24, Supp 25-26).

MIC was determined for compound 2 against VRSA, yielding a MIC value of 0.3125 mg/mL (Figure 3). MBC assessment confirmed its bactericidal nature, with an MBC value of 0.625 mg/mL (Figure 4). Studies have shown that palmatine exhibited significant antibacterial activity against MRSA and *Helicobacter pylori* (Long *et al.,* 2019). Palmatine and tetrahydropalmatine demonstrate bactericidal effects with MIC values of 0.312 µg/mL and 0.156 µg/mL, respectively (Shi *et al.,* 2015).

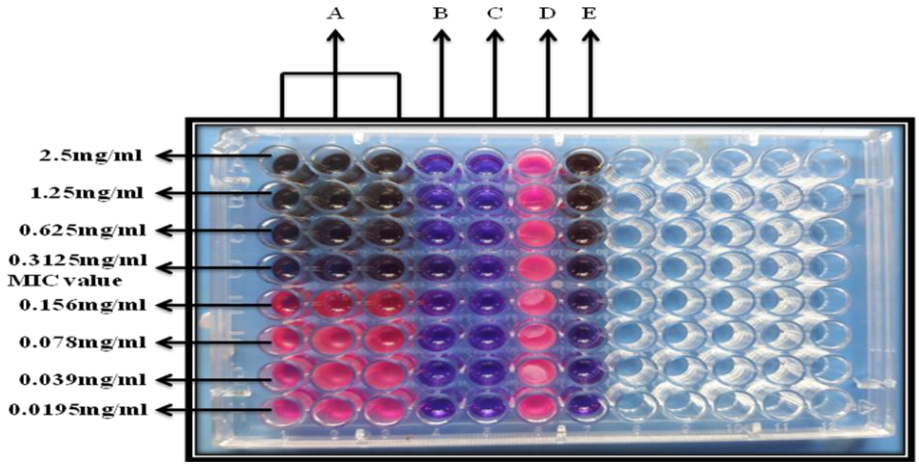

Figure 3. Determination of MIC of compound 2 against VRSA.

A= Triplicate sample, B= MHB, C= positive control (neomycin), bacteria and MHB, D= Bacteria and MHB and E= Compound with MHB

Figure 4. Determination of MBC of compound 2 against VRSA.

DPPH Free Radical Scavenging Activity

Antioxidant properties of extracts, compounds, and the standard antioxidant quercetin were assessed using DPPH, a stable free radical with maximum absorption at 517 nm. DPPH is initially purple but turns yellow when it accepts a hydrogen atom from a free radical scavenging antioxidant, forming reduced DPPH-H (Ainsworth *et al.,* 2007). A calibration curve for quercetin was established (Y=0.0118x+0.0052, R^2=0.9892) as shown in Supp 28. Absorbance at 517 nm was measured for various dilutions of 0.1 mg quercetin. Compound **3** and **5** demonstrated superior percentage scavenging activity at 72.67% and 66.46%, respectively. Their IC_{50} values were calculated as 0.1384 mg/mL and 0.2944 mg/mL, respectively (Fig. 5). Methanol extract and acidic fraction also exhibited significant scavenging activity at 71% and 57%, respectively. Their respective IC_{50} values are summarized in Figure 5. Fangchinoline and cepharanthine from *Stephania rotunda* Lour showed antioxidant activity (Semwal *et al.,* 2010a). The ethanolic extract of *Stephania rotunda* Engl tubers demonstrated antioxidant activity with an IC_{50} value of 212.29 µg/mL (Singh *et al.,* 2019). Palmatine and stepharanine from *Caulis mahoniae* exhibited antioxidant activity with IC_{50} values of 0.20 mg/mL and 0.59 mg/mL, respectively (Zhu *et al.,* 2016).

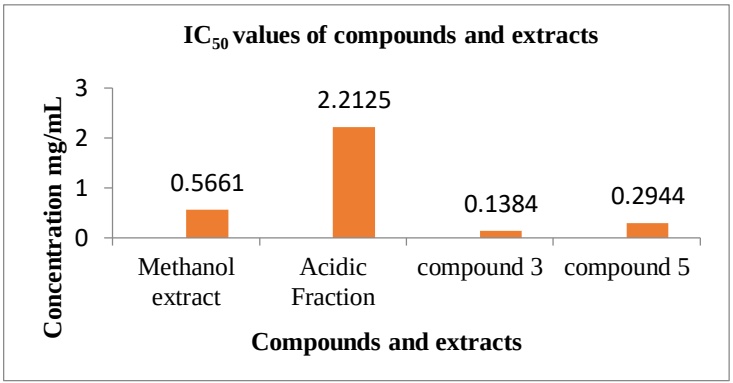

Figure 5. IC_{50} values of compounds and extracts in mg/mL

CONCLUSIONS

This study successfully standardized and quantified four alkaloids from the caudex extract of *Stephania glandulifera* using a validated HPLC method, ensuring accuracy, reliability, and linearity of detection. Among the isolated compounds, palmatine (2) demonstrated notable antibacterial activity, particularly against VRSA, highlighting its potential as a promising compound for developing new antibacterial agents. Similarly, stepharanine (3) and stepharine (5) exhibited strong antioxidant properties, suggesting their possible application in antioxidant drug discovery. Further studies should include *in vivo*, evaluations of the isolated compounds to confirm their therapeutic potential. Additionally, expanding toxicological and pharmacokinetic assessments will help determine their suitability for future pharmaceutical development.

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AUTHOR CONTRIBUTIONS

Conceptualization: JD, SLS; Investigation: JD, SLS; Methodology: JD, SLS; Data curation: SLS; Data analysis: JD, SLS; Writing - original draft: JD, SLS; Writing - review and editing: JD, SLS.

CONFLICT OF INTEREST

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

DATA AVAILABILITY STATEMENT

The corresponding author can provide the data used to prepare figures and tables on the request of publisher.

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