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RESEARCH ARTICLE

# Phytochemical and Bioactivity Analysis of *Colebrookea oppositifolia* Sm. from Nepal's Upper Hills

**Bimal G.C.[1], Indra Ojha[1], Ram Chandra Basnyat[1] and Khaga Raj Sharma[1]***

[1]*Central Department of Chemistry, Tribhuvan University, Kirtipur, Kathmandu, Nepal*

**Corresponding E: mail: khaga.sharma@cdc.tu.edu.np*

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## Abstract

Traditional medicine has long utilized herbal medicine to treat a wide range of illnesses. The purpose of this work was to quantify the levels of flavonoids and phenolic compounds in various solvent extracts and to analyze their antioxidant and antibacterial properties. Ethanol had the higher total flavonoid content (TFC) at 10.58 ± 0.002 mg QE/g and total phenolic content (TPC) at 142.88 ± 0.06 mg GAE/g among the extracts tested. The methanolic extract exhibited stronger antioxidant activity compared to the ethanolic extract, with an $IC_{50}$ of 10.34 ± 2.31 µg/ml. Both methanolic and ethanolic extracts effectively inhibited *Staphylococcus aureus*, showing a zone of inhibition of 15 mm and 16 mm, respectively, which is nearly closer to the positive control, neomycin (22 mm), according to antibacterial testing. In contrast to the control (0.0152 mg/mL and 0.0313 mg/mL), the methanolic extract displayed significant minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values against *Staphylococcus aureus* (12.5 mg/mL and 25 mg/mL, respectively). These results indicate that the examined plant extracts possess substantial bioactive potential, particularly the methanolic and ethanolic extracts, making them promising sources for the isolation of active compounds that could be drug candidates in the future drug development process against infectious diseases.

Keywords

**Keywords:** *Colebrookea oppositifolia* Sm., TPC, TFC, DPPH, Antimicrobial, MIC, MBC.

## Introduction

Since ancient times, medicinal plants have been utilized to manage and treat illnesses because of therapeutic ingredients that have long been vital to human health. These plants either act as direct therapeutic agents or serve as foundational sources for drug development, owing to the presence of bioactive compounds distributed across different plant organs [1]. Medicinal plants can be categorized based on scientifically validated therapeutic properties or on an ethnopharmacological basis, which often lacks comprehensive scientific evaluation. This underscores the need for further research into these natural resources [2]. Natural substances derived from plants, animals, and microorganisms have a long-standing history in traditional medicine. Fossil records suggest that humans have used plants for medicinal purposes for at least 60,000 years. Over millions of years, the evolutionary development of chemical diversity in these natural compounds has resulted in a wide range of biologically active substances with promising pharmacological potential [3]. Among these, phytochemicals produced by plants serve essential functions in plant physiology, including growth, pollination, and defense against pathogens [4]. In addition, they help to

protect plants from environmental stressors such as diseases and ultraviolet (UV) radiation [5]. Primary and secondary metabolites are two categories into which phytochemicals can be divided. Primary metabolites, such as proteins, carbohydrates, and nucleic acids, are vital for the basic metabolic processes of plants. In contrast, secondary metabolites such as phenolics, flavonoids, and terpenoids are synthesized through specialized pathways and are often linked to protective functions, including antibacterial, antifungal, and antiviral activities [6, 7]. These secondary compounds are of significant interest in pharmacological research due to their ability to combat oxidative stress, a factor associated with the progression of degenerative diseases like cancer, atherosclerosis, and gastric ulcers [8]. Despite its widespread use, very few studies look at the biological characteristics and phytochemical composition of *Colebrookea oppositifolia* Sm. stem portion. To fill this research gap and expand our knowledge of *Colebrookea oppositifolia* Sm. therapeutic potential, this study analyzed the phytochemical contents and assessed the biological activities of the stem section. *C. oppositifolia* Sm., commonly known as Dhursul, Binmeuli, or Indian squirrel tail, is predominantly found in Nepal as well as Bhutan and India. It is characterized by a densely branched, white tomentose stem and ternately whorled, elliptic-lanceolate leaves. The plant bears white panicled spikes as inflorescences, with a hairy calyx, a short corolla tube, and four stamens. The ovary is bilocular (with two locules), and the plant is typically found at elevations up to 1500 meters. It is noted for its bitter taste and lack of odor [9]. The present study focuses on the collection, identification, and phytochemical and biological activities of the plant extracts.

## Materials and Methods

### Chemicals

For this study, high-purity, analytical-grade solvents, methanol, and ethanol, were procured from Merck and Fischer Scientific to ensure efficient and consistent extraction. Essential reagents, including Folin-Ciocalteu (FC) reagent and resazurin, along with microbial culture media such as Mueller-Hinton Broth, Nutrient Agar, and Mueller-Hinton Agar, were sourced from HiMedia and LOBA CHEMI Pvt. Ltd. The use of high-quality solvents, reagents, and culture media was critical to ensuring accuracy and reliability in both the phytochemical analyses and microbiological assays.

### Plant Collection and Identification

The stem portion of *C. oppositifolia* Sm. was collected from the Far Western region of Nepal (Sayal Gaupalika-02, Doti). The specimen was authenticated at the National Herbarium and Plant Laboratories, Godawari, Lalitpur, Nepal, and received a voucher code KATH163317. A visual representation of the plant is provided in **Figure 1.**

**Figure 1.** *Colebrookea oppositifolia* Sm.

### Extract Preparation

To preserve its phytoconstituents, the plant material was washed carefully after collection, and then shade-dried. To ensure a uniform consistency for future use, it was ground into a fine powder using the grinding mill. Approximately 20 grams of *C. oppositifolia* Sm. powder was extracted with 200 mL of methanol and ethanol. The mixtures underwent maceration, with intermittent shaking every 24 hours over three days. After maceration, the extracts were filtered, and the filtrates were concentrated using a rotary evaporator at a controlled temperature of 40-45 °C [10]. The extracts obtained were in a semi-solid form (sticky), which was used for further analysis.

**Qualitative Phytochemical Analysis**

Qualitative phytochemical screening was performed using standard protocols to identify various metabolites present in *C. oppositifolia* Sm. [11]. Phytochemicals such as glycosides, flavonoids, alkaloids, phenolic compounds, terpenoids, steroids, carbohydrates, saponins, fixed oils, and lipids were targets of the screening. This analysis provided an overview of the bioactive constituents within the plant extract.

**Estimation of Total Phenolic Content (TPC)**

The total phenolic content (TPC) of the plant extracts was determined using the Folin-Ciocalteu colorimetric method, as described by Lu et al [12]. 20 µL of plant extract, 100 µL of 10 % Folin-Ciocalteu reagent (diluted 1:10), and 80 µL of 1M $Na_2CO_3$ were used to construct 96-well plates in triplicate. At room temperature, the reaction mixture was incubated for half an hour until a blue color appeared. The absorbance was then measured with a spectrophotometer at 765 nm. Using a gallic acid standard curve that ranged from 7.5 to 100 µg/mL, TPC was measured in milligrams of gallic acid equivalent per gram of extract dry weight (mg GAE/g).

**Estimation of Total Flavonoid Content (TFC)**

The total flavonoid content of the plant extracts was assessed using the aluminum chloride method as described by Ahmed et al. [13]. 96-well plates were filled with 20 µL of plant extract, 100 µL of distilled water, and 60 µL of ethanol in triplicate. 10 µL of a 10 % aluminum chloride ($AlCl_3$) solution and 10 µL of a 1M potassium acetate ($CH_3COOK$) solution were then added. For half an hour, the reaction mixture was incubated at room temperature. A spectrophotometer was then used to detect absorbance at 415 nm. The total flavonoid content was written as milligrams of quercetin equivalent per gram of extract dry weight (mg QE/g), with quantification based on a standard calibration curve for standard quercetin having the value from 7.5 to 100 µg/mL.

**Evaluation of Antioxidant Activity**

The positive control, quercetin, was serially diluted from the original concentration of 20 µg/mL down to 0.625 µg/mL, while the crude plant extract was diluted from 640 µg/mL to 5 µg/mL. In triplicate, 100 µL of each diluted plant extract and the positive control were added to a 96-well plate. The initial absorbance was recorded at 517 nm. Following this, each well received 100 µL of DPPH reagent, and the mixture was incubated for 30 minutes. The final absorbance was measured again at 517 nm. Methanol and 50% DMSO served as negative controls. The formula given below quantifies the ability of the extracts to scavenge free radicals, providing insight into their antioxidant potential [14].

Free radical scavenging capacity can be calculated as

$$\frac{Abs\ control - Abs\ sample}{Abs\ control} \times 100\ \% \quad \ldots\ldots (1)$$

Where, Abs control = absorbance of the control, Abs sample = absorbance of a sample

Inhibitory concentration ($IC_{50}$) was calculated using GraphPad Prism (version 8.0.2.263)

**Antibacterial Activity**

Antibacterial activity was assessed using the agar well diffusion method on Mueller-Hinton Agar (MHA) plates [15]. The test microorganisms, which included *Escherichia coli* (ATCC 25312), *Klebsiella pneumoniae* (ATCC 700603), *Staphylococcus aureus* (ATCC 43300), and *Shigella sonnei* (ATCC 25931), were grown in Mueller Hinton Broth (MHB) and incubated for 24 hours at 37 °C. To ensure consistent bacterial density, the broth's turbidity was adjusted to a 0.5 McFarland standard. A cork borer created wells in the agar plates, which were then filled with 50 mL of the plant extract. Negative control wells received 50% DMSO, while positive control wells were filled with 50 % neomycin. The Petri dishes were allowed to diffuse for 15 minutes before being

incubated at 37 °C for 18 to 24 hours. The plant extract's antibacterial effectiveness was assessed by measuring and observing the zones of clearance after incubation.

**Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)**

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined following the standard procedure described by Sarker et al. [16]. The 0.5 McFarland turbidity culture was diluted 1:100 in Mueller-Hinton Broth (MHB) to create the bacterial inoculum, which was then diluted to a final concentration of 106 CFU/mL. A 5 µL inoculum was then introduced into each well of a 96-well plate. Neomycin served as the positive control in this assay. After sealing the plate with a sterile lid, it was incubated at 37 °C for 20 to 24 hours. The plates were incubated for three to four further hours at 37 °C after adding 0.003 % resazurin to each well. Wells that remained blue indicated no bacterial growth, while those that turned pink signified the presence of bacterial growth. The minimum inhibitory concentration (MIC) was the lowest concentration of the extract at which bacterial growth was inhibited. To determine the MBC, the contents of the wells were streaked onto nutrient agar plates, which were then incubated for more than eighteen hours at 37 °C. The lowest bactericidal concentration of the crude plant extracts was demonstrated by the lack of bacterial colonies on the agar plates.

**Statistical Analysis**

Data collection and analysis from the Gen5 Mi-Microplate Reader were conducted using Microsoft Excel. Total flavonoid content (TFC) and total phenolic content (TPC) results were presented as mean ± standard deviation. The results for antioxidant activity were presented as mean ± standard error of the mean (SEM). The inhibitory concentration (IC) values were computed using GraphPad Prism software (version 8.0.2.263). This analytical approach ensured a comprehensive evaluation of the data obtained from the experiments.

## Results and Discussion

**Qualitative Phytochemical Analysis**

The results of preliminary phytochemical analysis of *C. oppositifolia* Sm. extract in two different solvents is displayed in **Table 1**.

**Table 1** Qualitative phytochemical screening of plant extract

| Phytochemicals | Methanolic extract | Ethanolic extract |
|---|---|---|
| Alkaloids | + | + |
| Flavonoids | + | + |
| Phenolic compounds | + | + |
| Steroidal compounds | + | - |
| Tannins | + | + |
| Cardiac glycosides | + | + |
| Saponins | + | - |
| Carbohydrates | + | + |
| Terpenoids | + | + |

**Total Phenolic Content (TPC)**

The phenolic content of the two solvent extracts of *C. oppositifolia* Sm. is presented in **Table 2**. According to the table below, the methanolic extract had a lower total phenolic content (TPC) value of 121.18 ± 0.04 mg GAE/g, however, the ethanolic extract had a higher TPC value of 142.88 ± 0.06 mg GAE/g. These results highlight the slight variation in phenolic compound concentrations among the two different solvent extracts.

**Table 2** TPC of methanolic and ethanolic extract

| Plant Extracts | TPC (mg GAE/g) |
|---|---|
| Methanolic | 121.18 ± 0.04 |
| Ethanolic | 142.88 ± 0.06 |

**Total Flavonoid content (TFC)**

The flavonoid content in the plant extracts of *C. oppositifolia* Sm. is shown in **Table 3**. The ethanolic extract demonstrated a slightly

higher total flavonoid content (TFC) value of 10.58 ± 0.002 mg QE/g, while the methanolic extract exhibited a lower TFC value of 8.26 ± 0.01 mg QE/g. These findings indicate no more variation in flavonoid concentrations between the two different solvent extracts.

**Table 3** TFC of methanolic and ethanolic extract

| Plant Extracts | TFC (mg QE/g) |
|---|---|
| Methanolic | 8.26 ± 0.01 |
| Ethanolic | 10.58 ± 0.002 |

**Antioxidant Potential**

The DPPH assay was used to evaluate the plant extracts' antioxidant activity, and Table 4 summarizes the $IC_{50}$ values for two different solvent extracts of *C. oppositifolia* Sm. The methanolic extract exhibited the lowest $IC_{50}$ value of 10.34 ± 2.31 µg/mL, indicating a strong antioxidant activity. In comparison to the ethanolic extracts, the methanolic extract demonstrated a much lower $IC_{50}$ value of 58.02 ± 1.13 µg/mL, indicating good antioxidant potential. These results highlight the varying effectiveness of two different solvents in extracting antioxidant compounds from the plant. The inhibition of radical scavenging activity is displayed in **Figure 2**.

**Table 4** Antioxidant potential ($IC_{50}$) values of different solvent extracts

| Plant Extracts | $IC_{50}$ (µg/mL) |
|---|---|
| Methanolic | 10.34 ± 2.31 |
| Ethanolic | 58.02 ± 1.13 |
| Quercetin | 3.355 ± 1.03 |

**Antibacterial Activity**

The agar well diffusion method was used to assess the plant extracts' antibacterial activity against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Shigella sonnei*, *Klebsiella pneumoniae*, and *Escherichia coli*) bacteria. The results of the antibacterial tests are presented in **Table 5** and illustrated in **Figure 3**. Both the extract, i.e., methanolic and ethanolic, exhibited a good zone of inhibition against *Shigella sonnei*, measuring 15 mm. Conversely, the aqueous extract demonstrated poor antibacterial activity against the tested bacteria. These findings suggest varying levels of antibacterial efficacy among the different extracts against the tested microorganisms.

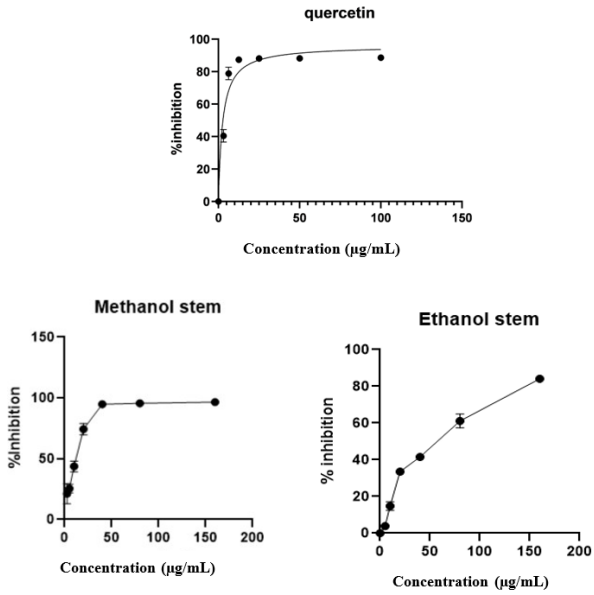


**Figure 2.** A plot showing the percentage inhibition against the concentration of methanolic and ethanolic extract.

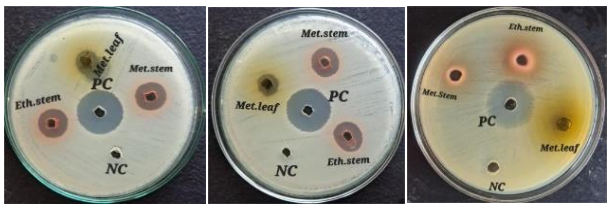


**Figure 3** Antibacterial test slides against different bacterial strains, KP = *Klebsiella pneumoniae*, SS = *Shigella sonnei,* SA = *Staphylococcus aureus, E. coli* = *Escherichia coli*

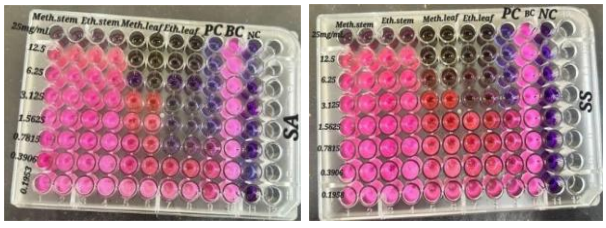


**Figure 4** Well-plates showing MIC against (a) *S. aureus* and (b) *S. sonnei*, Met = Methanol extract, PC = Positive

control, Eth = Ethanol extract, and NC = Negative control

**Table 5** Zone of Inhibition (ZOI) of plant extracts against different bacterial strains

| Plant Extracts | Bacteria | ZOI of Sample (mm) | Positive control (Neomycin) | Negative control |
|---|---|---|---|---|
| Methanolic | *Klebsiella pneumoniae* | 13 | 25 | 6 |
| | *Escherichia coli* | 12 | 24 | 5 |
| | *Shigella sonnei* | 15 | 22 | 5 |
| | *Staphylococcus aureus* | 15 | 24 | 5 |
| Ethanolic | *Klebsiella pneumoniae* | 13 | 25 | 6 |
| | *Escherichia coli* | 13 | 24 | 5 |
| | *Shigella sonnei* | 15 | 22 | 5 |
| | *Staphylococcus aureus* | 16 | 24 | 5 |

## MIC and MBC

The study revealed that the methanolic plant extract exhibited stronger antibacterial effects than the ethanolic extract, demonstrated by lower minimum bactericidal concentration (MBC) values for both *Shigella sonnei* (Gram-negative) and *S. aureus* (Gram-positive). In particular, as indicated in **Table 6**, the MIC and MBC values for methanolic extracts against *S. aureus* were 12.5 mg/mL and 25 mg/mL, while the corresponding values for ethanolic extracts were 25 mg/mL. This suggests that the methanolic extract was more effective at a lower concentration. However, the MIC and MBC values for both bacterial strains were significantly lower for the positive control, neomycin, which was 0.0152 mg/mL and 0.0313 mg/mL, respectively. This indicates that while the plant extracts do exhibit antibacterial activity, they are much less potent compared to neomycin. However, the methanolic extract still presents potential for antibacterial applications due to its relatively lower MIC than the methanolic extract. The experimental results are photographed and displayed in **Figures 4** and **5**.


**Figure 5.** Petri plates showing MBC of plant extract against *S. aureus* and *S. sonnei,* Met = Methanol extract, SA = *Staphylococcus aureus,* Eth = Ethanol extract, SS = *Shigella sonnei*

**Table 6** Values of MIC and MBC for plant extract

| Plant Extracts | *Staphylococcus aureus* | | *Shigella sonnei (SS)* | |
|---|---|---|---|---|
| | MIC (mg/mL) | MBC (mg/mL) | MIC (mg/mL) | MBC (mg/mL) |
| Methanol | 12.5 | 25 | 25 | >25 |
| Ethanol | 25 | 25 | 25 | >25 |
| Positive control | 0.0152 | 0.0313 | 0.0152 | 0.0313 |

## Discussion

According to the study, the ethanolic extract of *C. oppositifolia* Sm. has a considerably larger concentration of flavonoid (10.58 ± 0.002 mg QE/g) and phenolic (142.88 ± 0.06 mg GAE/g) chemicals than the methanolic extract. This elevated phenolic content in the ethanolic extract may be attributed to its ability to extract high-molecular-weight phenolic compounds more effectively than methanol [17]. Similarly, the higher flavonoid content observed in the ethanolic extract may be linked to the overall abundance of phenolic compounds, as flavonoids are a subclass of phenolics [18]. The $IC_{50}$ values obtained from the antioxidant assay demonstrate notable radical scavenging potential, showing minimal difference from the standard quercetin. This finding indicates that *C. oppositifolia* Sm. exhibits strong antioxidant activity, capable of neutralizing free radicals and mitigating oxidative damage. Previous studies reported an $IC_{50}$ value of 32.50 µg/mL for the methanolic extract, supporting the current findings [19]. While total phenolic and flavonoid contents often correlate strongly with antioxidant activity, other bioactive compounds such as carotenoids, tocopherols, and vitamin C also contribute significantly to antioxidant effects [20]. Additionally, environmental and climatic factors can influence antioxidant potential and the levels of total phenolics and flavonoids [21].

The Pearson correlation coefficients provide valuable insights into the relationships

between Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and Antioxidant Activity (AA), allowing us to better understand how these factors interact with one another. TPC and TFC have a strong positive correlation ($r = 0.923$, $p = 0.009$), which implies that flavonoids and phenolics may have comparable biosynthesis processes. Interestingly, negative correlations were found between TPC and antioxidant activity ($r = -0.835$, $p = 0.038$) and between TFC and AA ($r = -0.922$, $p = 0.009$), indicating that factors beyond phenolic and flavonoid contents may influence antioxidant capacity. The TPC and antioxidant activity relationship was significant at the 0.05 level, while the TFC and antioxidant activity relationship was significant at the 0.01 level.

The crude methanolic and ethanolic extracts also demonstrated strong antibacterial activity, particularly against *Shigella sonnei* and *Staphylococcus aureus*, as evidenced by significant zones of inhibition. These results are consistent with previous findings on the methanolic extract of *C. oppositifolia* Sm., which exhibited comparable antibacterial effects against the same bacterial strains as shown by Ahmed *et.al.* The antibacterial activity is likely attributed to the presence of secondary metabolites such as rutin and flavonols [22]. Additionally, compounds including vitamins, carotenoids, saponins, enzymes, and minerals may also contribute to the observed antibacterial effects [23].

With MIC and MBC values of 12.5 mg/mL and 25 mg/mL, respectively, for the ethanolic extract and 25 mg/mL for the methanolic extract, the study also examined the antibacterial potency of both extracts against *S. sonnei* and *S. aureus*. These results demonstrated a strong antibacterial effect.

## Conclusion

In conclusion, *Colebrookea oppositifolia* Sm. demonstrates significant therapeutic potential due to its rich phytochemical composition, particularly its high content of phenolics, flavonoids, and other bioactive compounds. Among the solvent extracts tested, the ethanolic extract exhibited the highest total phenolic content (TPC) and total flavonoid content (TFC), though it showed relatively lower antioxidant activity. Conversely, the methanolic extract, despite having lower TPC and TFC, displayed superior antioxidant potential. Both extracts exhibited effective antibacterial activity against *Staphylococcus aureus*, with the methanolic extract presenting a more potent minimum inhibitory concentration (MIC), highlighting its strong antibacterial efficacy.These findings suggest that *C. oppositifolia* Sm. possesses a promising phytochemical and biological profile, supporting its potential as a candidate for drug discovery. It demonstrated antioxidant and antibacterial properties, positioning it as a valuable source for the development of novel therapeutic agents. Further in-depth studies are warranted to elucidate the pharmacological mechanisms and broaden the clinical relevance of this plant in modern medicine.

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## Author's Contribution Statement

**B. G. C:** Methodology, Data Curation, Investigation, Writing: Original Draft **I. Ojha:** Writing: Review & Editing, **R. C. Basnyat:** Supervision, Writing: Review & Editing, **K. R. Sharma:** Conceptualization, Data Curation, Formal Analysis, Writing: Review & Editing and Supervision.

## Conflict of Interest

The authors hereby declare that there are no conflicts of interest associated with this research.

## Data availability statement

The data supporting this study's findings are available from the corresponding authors and supplementary file upon reasonable request.

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