ISSN: 3021-7636
International Journal of Science Technology and Health

Research Article
Vol. 4 (1), 2026, page 01-10
DOI: https://doi.org/10.69930/ijsth.v4i1.56

Phytochemical Screening of Secondary Metabolite Classes in the *n*-Hexane Fraction of *Cleome viscosa* L. and Its Antibacterial Activity against *Escherichia coli* ATCC 35219

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Received : 21 November 2025	Accepted: 30 December 2025	Available online: 31 December 2025

Abstract: Infectious diseases are one of the major challenges in human life because they can disrupt daily activities and even lead to death. *Cleome viscosa* L. is an erect, branched annual herb with yellow flowers and grooved stems. This plant thrives in humid and warm habitats and is commonly found as a weed in cleared areas prepared for rice planting, particularly along roadsides near rice fields. Traditionally, *Cleome viscosa* L. has been used as an antidiarrheal, antibiotic, anti-inflammatory, antitumor, antiseptic, and anti-leprosy agent. The aim of this study is to identify the secondary metabolite compounds present in the n-hexane fraction extract of the *Cleome viscosa* L. plant and to assess the antibacterial activity of this extract against *Escherichia coli* ATCC 35219. The secondary metabolites identified in the n-hexane extract of *Cleome viscosa* L. are alkaloids and steroids. The results of the antibacterial activity test of the n-hexane fraction showed positive results against *Escherichia coli* ATCC 35219, with an average inhibition zone diameter of 5.67 mm and 6.83 mm at concentrations of 750 ppm and 1000 ppm, indicating moderate antibacterial activity. At concentrations of 250 ppm and 500 ppm, the antibacterial activity was weak, with inhibition zone diameters of 3.50 mm and 4.50 mm, respectively.

Keywords: *Cleome viscosa* L., Secondary Metabolites, Antibacterial, and *Escherichia coli*.

1. Introduction

Infectious diseases are a major concern in human life because they can disrupt daily activities and even lead to death. These diseases are often caused by various types of microorganisms, such as bacteria, fungi, or viruses. One of the bacteria responsible for infectious diseases is *Escherichia coli (E. coli)*. *E. coli* is a normal flora found in the human intestine, where it plays a role in breaking down food residues in the large intestine, which are then excreted in feces. However, *E. coli* can also have a negative impact, as it is capable of causing a range of infectious diseases. Some of the infectious diseases caused by *E. coli* include gastrointestinal infections (such as diarrhea), urinary tract infections, and meningitis (Ramos et al., 2020).

The treatment of diseases caused by microorganism infections with antibiotics often leads to resistance, highlighting the need for new products with high potential as antibiotics (Nwobodo et al., 2022). Despite this, antibiotics still account for a large percentage of

medication use. The emergence of various infections and the development of microorganism resistance to existing antibiotics have prompted research into the development of new antibiotics that are more effective at combating disease-causing microorganisms (Brüssow, 2024). In response to this issue, efforts are being made to utilize local natural resources, particularly medicinal plants, which possess antibiotic-like properties and could potentially replace traditional antibiotics (Imran et al., 2024). One such medicinal plant with promising antibiotic potential is *Cleome viscosa* L.

Cleome viscosa L. is a weed found throughout the tropical regions of the world. This plant typically grows to a height of about 30-90 cm and is branched. Its leaves are 3-5 foliolate, obovate, and gradually become shorter towards the top. The flowers are yellow, and the fruit is capsule-shaped, flat, and ribbed throughout, while the seeds are finely transversely ridged (Mali, 2010). According to research, the leaves, bark, roots, and seeds of the *Cleome* genus plant are used for various medicinal purposes, including as an anti-tumor, antibiotic, anti-leprosy, and malaria fever remedy. It is also beneficial for blood diseases and uterine complaints, and its leaves are used for treating wounds and boils (Panduraju et al., 2011).

The method used to extract *Cleome viscosa* L. in this study is the maceration method. Maceration is a process in which the material (simplicia) is soaked in a suitable solvent at room temperature for a specified period of time. The advantages of the maceration method include its simplicity, ease of use, and the ability to prevent the degradation of thermolabile compounds (Zhang et al., 2023). The solvent used is chosen to dissolve the desired substances in the material, following the principle of "like dissolves like" in order to maximize the yield of the desired compounds and minimize the extraction of unwanted substances (Popova and Bankova, 2023).

Based on the above description, a study was conducted to test the phytochemical content of secondary metabolite compounds in the n-hexane fraction of the methanol extract of *Cleome viscosa* L., as well as its antibacterial activity against *Escherichia coli* 35219. According to the literature, the exploration of *Cleome viscosa* L. from Indonesia, particularly Southeast Sulawesi, has not been extensively studied, and therefore, further research is needed to gather data and information regarding the potential benefits of the *Cleome viscosa* L. plant.

2. Methods

2.1 Materials and Equipment

The research materials include yellow spider flower (*Cleome viscosa* L.), distilled water, methanol, n-hexane, sulfuric acid, 10% sodium hydroxide, 2N hydrochloric acid, chloroform, 1% ferric chloride, Dragendorff's reagent, aluminum foil, tissue, clear plastic wrap, plastic wrap, Whatman 42 filter paper, sodium chloride (NaCl), Nutrient Agar (NA) medium, and *Escherichia coli* ATCC 35219 bacterial culture.

The equipment includes a 270-mesh sieve, rotary evaporator, Miyako blender, Pyrex Erlenmeyer flask, incubator, Pyrex measuring glass, Pyrex beaker, stirring rod, Ohaus Explorer analytical balance, volumetric pipette, capillary pipette, stopwatch, measuring pipette, Pyrex volumetric flask, dropper pipette, funnel, stand, clamp, scissors, knife, gloves, glass bottle, container bottle, vial bottle, ruler, strainer, and Petri dishes.

2.2 Research Procedure

a. Sample Preparation

The *Cleome viscosa* L. sample was collected by uprooting the entire plant, then cleaned and dried by sun-drying at room temperature for two weeks. After drying, the sample was blended into a fine simplicia powder.

b. Maceration Process

Three hundred grams of coarse yellow spider flower powder were macerated with methanol three times consecutively, with each extraction lasting 72 hours. The liquid macerate was then filtered. The resulting macerate was concentrated using a vacuum rotary evaporator to obtain a thick, green-colored concentrated extract. The concentrated macerate was then weighed to determine its weight.

c. Fractionation

The obtained extract was dissolved in 25 mL of methanol and placed in a separating funnel. Seventy-five milliliters of n-hexane solvent were added, and the mixture was shaken until separation between the n-hexane and methanol fractions occurred. After separation, each fraction was carefully separated, and the methanol fraction was further partitioned with n-hexane up to three times. The n-hexane fractions obtained from the partition were combined and concentrated using a rotary vacuum evaporator at 64°C until a thick n-hexane fraction was obtained. The thick n-hexane fraction was then weighed to determine the weight of the concentrated extract.

d. Phytochemical Screening

1) Alkaloid Test

A 2.0 mL test solution is placed into a test tube and dissolved in 5 mL of 2.0 N HCl, followed by the addition of Dragendorff's reagent. A positive alkaloid test is indicated by the formation of an orange precipitate (Das et al., 2014).

2) Flavonoid Test

A 2 mL sample is placed into a test tube and heated for approximately 5 minutes. Then, 0.1 g of magnesium metal and 5 drops of concentrated HCl are added. The formation of an orange to red color indicates a positive result for the flavonoid test (Hardininsih et al., 2025).

3) Saponin Test

A 10 mL of the test solution is placed into a test tube and shaken for 10 seconds. The presence of saponins is indicated by the formation of foam 1-10 cm high that persists for 10 minutes and does not disappear upon the addition of 1 drop of 2 N HCl (Febriani et al., 2024).

4) Tannin Test

A 2 mL of the test solution is divided into two test tubes: test tube A (as a blank) and test tube B, which is reacted with 10% $FeCl_3$ solution. A positive test for tannins and polyphenols is indicated by the formation of a dark blue to greenish-black color (Usman et al., 2009).

5) Steroid and Terpenoid Test

A 2 mL test solution is placed into a test tube, dissolved in 0.5 mL chloroform, then 0.5 mL acetic acid and 2 mL concentrated sulfuric acid are added along the wall of the tube. A positive result for terpenoids is indicated by the formation of a brownish or violet ring at the interface, while a bluish-green ring indicates the presence of steroids (Pant et al., 2017).

e. Thin Layer Chromatography (TLC) Test for Steroids

The extract is tested using thin layer chromatography (TLC) to identify and determine the eluent ratio that can effectively separate the compounds. The best eluent

ratio is indicated by the specific separation of compounds on the TLC plate. The stationary phase is prepared using silica gel G60 F254/TLC plates measuring 4 cm in length and 1 cm in width, which are then washed with methanol and activated in an oven at 100°C for 10 minutes. Ten milligrams of the n-hexane fraction is dissolved in 1 mL of n-hexane and then spotted onto the stationary phase. The mobile phase used is n-hexane: ethyl acetate (7:3), and spot visualization is achieved using the Liebermann-Burchard reagent, followed by heating at 105°C for 5 minutes. A positive steroid reaction is indicated by the presence of blue-violet colored spots (Sunarto et al., 2022).

f. Antibacterial Activity Test

Antibacterial activity is tested using the agar diffusion method with paper discs. The concentrations used are 250, 500, 750, and 1000 ppm, with n-hexane as the solvent, sterilized by UV irradiation for 30 minutes. Filter paper discs with a 6 mm diameter are soaked in the sample solution and then placed onto Petri dishes containing *E. coli* culture. The bacteria, suspended in sterile 0.9% NaCl physiological solution, are spread over Nutrient Agar (NA) media in sterile Petri dishes and incubated at 37°C for 24 hours. Chloramphenicol is used as the antibiotic standard. The clear zones formed are measured as inhibition zones after the incubation period is complete (Thakurta et al., 2017).

3. Results and Discussion

3.1. Sample Preparation Results

The *Cleome viscosa* L. sample was collected, cleaned to remove contaminants, and thus maximizing the extraction process. The cleaned sample was then dried at room temperature to reduce water content and prevent microbial growth, allowing the sample to be stored for an extended period. Subsequently, the sample was cut into small pieces and ground using a blender to increase its surface area, facilitating better interaction with the solvent during the extraction process. The result of the grinding process yielded 300 g of powder.

3.2. Maceration Results (Solid-Liquid Extraction)

The methanol extract of *Cleome viscosa* L. obtained from maceration weighed 56 g. The yield of the methanol extract was then calculated to determine the amount of extract obtained during the extraction process. The yield was found to be 18.6%. Natural extracts containing both polar (OH) and non-polar (CH_3) groups can be used to extract polar and semi-polar compounds. The polar groups extract polar and semi-polar compounds, while non-polar groups extract non-polar compounds. During the maceration process, stirring was performed to maximize contact between the solvent and the sample, thus increasing the extract yield. During the soaking process, the organic compounds in the sample diffuse through the cell walls, dissolving the constituents inside the cells, while also encouraging the intracellular solution to diffuse out (Chan, et al., 2015).

3.3 Fractionation (Liquid-Liquid Partition)

Fractionation using the liquid-liquid partition method was carried out with a separatory funnel and two immiscible solvents. The methanol extract was dissolved in methanol and placed in the separatory funnel. Then, 75 mL of n-hexane solvent was added and gently shaken until mixed. During shaking, the separatory funnel tap was occasionally opened to release any formed gas and reduce pressure, preventing the funnel from breaking. The mixture was then left to stand until two layers formed: the upper layer being the n-hexane phase and the lower layer being the methanol phase (Abu et al., 2017).

Figure 1. Fractionation Process

The result of the fractionation yielded a thick n-hexane fraction weighing 21.7 g. The yield of the thick n-hexane fraction was then calculated to determine the amount of fraction obtained during the fractionation process (Zhang et al., 2023). The result obtained is shown in Table 1.

Table 1. Results of the Soaking of the n-Hexane Fraction

Weight of Extract (g)	Weight of Fraction (g)	Extract Yield (%b/b)
56 g	21.7 g	38.75 %

3.4 Phytochemical Screening Results

The n-hexane fraction obtained was subsequently subjected to phytochemical screening to identify the types of compounds present in the extract and to determine the compound groups that may exhibit inhibitory activity against bacteria. The compounds identified include alkaloids, flavonoids, saponins, tannins, and terpenoids (Dia et al., 2019). The identification results are presented in Table 2.

The phytochemical analysis of the n-hexane fraction from *Cleome viscosa* L. revealed the presence of secondary metabolite compounds, including alkaloids and steroids. The secondary metabolites obtained tend to be non-polar, as they were extracted using a non-polar solvent. The alkaloid test yielded positive results, indicated by the formation of an orange precipitate, which is potassium alkaloid. The steroid test also yielded positive results, indicated by the formation of a greenish-blue ring. This suggests an oxidation reaction in the steroid group, resulting in the formation of conjugated double bonds.

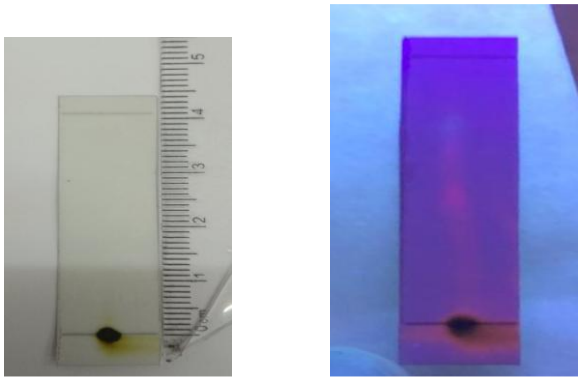
Table 2. Phytochemical Screening Results

Compound	Positive Test	Observation Results	Notes
Alkaloids	Formation of an orange precipitate (Dragendorff test)	Formation of orange precipitate	+
Flavonoids	Formation of yellow color	Formation of yellow color up to red	-
Saponins	Foam formation with a height of 1-10 cm	No foam formation with a height of 1-10 cm	-
Tannins	Formation of a brownish-black color	Formation of brownish-black color	-
Terpenoids	Formation of a red color	No formation of red color	-

Compound	Positive Test	Observation Results	Notes
Steroids	Formation of a bluish-green ring	Formation of bluish-green ring	+

3.5 Thin Layer Chromatography (TLC) Results for Steroids

The Thin Layer Chromatography (TLC) method can be used to qualitatively identify compounds in the n-Hexane fraction of *Cleome viscosa* L. The identification of separated compounds is based on the Rf value (retardation factor), which describes the distance traveled by a compound relative to the total distance traveled by the solvent. Steroid compounds are identified using a mobile phase ratio of n-Hexane to ethyl acetate (7:3), and spot visualization is achieved with the Lieberman-Burchard reagent (Frederick et al. 2021). The results obtained are shown in Figure 2.



Visible Light UV Light 366 nm

Figure 2. TLC results of the n-Hexane fraction of *Cleome viscosa* L. for steroid compounds (a) visible light, (b) UV 366 nm.

Table 3. Elution Results of the n-Hexane Fraction with a Mobile Phase of n-Hexane: Ethyl Acetate (7:3).

Rf	**Visible Light**	**UV 366 nm**
0.775	No Spot	Blue
0.7	No Spot	Blue-red
0.55	No Spot	Red-Violet
0.5	No Spot	Red-Violet
0.45	No Spot	Red-Violet
0.2	Yellowish-green	Red-Violet

The TLC results were used to reinforce the phytochemical screening for steroid compounds with a mobile phase of n-Hexane: ethyl acetate (7:3). The elution results yielded six spots with Rf values of 0.775, 0.7, 0.55, 0.5, 0.45, and 0.2. The Rf values of 0.775 and 0.7 are suspected to correspond to steroid compounds, as indicated by the blue stains observed after spraying with Lieberman-Burchard reagent, as shown in Figure 2.

3.6 Antibacterial Activity Test

This study aims to evaluate the potential of the n-Hexane fraction of *Cleome viscosa* L. in inhibiting the growth of *E. coli* bacteria. The parameter used to assess the potential of the n-Hexane fraction in inhibiting bacterial growth is the result of the diffusion test, expressed as

the diameter of the inhibition zone (mm), which is analyzed statistically. The effect of the concentration of the n-Hexane fraction of *Cleome viscosa L.* (ppm) on antibacterial activity against *E. coli* is indicated by the formation of a growth inhibition zone around the disc, due to the presence of antibacterial compounds (Warsina et al., 2022). The results of the antibacterial activity test are shown in Figure 3.

Figure 3. Results of the antibacterial activity test against *E. coli* (a) Chloramphenicol, (b) n-Hexane, (c) n-Hexane Fraction 250 ppm, and (d) n-Hexane Fraction 500 ppm.

Figure 3 can be explained as follows: chloramphenicol exhibits a very strong inhibition zone. Chloramphenicol is used as a positive control solution with antibacterial activity, indicated by an average inhibition zone of 24.17 mm for *E. coli* bacteria. Chloramphenicol works by inhibiting microbial protein synthesis, specifically by targeting the peptidyl transferase enzyme, which acts as a catalyst in forming peptide bonds during microbial protein synthesis (Sood, 2016).

The negative control used in the antibacterial test, n-Hexane, shows no formation of a clear zone. This is because n-Hexane's structure lacks functional groups or chemical properties that would make it an antibacterial agent. The n-Hexane fraction, as a test solution at concentrations of 250 and 500 ppm, shows weak inhibition zones with average diameters of 3.50 mm and 4.50 mm, respectively (Rahima et al., 2025).

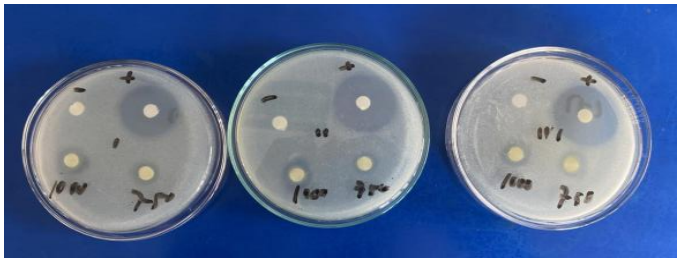


Figure 4. Results of the antibacterial activity test against *E. coli* (a) Chloramphenicol, (b) n-Hexane, (c) n-Hexane Fraction 750 ppm, and (d) n-Hexane Fraction 1000 ppm.

Figure 4 shows that the test solution of the n-Hexane fraction of *Cleome viscosa* L. at concentrations of 750 and 1000 ppm has the ability to inhibit bacterial growth, as evidenced by the clear zones formed around the discs, with average inhibition zones of 5.67 mm and 6.83 mm, respectively. This level of bacterial growth inhibition is categorized as moderate. This is supported by the research of Muharni et al. (2017), which reports that if the bacterial inhibition zone is less than 5 mm, it is considered weak; if the inhibition zone is between 5-10 mm, it is categorized as moderate; 10-20 mm as strong; and greater than 20 mm as very strong. Furthermore, it can be seen that the 1000 ppm n-Hexane fraction has a larger clear zone around the disc compared to the 750 ppm n-Hexane fraction. This suggests that as the concentration

increases, the antibacterial properties improve, and the ability to inhibit bacteria becomes greater (Nendissa and Nendissa, 2021).

Table 4. Results of the Inhibition Zone Test for the n-Hexane Fraction of *Cleome viscosa L.* against *Escherichia coli* Bacteria.

Extract Concentration (ppm)	Average Inhibition Zone Diameter (mm)	Notes
250 ppm	3.50	Weak
500 ppm	4.50	Weak
750 ppm	5.67	Moderate
1000 ppm	6.83	Moderate
K+ Chloramphenicol	24.17	Very Strong
K- n-Hexane	0	No Inhibition

Note : K+ (Positive Control), K- (Negative Control)

Table 4 shows the average diameter of the inhibition zones formed in the inhibition zone test using the disc diffusion method, indicating an increase in the area of the inhibition zones from the lowest to the highest test solution concentration. The results of the inhibition zone areas suggest the presence of antibacterial compounds with weak inhibitory activity at concentrations of 250 ppm and 500 ppm, and moderate inhibitory activity at concentrations of 750 ppm and 1000 ppm (Wijerathna, 2018).

Conclusion

The phytochemical screening of the n-hexane fraction of *Cleome viscosa* L. revealed the presence of secondary metabolites, specifically alkaloids and steroids, as indicated by the formation of a green precipitate and a bluish-green ring, respectively. Furthermore, the antibacterial activity assay demonstrated that the n-hexane fraction exhibited inhibitory effects against *Escherichia coli*, with moderate antibacterial activity observed at concentrations of 750 ppm and 1000 ppm, producing average inhibition zone diameters of 5.67 mm and 6.83 mm, respectively, while weak antibacterial activity was observed at lower concentrations of 250 ppm and 500 ppm.

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