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Profile of Secondary Metabolite and Toxicity of the Acetone Fraction of *Cleome viscosa* L. using the Brine Shrimp Lethality Test (BSLT)

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Abstract: *Cleome viscosa* L. is a flowering plant belonging to the family Cleomaceae that has long been used in traditional medicine. This study aimed to identify the classes of secondary metabolites present in the acetone fraction of *C. viscosa* L. and to evaluate its toxicity against *Artemia salina* Leach larvae. Powdered *C. viscosa* L. was extracted by maceration using methanol as the extraction solvent, followed by solvent fractionation. Phytochemical screening was carried out based on characteristic color reactions, while thin-layer chromatography (TLC) was performed to confirm the presence of the detected metabolites. Toxicity was evaluated using the Brine Shrimp Lethality Test (BSLT) against *A. salina* larvae. The phytochemical screening and TLC analyses revealed that the acetone fraction of *C. viscosa* L. contained flavonoids and tannins. The BSLT assay demonstrated that the acetone fraction exhibited toxic activity against *A. salina* larvae, with an LC_{50} value of 110.66 µg/mL. These findings indicate that the acetone fraction of *C. viscosa* L. is a promising source of bioactive compounds and warrants further investigation through the isolation, structural characterization, and biological evaluation of its active constituents.

Keywords: *Artemia salina*; Brine Shrimp Lethality Test (BSLT); *Cleome viscosa* L.; phytochemical screening; toxicity

1. Introduction

Indonesia is one of the world's richest countries in terms of biodiversity and possesses enormous potential as a source of natural bioactive compounds for the development of plant-based medicines (Bappenas, 2016; Ministry of Environment and Forestry of the Republic of Indonesia, 2020). Numerous plant species have long been used in traditional medicine because they contain secondary metabolites that exhibit antioxidant, antimicrobial, anti-inflammatory, and anticancer activities (Dias et al., 2012; Atanasov et al., 2021). The exploration of secondary metabolites from medicinal plants continues to expand due to their potential to yield novel pharmaceutical compounds and support the development of health products derived from biological resources (Newman & Cragg, 2020; Atanasov et al., 2021).

One plant with considerable potential is yellow spider flower (*Cleome viscosa* L.), a member of the family Cleomaceae that is widely distributed throughout tropical regions (Kirtikar & Basu, 1999). This plant has been traditionally used to treat fever, diarrhea, skin infections, bronchitis, rheumatism, and various infectious diseases (Parrotta, 2001; Amponsah et al., 2022). Previous studies have reported that *C. viscosa* exhibits antioxidant, antibacterial, anti-inflammatory, hepatoprotective, antidiabetic, and antitumor activities (Savioli et al., 2022; Arun et al., 2026). These biological properties are associated with its rich content of secondary metabolites, including flavonoids, alkaloids, tannins, saponins, terpenoids, steroids, and phenolic compounds (Savioli et al., 2022).

The characterization of secondary metabolites is an essential step in natural products research (Harborne, 1998) (Imran, et al., 2026). Phytochemical screening provides preliminary information regarding the classes of compounds present in a plant extract, while thin-layer chromatography (TLC) is employed to confirm the presence of these compounds based on their separation patterns and retention factor (Rf) values (Wagner & Bladt, 2009). In addition to compound identification, preliminary evaluation of biological activity is necessary to assess the bioactive potential of plant extracts. One of the most widely used methods for this purpose is the Brine Shrimp Lethality Test (BSLT), which is simple, rapid, cost-effective, and has demonstrated a strong correlation as a preliminary screening method for the cytotoxic activity of various bioactive compounds (Meyer et al., 1982; Hamidi et al., 2014).

Although numerous studies have reported the phytochemical constituents and pharmacological activities of *C. viscosa*, most have focused on crude extracts (Savioli et al., 2022). Information regarding the characteristics of secondary metabolites in the acetone fraction, confirmed by TLC analysis, and their relationship with toxicity as determined by the BSLT method remains limited. Furthermore, studies on C. viscosa originating from Indonesia are still relatively scarce compared with those conducted in other countries. These limitations highlight the need for further investigation to obtain comprehensive information on the secondary metabolite profile and bioactivity potential of the acetone fraction of this plant.

Based on this background, the present study aimed to identify the classes of secondary metabolites present in the acetone fraction of yellow spider flower (*Cleome viscosa L.*) through phytochemical screening and TLC analysis, as well as to evaluate its toxicity using the Brine Shrimp Lethality Test. The findings of this study are expected to provide scientific evidence regarding the potential of the acetone fraction of *C. viscosa* as a source of bioactive compounds, thereby supporting further research in natural products chemistry and the development of plant-derived pharmaceuticals.

2. Methods

2.1. Materials and Tools

The primary plant material used in this study was yellow spider flower (*Cleome viscosa* L.), collected from Ranomeeto District, South Konawe Regency, Southeast Sulawesi, Indonesia. The chemicals used included methanol, acetone, n-hexane, ethyl acetate, dimethyl sulfoxide (DMSO), distilled water, hydrochloric acid (HCl), Dragendorff's reagent, Liebermann–Burchard reagent, magnesium powder (Mg), ferric chloride ($FeCl_3$), chloroform ($CHCl_3$), 10% sulfuric acid (H_2SO_4), non-iodized sea salt, and *Artemia salina* Leach eggs.

The principal equipment used in this study included a rotary vacuum evaporator, an analytical balance, a blender, a separatory funnel, a thin-layer chromatography (TLC)

chamber, silica gel 60 F_{254} TLC plates, a 366 nm UV lamp, micropipettes, a microplate, and standard laboratory glassware.

2.2. Preparation of Plant Material

The *C. viscosa* plant material was cleaned to remove adhering impurities, cut into small pieces, and air-dried at room temperature until a low moisture content was achieved. The dried material was then ground using a blender and sieved to obtain a homogeneous powdered sample. A total of 500 g of the powdered plant material was used for the extraction process.

2.3. Extraction and Fractionation

The powdered plant material was extracted by the maceration method using methanol as the extraction solvent for 3 × 72 h at room temperature with occasional stirring. The resulting filtrates were combined and concentrated under reduced pressure using a rotary vacuum evaporator to obtain a crude methanolic extract.

The crude methanolic extract was subsequently subjected to sequential liquid–liquid fractionation using a separatory funnel. The first partitioning step was carried out with n-hexane to remove nonpolar constituents. The remaining methanolic fraction was then partitioned with acetone to obtain the acetone fraction, which was concentrated using a rotary vacuum evaporator to yield a concentrated fraction for subsequent analyses (Azwanida, 2015; Zhang et al., 2018).

2.4. Phytochemical Screening (Harborne, 1998)

2.4.1. Terpenoid Test

Two milliliters of the sample were transferred into a test tube, followed by the addition of two drops of chloroform ($CHCl_3$) and three drops of Liebermann-Burchard reagent. The mixture was observed for color changes. The appearance of a red or purple color indicated a positive result for terpenoids.

2.4.2. Steroid Test

Two milliliters of the sample were placed in a test tube, followed by the addition of two drops of chloroform ($CHCl_3$) and three drops of Liebermann-Burchard reagent. The formation of a green color indicated the presence of steroids.

2.4.3. Alkaloid Test

Two milliliters of the sample were transferred into a test tube, followed by the addition of three drops of concentrated hydrochloric acid (HCl) and five drops of Dragendorff's reagent. The formation of an orange-brown precipitate indicated a positive result for alkaloids.

2.4.4. Flavonoid Test

Two milliliters of the sample were placed in a test tube and heated for approximately 5 min. Subsequently, 0.1 g of magnesium powder (Mg) and five drops of concentrated hydrochloric acid (HCl) were added. The development of an orange color indicated the presence of flavonoids.

2.4.5. Tannin Test

Two milliliters of the sample were transferred into a test tube and heated for approximately 5 min. After heating, 1 mL of ferric chloride ($FeCl_3$) solution was added. The appearance of a dark green to blackish-green color indicated the presence of tannins.

2.4.6. Saponin Test

The presence of saponins was determined using the foam test. Two milliliters of the extract were placed in a test tube, mixed with 1 mL of hot distilled water, and shaken vigorously ten times. The formation of a stable foam measuring 1–10 cm in height indicated a positive result for saponins.

2.5. Thin-Layer Chromatography (TLC)

Thin-layer chromatography (TLC) analysis was carried out using silica gel 60 F_{254} plates as the stationary phase. The TLC plates were cut to dimensions of 5 × 5 cm. TLC analysis was performed to confirm the presence of flavonoids and tannins in the acetone fraction of C. viscosa.

2.5.1. Flavonoid Analysis

The acetone fraction of *C. viscosa* was dissolved in acetone and spotted onto a silica gel 60 F_{254} TLC plate. The plate was developed in a saturated TLC chamber containing an n-hexane:ethyl acetate (8:2, v/v) mobile phase until the solvent front reached the predetermined limit. After development, the plate was air-dried, sprayed with 10% sulfuric acid (H_2SO_4) as the visualizing reagent, and observed under ultraviolet (UV) light at a wavelength of 366 nm (Forestryana & Arnida, 2020).

2.5.2. Tannin Analysis

The acetone fraction of *C. viscosa* was dissolved in acetone and applied onto a silica gel 60 F_{254} TLC plate. The plate was developed in a saturated chamber using an n-hexane:ethyl acetate (8:2, v/v) mobile phase until the solvent front reached the designated limit. The developed plate was dried, sprayed with 5% ferric chloride ($FeCl_3$) solution as the detecting reagent, and examined under UV light at 366 nm.

2.6. Toxicity Assay Using the Brine Shrimp Lethality Test (BSLT)

2.6.1. Preparation of Artificial Seawater

Artificial seawater was prepared by dissolving 38 g of non-iodized sea salt in 1,000 mL of distilled water.

2.6.2. Hatching of Artemia salina Leach Eggs

The hatching of *Artemia salina* Leach eggs was initiated by placing the eggs in a partitioned hatching container filled with artificial seawater. One compartment containing the eggs was covered with aluminum foil to provide a dark environment, while the opposite compartment was left uncovered and exposed to light. Continuous illumination was provided using a lamp, and the eggs were allowed to hatch for 48 h. After hatching, the larvae (nauplii) migrated toward the illuminated compartment. Actively swimming 48-h-old nauplii were used for the toxicity assay (Meyer et al., 1982).

2.6.3. Preparation of Test Solutions

The test solutions were prepared by dissolving 5 mg of the acetone fraction in dimethyl sulfoxide (DMSO), followed by dilution with artificial seawater to obtain a stock solution with a concentration of 1,000 µg/mL in a final volume of 5 mL. The stock solution was subsequently diluted to prepare test solutions with concentrations of 500, 250, 125, 62.5, 31.25, and 15.625 µg/mL.

2.6.4. Toxicity Assay

The toxicity of the acetone fraction was evaluated using the *Brine Shrimp Lethality Test* (BSLT). Ten *Artemia salina* Leach nauplii were transferred into each well of a microplate containing artificial seawater, followed by the addition of the test solution to obtain final concentrations of 15.625, 31.25, 62.5, 125, 250, 500, and 1,000 µg/mL. Each concentration was tested in triplicate, while artificial seawater containing DMSO served as the negative control. After 24 h of incubation, the numbers of dead and surviving larvae were recorded (Meyer et al., 1982)..

The percentage mortality was calculated using the following equation:

$$\text{Mortality (\%)} = \frac{\text{Number of dead larvae}}{\text{Number of dead larvae + Number of surviving larvae}} \times 100\%$$

The LC_{50} value was determined by probit analysis based on the mortality data obtained after 24 h of exposure (Meyer et al., 1982).

3. Results and Discussion

3.1. Fraction Yield

The fractionation of the methanolic extract of *Cleome viscosa* L. using acetone as the partitioning solvent produced two fractions, namely the acetone-soluble fraction and the acetone-insoluble fraction. The fractionation results showed that the acetone-insoluble fraction weighed 4.39 g, corresponding to a yield of 43.6%, whereas the acetone-soluble fraction weighed 3.40 g, corresponding to a yield of 34.0% (Table 1).

Table 1. Weight and yield of fractions obtained from the fractionation of the methanolic extract of *Cleome viscosa* L.

Fraction	Fraction weight (g)	Yield (%)
Acetone-soluble fraction	3.40	34.0
Acetone-insoluble fraction	4.39	43.6

The higher yield of the acetone-insoluble fraction indicates that the majority of the constituents present in the methanolic extract exhibited relatively low solubility in acetone. In contrast, the acetone-soluble fraction contained semipolar compounds that were readily soluble in acetone and was subsequently selected for phytochemical screening, thin-layer chromatography (TLC) analysis, and toxicity evaluation. The differences in fraction yields can be attributed to the chemical composition of the crude extract and the differences in solvent polarity employed during the fractionation process. Fractionation based on solvent polarity is intended to enrich specific groups of compounds, thereby facilitating the characterization of secondary metabolites and the evaluation of their biological activities (Azwanida, 2015; Zhang et al., 2018).

3.2 Phytochemical Screening of the Acetone Fraction of *Cleome viscosa* L.

Phytochemical screening was conducted to qualitatively identify the classes of secondary metabolites present in the acetone fraction of *Cleome viscosa* L. using specific chemical reagents. The results showed that the acetone fraction tested positive for flavonoids and tannins, whereas alkaloids, saponins, steroids, and terpenoids were not detected (Table 2).

Table 2. Phytochemical screening results of the acetone fraction of *Cleome viscosa* L.

Compound class	Observation	Result
Alkaloids	No orange-brown precipitate formed	(-)
Flavonoids	Orange coloration developed	(+)
Tannins	Dark green to blackish-green coloration developed	(+)
Saponins	No persistent foam formed	(-)
Steroids	No green or violet coloration developed	(-)
Terpenoids	No red or purple coloration developed	(-)

The development of an orange color in the flavonoid test indicates the reaction of flavonoid compounds with the magnesium–hydrochloric acid reagent, resulting in the formation of flavilium salts, which serve as a positive indicator for flavonoids. Meanwhile, the appearance of a dark green to blackish-green color following the addition of ferric chloride ($FeCl_3$) indicates the formation of a complex between Fe^{3+} ions and the phenolic hydroxyl groups of tannins. These findings suggest that the acetone fraction of *C. viscosa* L. is predominantly composed of semipolar phenolic compounds (Harborne, 1998; Wagner & Bladt, 2009).

3.3 Thin-Layer Chromatography (TLC) Analysis of the Acetone Fraction of *Cleome viscosa* L.

Thin-layer chromatography (TLC) analysis was performed to confirm the results of the phytochemical screening of the acetone fraction of *Cleome viscosa* L. Two mobile phase systems, namely *n*-hexane:ethyl acetate (4:6, v/v) and *n*-hexane:ethyl acetate (8:2, v/v), produced different separation patterns. The *n*-hexane:ethyl acetate (8:2, v/v) mobile phase provided better chromatographic separation than the *n*-hexane:ethyl acetate (4:6, v/v) system, as evidenced by well-resolved, distinct, and circular spots. Using this mobile phase, four spots were observed with Rf values of 0.10, 0.17, 0.32, and 0.47**.** Therefore, the *n*-hexane:ethyl acetate (8:2, v/v) system was selected for subsequent TLC analysis (Wagner & Bladt, 2009).

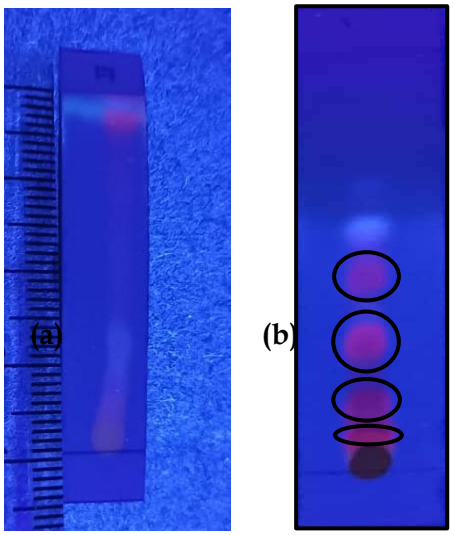


Figure 1. Thin-layer chromatography (TLC) chromatograms of the acetone fraction of *Cleome viscosa* L. using different mobile phase compositions under UV light (366 nm): (a) n-hexane:ethyl acetate (4:6) and (b) n-hexane:ethyl acetate (8:2).

Flavonoid identification was carried out using 10% sulfuric acid (H_2SO_4) as the detecting reagent, followed by observation under 366 nm ultraviolet (UV) light**.** The analysis revealed the presence of two orange-colored spots with Rf values of 0.42 and 0.47, indicating the presence of flavonoid compounds. These results confirm the phytochemical screening findings, which demonstrated the presence of flavonoids in the acetone fraction, and are consistent with the studies reported by Setyaningrum et al. (2020).

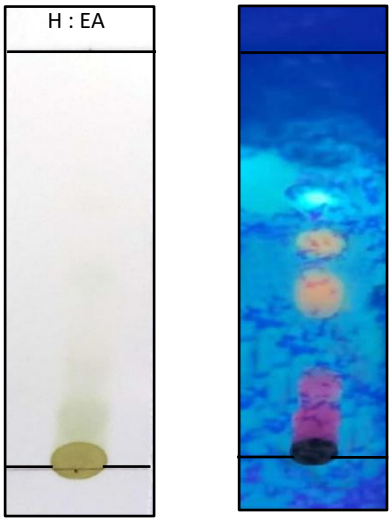
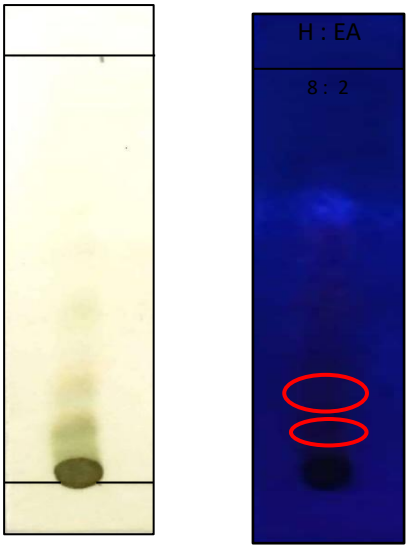


Figure 2. TLC Analysis of Flavonoid Compounds: (a) Observation under visible light after spraying with 10% H_2SO_4**;** (b) Observation under 366 nm UV light after spraying with 10% H_2SO_4.

Tannin identification was performed using 5% ferric chloride ($FeCl_3$) as the detecting reagent. The TLC analysis revealed two dark-colored spots with Rf values of 0.15 and 0.25, indicating the presence of tannin compounds in the acetone fraction. These findings further support the results of the phytochemical screening and are consistent with the study by Shafodino et al. (2022), who reported that the appearance of black or dark green spots after spraying with $FeCl_3$ is a positive indicator of the presence of tannins.



(a) (b)

Figure 3. TLC Analysis of Tannin Compounds: (a) Observation under visible light after spraying with 5% $FeCl_3$; (b) Observation under 366 nm UV light after spraying with 5% $FeCl_3$

The TLC analysis confirmed the presence of flavonoids and tannins in the acetone fraction of *Cleome viscosa* L. The agreement between the TLC results and the phytochemical screening findings indicates that fractionation using acetone was effective in enriching semipolar compounds, particularly flavonoids and tannins. The presence of these two classes of secondary metabolites is likely to contribute to the biological activity of the acetone fraction and provides a basis for further evaluation through toxicity assessment using the Brine Shrimp Lethality Test (BSLT) (Meyer et al., 1982).

3.4. Toxicity Assessment Against *Artemia salina* L. Larvae

The toxicity of the acetone fraction of *Cleome viscosa* L. was evaluated using the *Brine Shrimp Lethality Test* (BSLT). As shown in **Table 3**, larval mortality increased with increasing extract concentration, ranging from 3.81% at 15.625 µg/mL to 100% at 1,000 µg/mL, indicating a clear dose-dependent toxic effect.

Table 3. Toxicity of the acetone fraction of *Cleome viscosa* L. against *Artemia salina* Leach larvae

Sample	K (µg/mL)	Log K	Mortality	LC_{50} (µg/mL)	Standard (Nhamussua et al., 2025)
Acetone fraction of *C.viscosa* L.	15.625	1.19	3,81	110,66 µg/mL	>1000 (Non-toxic)
	31.25	1.49	11,76		
	62.5	1.79	27,14		500-1000 (Weakly toxic)
	125	2,09	51,61		<500 (Toxic)
	250	2,39	80,30		

	500	2,69	95,18	≤ 30 (Highly toxic)
	1000	3	100	

Probit analysis revealed an LC_{50} value of 110.66 µg/mL. According to Meyer et al. (1982), extracts with LC_{50} values below 1,0 µg/mL are considered toxic. Therefore, the acetone fraction of *C. viscosa* exhibited significant toxicity toward *A. salina* larvae. This finding is consistent with the review by Savioli et al. (2022), which reported that *C. viscosa* contains flavonoids, tannins, and other phenolic compounds associated with various biological activities. The observed toxicity is likely related to the presence of flavonoids and tannins identified in the phytochemical screening and confirmed by TLC analysis.

The toxicity observed in this study suggests that the acetone fraction of *C. viscosa* contains bioactive constituents suitable for further investigation. Although the BSLT is only a preliminary screening assay, extracts with low LC_{50} values are considered promising candidates for subsequent isolation of active compounds and evaluation using more specific biological assays (Meyer et al., 1982; Hamidi et al., 2014).

Conclusions

The acetone fraction of *Cleome viscosa* L. contained flavonoids and tannins, as confirmed by phytochemical screening and thin-layer chromatography (TLC). Toxicity evaluation using the *Brine Shrimp Lethality Test* (BSLT) showed an LC_{50} value of 110.66 µg/mL, indicating that the acetone fraction is toxic and contains bioactive compounds with potential for further investigation.

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