JNCS Journal of Nepal Chemical Society January 2026, Vol. 46, No. 1, 83-93
ISSN: 2091-0304 (print)
DOI: https://doi.org/10.3126/jncs.v46i1.91125
RESEARCH ARTICLE

Phytochemical Analysis and Bioactivity Assessment of Dichloromethane and Hexane Extracts of *Catunaregam spinosa* (Thunb.) Tirveng

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(Received: December 17, 2025, revised: January 23, 2026 accepted: January 30, 2026)

Abstract

Medicinal herbs have long been used by Nepalese communities to cure a variety of ailments, from mild to lethal. The purpose of this study is to estimate the phytochemicals and determine the antioxidant and antibacterial properties of dichloromethane (DCM) and hexane stem bark extracts of *Catunaregam spinosa* (Thunb.) Triveng. Thin-layer chromatography of extracts using different ethyl acetate/DCM ratios revealed multiple spots, indicating the presence of several non-polar molecules with changing affinity for the mobile phase as well as their chemical variety. The total phenolic content (TPC), total tannin content (TTC), and total flavonoid content (TFC) were determined using the Folin-Ciocalteu phenol reagent and the aluminum chloride colorimetric technique. The DCM crude extract of the stem bark had greater TPC and TTC values of 54.83 ± 1.80 mg GAE/g and 167.58 ± 6.70 mg TAE/g, respectively, but a lower TFC value of 7.65 ± 1.16 mg QE/g compared to the hexane extract. The antioxidant activity of plant extracts was tested in vitro using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. DCM (IC_{50} 60.18 ± 0.49 µg/mL) showed stronger antioxidant activity than the hexane extract (IC_{50} 72.68 ± 0.26 µg/mL). The agar well diffusion method was used to test the antibacterial activity against *Shigella sonnei* (ATCC 25931), *Escherichia coli* (ATCC 25912), *Klebsiella pneumoniae* (ATCC 700603), and *Staphylococcus aureus* (ATCC 43300). Since the study found that plant extracts contain an abundance of phenolic and flavonoid compounds, this medicinal plant could be the source of a natural antioxidant and antibacterial agent for future drug development processes.

Keywords: Antibacterial; Antioxidant; *Catunaregam spinosa*; Phytochemicals; Plant extract.

Introduction

The plant kingdom comprises a diverse spectrum of species that contain physiologically active compounds with therapeutic potential. Many plant-based compounds have been isolated, identified, and brought into international markets by pharmaceutical corporations with success. Several scientific investigations have shown that phytocomponents play an important role in the prevention of a wide range of illnesses [1]. Plant chemicals like polyphenols, flavonoids, and carotenoids have strong antioxidant and anti-inflammatory properties. Polyphenols are plant-derived chemicals distinguished by their phenolic structure. Their medicinal properties are increasingly exploited in food manufacturing and fortification [2]. These compounds protect cells from oxidative stress and inflammation-related damage by neutralizing harmful free radicals, which can protect nerve cells, reduce inflammation in the brain, and improve neural plasticity, potentially benefiting people with chronic diseases such as Alzheimer's and Parkinson's [3,4].

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Certain plant compounds, such as alkaloids and essential oils, are antiviral and antibacterial. These compounds can inhibit the growth and replication of bacteria, viruses, and fungi. They are serious in fighting infections and enhancing immune system function, which aids in the stoppage and treatment of a variety of infectious diseases. Certain plant compounds, such as Omega-3 fatty acids found in seeds and nuts, as well as flavonoids from fruits and vegetables, have been demonstrated to improve cardiovascular health. These compounds reduce cholesterol, blood pressure, and blood clot formation, all of which minimize the risk of heart disease and stroke [5]. Many studies have been conducted in recent years to study the healing properties of higher plants with ethnobotanical histories for a variety of reasons, including (1) concern about the potential side effects of allopathic medicine, (2) the lower cost of phytotherapy, and (3) the fact that many herbal remedies have been successful in replacing allopathic medicines in relieving disease symptoms. As the rise of multidrug-resistant and pan-drug-resistant microorganisms, as well as antibiotic abuse, has posed a significant challenge to synthetic drug researchers, they are now focusing on the advancement of safe biologically active plant-derived chemicals for use in the development of innovative medications [6,7].

Nepal is a Himalayan country with a diverse ecosystem due to its topography, geography, and climate changes within a short geographical area [8]. It is believed that diverse ethnic groups in Nepal employ about 2000 plant species to address their primary healthcare needs [9, 10]. In many cases, the type of plant used for therapy, the extract derived from it, and the manner of application are all based on Eastern Hemisphere folk wisdom passed down through generations [10, 11].Additionally, the geographical distribution of these plants has been seen among ethnic groups. Such communities have used medicinal plants to treat human diseases, and some of these practices are part of Ayurveda [12].

Catunaregam spinosa (*C. spinosa*) is a *Rubiaceae* plant also known as Mainphal or Emetic nut [13]. The species was originally common as undergrowth in the Terai region, but its numbers are quickly dwindling. *C. spinosa* fruits are edible and have a strong astringent taste due to their high tannin content. The fresh fruits are high in carbohydrates and saponins, which are used as a tonic, alternative, demulcent, diuretic, and restorative [14–16]. The seeds contain a high concentration of organic acids and essential oils. The dried and powdered fruit pulp has been associated with emetic properties. Dysentery and diarrhea can be treated using the bark. *C. spinosa* also had hypoglycemic, Piscicidal, insecticidal, nauseating, expectorant, antihelmintic, abortifacient, and anticancer activities [16]. Many compounds have been found from various parts of *C. spinosa*, such as fruit and stem bark, but they have not been properly investigated for prospective drug discovery paths [17, 18]. It is crucial to comprehend this medicinally significant plant and its scientific advancement. Thus, the goal of this research is to collect and study the phytochemicals and pharmacological activity of *C. spinosa* stem bark in two non-polar solvents: DCM and hexane, gathered in Nepal's Terai region, where research is somewhat downplayed in comparison to the Himalaya and mountain regions. The study also employs principal component analysis (PCA) to link phytochemical composition with antioxidant and antibacterial activity. Some compounds reported in *C. spinosa* (Thunb.) Triveng has been shown in **Figure 1**.

Materials and Methods

Chemicals and Equipment

Merck and Sigma-Aldrich supplied the analytical-grade chemicals and reagents used in the experimental section of this investigation. The major pieces of equipment used included a microplate reader (Epoch 2, Biotech

Instrument, Inc., USA), a micropipette (Erba Biohits), a water bath (Clifton), an incubator, and a Buchi RE111 rotavapor.

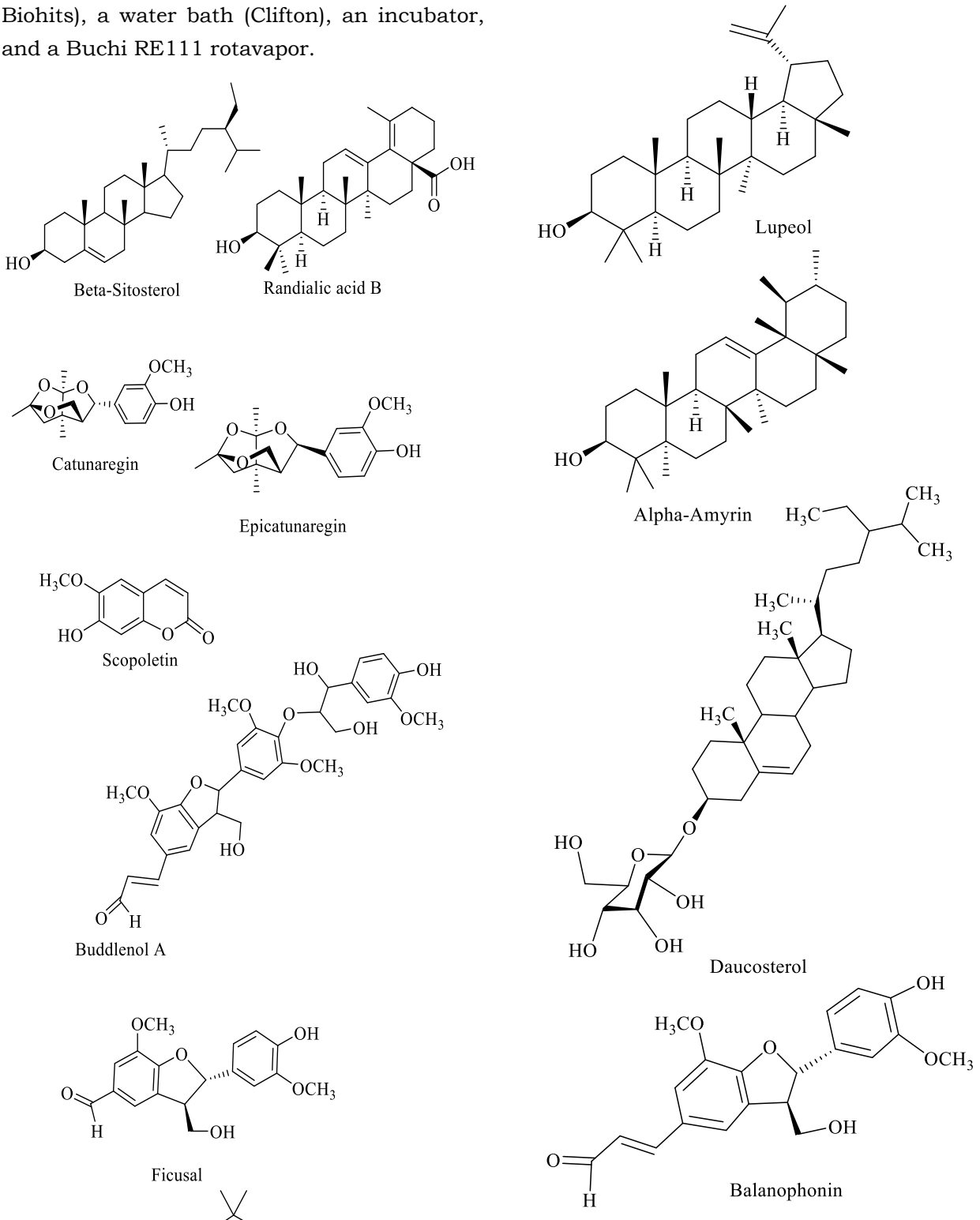

Figure 1: Some compounds reported in *C. spinosa* (Thunb.) Triveng

Plant Sample Collection and Identification

A plant sample was collected from the Maanjhundi community forest, Badhaiyatal-3, Bardiya, Nepal, in their original, wild habitat. The local population recognized the herb's healing properties. The herbarium was

submitted for identification at the National Herbarium and Plant Laboratories in Godavari, Lalitpur, Nepal (Voucher code RB002). **Figure 2** depicts *Catunaregam spinosa* (Thunb.) Triveng.

Figure 2: *Catunaregam spinosa* (Thunb.) Triveng

Extraction of Plant Metabolites

The sample was washed with tap water and allowed to dry in the shade before being crushed into a fine powder that was utilized immediately for the extraction process. Cold percolation was employed to convert powdered plant components into extracts based on solvent polarity. Each sample was weighed, then dipped into 500 mL conical flasks containing a 1:6 ratio of solvents (DCM and Hexane), which was filtered, and subsequently dried in a rotary evaporator at a pressure of approximately 650 mmHg and a temperature of approximately 40°C. Extracts were then further dried using a hot water bath at approximately 27°C, as described in [19], and stored in the refrigerator for future tests.

Phytochemical Analysis

The initial qualitative phytochemical analysis of plant extracts was conducted using color differentiation techniques [20].

TLC separation of the secondary metabolites

Thin-layer chromatography was performed to separate secondary metabolites present in the plant extracts based on a standard procedure [21]. For this, the generated DCM and hexane extract (1 mg) were transferred to a vial and diluted with 1 mL of acetone. The mixture was then vortexed, and TLC profiling was carried out using a particle-free solution. Spotting and development spots were manually placed on TLC plates using a capillary tube. The plates were subsequently developed in a developing chamber with various solvent systems containing ethyl acetate/hexane mixtures (50, 30, and 20%). The produced plates were dried and examined in an iodine chamber.

Estimation of Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and Total Tannin Content (TTC)

The total phenolic, flavonoid, and tannin contents of the plant extracts were determined using standard spectrophotometric colorimetry methods based on standard procedures [22,23]. Total phenolics were determined using the Folin-Ciocalteu test. For this, 20 µL of each plant extract (1 mg/mL) solution was added with 100 µL of diluted Folin-Ciocalteu reagent at varying doses of 3.75, 7.5, 15, 30, 60, 80, and 100 µg/mL, and 80 µL of a 1 M sodium carbonate (Na_2CO_3) solution. The absorbance was measured at 765 nm, and results were reported in mg gallic acid equivalents per gram of extract (mg GAE/g). The $AlCl_3$ technique was used to determine total flavonoid concentration. For TFC, 100 µL of distilled water, 60 µL of ethanol, 10 µL of potassium acetate, and 10 µL of a 10% $AlCl_3$ solution were mixed with 20 µL of each extract. The absorbance was measured at 415 nm, and the results were expressed as milligrams of quercetin equivalents per gram of dry weight (mg QE/g DW). Total tannins were determined using the Folin-Ciocalteu technique, with tannic acid as the standard. For TTC, 10 µL of extract was mixed with 70 µL of distilled water, followed by the addition of 50 µL of FCR and 70 µL of 1 M sodium carbonate. The absorbance was measured at 725 nm, and the results were reported in mg tannic acid equivalents per gram of extract (mg TAE/g). All investigations employed appropriate calibration curves.

Antioxidant Activity

The DPPH assay was used to determine each extract's ability to scavenge free radicals consistent with the approved protocol [24, 25]. Quercetin was used as a positive control at

dilutions of 20, 10, 5, 2.5, 1.25, and 0.625 µg/mL in methanol. A mixture of 100 µL of each plant extract and 100 µL of 0.1 mM DPPH reagent was incubated in the dark for around 30 minutes. The absorbance at 517 nm was then measured. The concentration needed to neutralize 50% of DPPH radicals (IC_{50}) might be calculated by plotting scavenging activity against extract concentration. The DPPH potential (percent inhibition) for each extract sample was calculated using the formula below:

$$\text{Radical scavenging activity (\%)} = \frac{A0-As}{A0} \times 100$$

A_s stands for the absorbance of the sample extract, and A_0 for the absorbance of the control blank.

Antibacterial Assay

Four bacterial pathogens were investigated for antibacterial efficacy: one Gram-positive bacterium, *Staphylococcus aureus* (ATCC 43300), and three Gram-negative bacteria, *Escherichia coli* (ATCC 25912), *Shigella sonnei* (ATCC 25931), and *Klebsiella pneumoniae* (ATCC 700603). Antibacterial Activity was tested using the agar well diffusion method following standard protocol [26,27]. Following injection into Muller-Hinton Broth (MHB), the pathogens were cultured at 37°C. Adjusting the turbidity to the standard 0.5 McFarland yielded a final inoculum of 1.5×108 CFU/mL. Each set included 50 µL extract sample dissolved at 50 mg/mL in 50% DMSO, a positive control of 1 mg/mL neomycin, and a negative control of 50% DMSO. After 15 minutes of diffusion at room temperature, it was incubated for 24 hours at 37 °C. After incubation, the ZOI (mm) around the well was determined.

Statistical Analysis

The TPC, TFC, TTC, and IC_{50} values in the DPPH test were calculated using Microsoft Excel and GraphPad Prism 9.5.1 software. The relevant graphs were then generated. The triplicate findings were reported as mean ± standard deviation.

Results and Discussion

Phytochemical Analysis

Certain compounds or the synergistic combination of many chemicals in plants provide them with therapeutic capabilities. In this study, the phytochemical analysis found promising results for flavonoids, phenols, steroids, and tannins. Saponins are present in DCM, whereas anthraquinones are found in hexane. Both extracts lack alkaloids and terpenoids analogous to [28,29]. *C. spinosa* has the potential to be a fantastic source of phytonutrients as it possesses a wide range of pharmacological activity, including antioxidant, antimutagenic, antipyretic, anti-venom, anti-inflammation, anti-cancer, hepatoprotective, abortifacient, hemolytic mollisidial, and anthelmintic properties [30].

TLC Separation of the Secondary Metabolites

Ascending chromatography shows that different phytochemicals in the single extract produce different spots in various solvent systems. This study revealed that the increase in the number of TLC spots with decreasing ethyl acetate and increasing DCM indicates improved separation of non-polar compounds, as on TLC paper. The less polar mobile phase reduces solvent strength and limits hydrogen bonding with the stationary phase, allowing non-polar molecules to migrate based mainly on van der Waals interactions and minor differences in molecular size and polarizability [31], thereby resolving multiple non-polar constituents present in the DCM and hexane extracts. This difference in phytochemical spots provides an important clue to their polarity and aids in selecting a suitable solvent for further separation of pure components using column chromatography.

Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and Total Tannin Content (TTC)

Findings show that phenolic compounds occur in substantial amounts in the plant. The DCM crude extract had a TPC of 54.83 ± 1.80 mg GAE/g, while the value was 11.43 ± 0.78

mg GAE/g in the hexane crude extract. The DCM extract (167.58 ± 6.70 mg TAE/g) and the hexane extract (25.93 ± 2.10 mg TAE/g) had different TTC levels. Similarly, Hexane extract has a TFC (43.76 ± 2.63 mg QE/g) and the DCM extract (7.65 ± 1.16 mg QE/g). **Table 1** shows TPC, TFC, and TTC of DCM and hexane extracts of *C. spinosa*. **Figure 3** displays a bar diagram of TPC, TFC, and TTC of DCM and hexane extracts of *C. spinosa*. Here, the DCM stem bark extracts had higher TPC and TTC in the comparative analysis of *C. spinosa*. In contrast, the hexane extract had higher TFC, comparable to [32], where the DCM extract exhibited a higher TPC (84.63 ± 9.01 mg GAE/g) than the hexane extract (74.04 ± 7.07 mg GAE/g), but a considerably higher TFC (97.14 ±1.74 mg QE/g) than the DCM extract (59.88 ± 4.25 mg QE/g).

Table 1: TPC, TFC, and TTC of DCM and hexane extracts of *C. spinosa*

Phyto constituents	Extracts	
	DCM	hexane
Total phenolic content (mg GAE/g)	54.83 ± 1.80	11.43 ± 0.78
Total flavonoid content (mg QE/g)	7.65 ± 1.16	43.76 ± 2.63
Total tannin content (mg TAE/g)	167.58 ± 6.70	25.93 ± 2.10

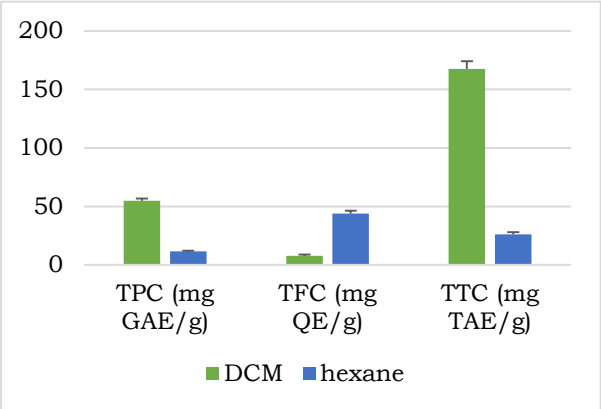

Figure 3: TPC, TFC, and TTC of DCM and hexane extracts of *C. spinosa*

Antioxidant Activity

Free radicals in the body can cause cell damage and contribute to a range of illnesses, while antioxidants can eliminate and repair them [33]. It is well known that plant metabolites, such as polyphenolic compounds, are potent antioxidants because they can function as reducing agents, hydrogen donors, and singlet oxygen scavengers [34]. The percentage results of radical scavenging achieved by conventional Quercetin and plant extracts at various doses are given in **Table 2** and **Figure 4**. **Figure 5** displays a bar diagram of the free radical scavenging by DCM and hexane extracts. The outcomes indicate that both stem bark plant extracts have the potential to be antioxidants due to their lower IC_{50} values. The IC_{50} value of the hexane extract was 72.68 ± 0.26 µg/mL, whereas the DCM extract was 60.18 ± 0.49 µg/mL. There is a significant positive correlation between TPC, TTC, and IC_{50}, showing that when TPC and TTC levels rise, antioxidant activity rises, and vice versa. These findings suggested that even nonpolar chemicals may have strong antioxidant activity based on their polarity, consistent with [35]. The investigation identified indicators of dose-dependent antioxidant power, and the results suggested that DCM extract is a superior DPPH free radical inhibitor than hexane extract, similar to [36], which showed that at 300 µg/mL, the DCM extract maintained its significant reducing activity (93.66%), outperforming the n-hexane extract (83.67%).

Table 2: IC_{50} of stem bark crude extracts of *C. spinosa* in two different solvents

Extracts/Standard	IC_{50} (µg/mL) Mean ± SD
Quercetin	3.83 ± 0.00
DCM	60.18 ± 0.49
Hexane	72.68 ± 0.26

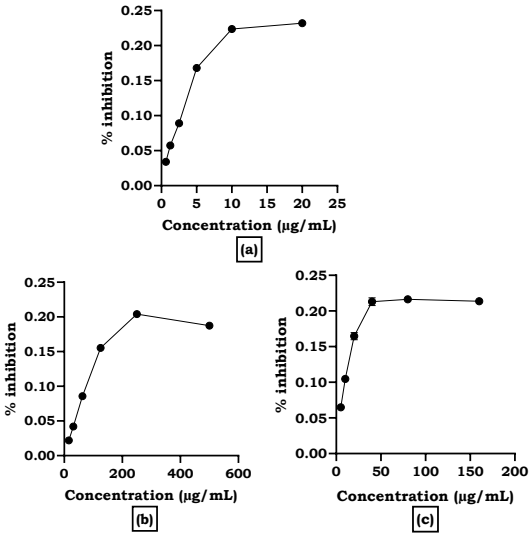

Figure 4: Standard curve of DPPH inhibition by (a) Quercetin, (b) DCM, and (c) hexane extract

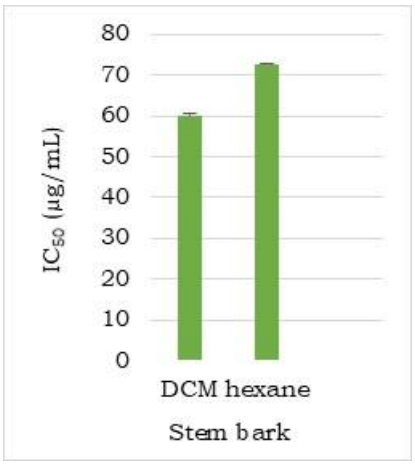

Figure 3: Free radical scavenging activity of DCM and hexane extract

Antibacterial Activity

Hexane extract had a ZOI of 10 mm/20 µL against *Staphylococcus aureus*, 13 mm/20 µL against *Shigella sonnei* and *Klebsiella pneumoniae*, and 9 mm/20 µL against *Escherichia coli*. DCM inhibited *Shigella sonnei* (18 mm/20 µL), *Klebsiella pneumoniae* (14 mm/20 µL), *Staphylococcus aureus* (11 mm/20 µL), and *Escherichia coli* (10 mm/20 µL) more effectively than hexane. Results of antibacterial screening of crude extracts against four distinct pathogens are shown in **Table 3** and **Figure 4**. **Figure 5** compares the ZOI of DCM and hexane extract against four distinct pathogens graphically. Furthermore, phenolic content has been directly linked to antibacterial activity, and it has been demonstrated that the active crude extract, that is, the DCM extract of stem bark, was a more potent antibacterial agent than the hexane crude extract, comparable to [37]. This study also indicates that gram-negative bacteria were inhibited more strongly than gram-positive bacteria, consistent with [38, 39]; however, the findings are quite opposite to [40], where dichloromethane extract was rarely effective against the microorganisms tested. Slight differences in the findings may depend on several variables such as plant species, collection time, geographical location, and other environmental factors.

Table 3: Results of antibacterial screening of crude extracts against four distinct *pathogens*

ZOI (mm)	Extracts/Positive control		
	DCM	hexane	PC
Staphylococcus aureus	11	10	28
Shigella sonnei	18	13	29
Klebsiella pneumoniae	14	13	29
Escherichia coli	10	9	28

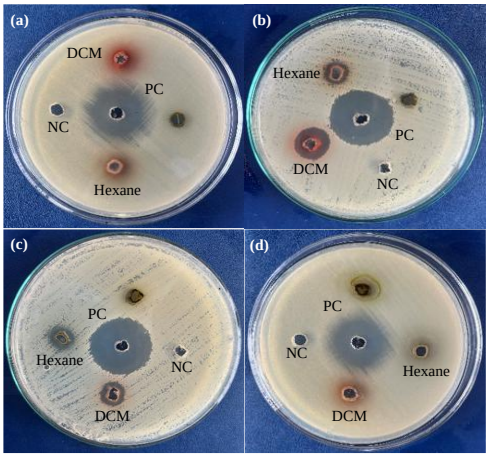

Figure 4: ZOI of crude extracts against four pathogenic microbes: (a) *Staphylococcus aureus*, (b) *Shigella sonnei*, (c) *Klebsiella pneumoniae*, and (d) *Escherichia coli* (Where DCM stands for dichloromethane, PC and NC stand for positive and negative control)

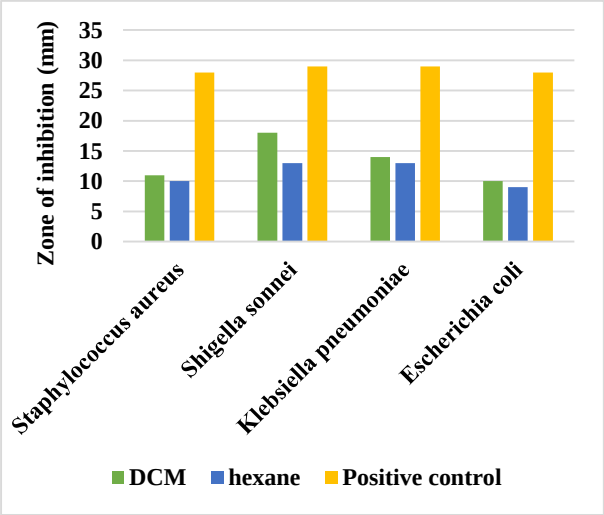

Figure 5: ZOI (mm) comparison of DCM and hexane extract against four distinct pathogens

Conclusions

The current study suggests the presence of medicinally relevant bioactive chemicals in *C. spinosa*, which could have major implications for innovative drug discovery. TLC study of phytochemicals showed great sensitivity and separation, allowing for the isolation, purification, and identification of active components in extracts using various chromatographic and spectroscopic techniques. Based on the findings, this study concludes that the plant has the potential to be employed as an antioxidant and antibacterial agent. As a result, several phytochemical screens and therapeutic effects of this medicinal plant must be investigated in toxicity studies in animal models, as well as elucidated for potential clinical applications in the treatment of human diseases.

Acknowledgements

We would like to express our sincere gratitude to the National Herbarium and Plant Laboratories, Godawari, Lalitpur, Nepal, for the identification of the plant. The Institute of Biomolecule Reconstruction at Sun Moon University, Republic of Korea, for supplying the bacterial strains.

Author's contribution statement

R. Baraili: Drafted the manuscript, contributed to reviewing and editing the text, and conducted the experiment. **K. R. Sharma**: Conceptualized and supervised the study, wrote and edited the manuscript, and prepared the final version of the draft.

Conflict of interest

We declare that we have no conflict of interest

Data availability statement

All of the data produced in this research will be provided if requested.

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