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Artemia salina bioassay as tool for determination of preliminary *Thunbergia alata* toxicity

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SUMMARY

Introduction: *Thunbergia alata* is considered an invasive species in Colombia and it is responsible for the decrease in native biodiversity and therefore represents a significant threat to flora. Hence, it is considered pertinent to evaluate its toxicological potential and, through the corresponding preliminary phytochemical analysis tests, determine which components are associated with such toxicity, thus conducting a search for possible pharmacological utility of the species under study. **Aim**: Starting at the floral parts of the *Thunbergia alata* species collected in the municipality of Tenjo Cundinamarca, obtain the ethanolic extract. To carry out a preliminary phytochemical analysis and a general lethality bioassay on *Artemia salina* with the extract free of hydroalcoholic solvent in the way to find its toxicity. **Results**: The prepared extract underwent a preliminary phytochemical analysis, where it was found that the floral parts of the *Thunbergia alata* species contain phenols, flavonoids, phenolic antioxidants, tannins, anthraquinones, flavones, and saponins. Finally, the lethality bioassay was conducted on *Artemia salina*, and the toxic potential of the extract of interest was statistically analyzed using Probit analysis, where the curve yielded a lethal concentration 50 (LC_{50}) of 2.036 μg/mL. **Conclusions:** The ethanolic extract of the *Thunbergia alata* species free of solvent has an extreme toxic potential.

Keywords: *Thunbergia alata*; toxicity; *Artemia salina*

RESUMEN

Bioensayo de *Artemia salina* como herramienta para la determinación preliminar de la toxicidad de *Thunbergia alata*

Introducción: *Thunbergia alata* se considera una especie invasora en Colombia, responsable de la disminución de la biodiversidad nativa y, por lo tanto, representa una amenaza significativa para la flora. Por lo tanto, se considera pertinente evaluar su potencial toxicológico y, mediante los análisis fitoquímicos preliminares correspondientes, determinar qué componentes están asociados con dicha toxicidad, buscando así la posible utilidad farmacológica de la especie en estudio. **Objetivo:** A partir de las partes florales de la especie *Thunbergia alata* recolectadas en el municipio de Tenjo, Cundinamarca, se obtuvo el extracto etanólico. Se realizó un análisis fitoquímico preliminar y un bioensayo de letalidad general en *Artemia salina* con el extracto libre de solvente hidroalcohólico para determinar su toxicidad. **Resultados:** El extracto preparado se sometió a un análisis fitoquímico preliminar, donde se encontró que las partes florales de la especie *Thunbergia alata* contienen fenoles, flavonoides, antioxidantes fenólicos, taninos, antraquinonas, flavonas y saponinas. Finalmente, se realizó un bioensayo de letalidad en *Artemia*

salina, y se analizó estadísticamente el potencial tóxico del extracto de interés mediante análisis Probit, donde la curva arrojó una concentración letal 50 (CL50) de 2,036 µg/mL. **Conclusiones:** El extracto etanólico de la especie *Thunbergia alata* libre de solvente presenta un potencial tóxico extremo.

Palabras clave: *Thunbergia alata*; toxicidad; *Artemia salina*

RESUMO

Bioensaio de *Artemia salina* como ferramenta para determinação da toxicidade preliminar de *Thunbergia alata*

Introdução: *Thunbergia alata* é considerada uma espécie invasora na Colômbia e responsável pela diminuição da biodiversidade nativa, representando, portanto, uma ameaça significativa à flora. Portanto, considera-se pertinente avaliar seu potencial toxicológico e, por meio dos correspondentes testes de análise fitoquímica preliminar, determinar quais componentes estão associados a tal toxicidade, buscando assim a possível utilidade farmacológica da espécie em estudo. **Objetivo:** A partir das partes florais da espécie *Thunbergia alata* coletadas no município de Tenjo Cundinamarca, obter o extrato etanólico. Realizar uma análise fitoquímica preliminar e um bioensaio de letalidade geral em *Artemia salina* com o extrato livre de solvente hidroalcoólico, a fim de determinar sua toxicidade. **Resultados:** O extrato preparado foi submetido a uma análise fitoquímica preliminar, onde se constatou que as partes florais da espécie *Thunbergia alata* contêm fenóis, flavonoides, antioxidantes fenólicos, taninos, antraquinonas, flavonas e saponinas. Por fim, o bioensaio de letalidade foi conduzido em *Artemia salina*, e o potencial tóxico do extrato de interesse foi analisado estatisticamente por meio da análise de Probit, onde a curva apresentou uma concentração letal 50 (CL50) de 2,036 µg/mL. **Conclusões:** O extrato etanólico da espécie *Thunbergia alata*, livre de solvente, apresenta um potencial tóxico extremo.

Palavras-chave: *Thunbergia alata*; toxicidade; *Artemia salina*

1. INTRODUCTION

Thunbergia alata is considered an aggressive invasive species [1]. Specifically in Colombia, this species is responsible for the decrease in native biodiversity and therefore represents a significant threat to fauna. Hence, it is considered pertinent to evaluate its toxicological potential and, through the corresponding preliminary phytochemical analysis tests, determine which components are associated with such toxicity, thus conducting a search for possible pharmacological utility of the species under study [2, 3].

Invasive species are considered the second leading cause of biodiversity loss worldwide [4]. It is estimated that 40% of animal extinctions in the last five centuries are attributable to them. The presence of these species can lead to the extinction of native species through predation, competition for resources, or transmission of deadly diseases. Invasive species also have the ability to hybridize with similar native species, altering the genetic heritage and endangering species survival. Their presence can have serious consequences for human activities, thereby affecting the economy of a region or country. They can damage forests, impact fishing and livestock, degrade water quality, and harm industry. Moreover, many agricultural pests, such as weeds and invertebrates, are invasive exotic species that can cause significant damage to production, and their chemical control can have negative effects on other species and human health [5]. The magnitude of environmental and economic impacts resulting from biological invasions makes this issue transcend national borders and become an international concern, implying the need for collaboration between countries to coordinate strategies, control species trafficking, and monitor communication routes [6]. *Thunbergia alata* is a plant species classified

as a highly invasive organism, widely used for ornamentation and demarcation of various areas due to its rapid growth. Its use on a national level in Colombia is significant, to the extent that it has caused a high-risk invasion in various regions. Its harmful impact has been evidenced through ecosystem degradation and alteration, along with the reduction of native biodiversity, affecting biogeochemical cycles and the food chain, and creating significant social and economic problems [2]. This plant is commonly found at forest edges, where it tends to climb over other plants and hinder their growth, sometimes completely smothering them. Such behavior negatively impacts the germination and establishment of native species; it could even be categorized as one of the top ten most challenging invasive species in Colombia.

Due to the high proliferation of this species (*Thunbergia alata*), it is of great importance to determine and quantify the toxic effect of the ethanolic extract from the floral parts of *Thunbergia alata* using the general lethality bioassay in *Artemia salina*. This study aims to assess if the species possesses pharmacological potential that could allow its pharmaceutical use, while also contributing to species control due to its harmful activities in ecosystems [7]. Additionally, it is relevant to note that there is currently no literature specifically addressing toxicity studies regarding the potential toxicity present in the floral parts of this species. Previous studies have only focused on the pharmacological activity found in stems and leaves of *Thunbergia alata*, such as the study conducted by the Pontificia Universidad Javeriana, which aimed to evaluate the chemical and antifungal potential of *Thunbergia alata* in leaves and stems [8].

2. MATERIALS AND METHODS

For the sample collection, 50 grams of plant material, specifically floral parts of the species *Thunbergia alata*, were collected in the region of Cundinamarca, municipality of Tenjo.

For the treatment of the collected plant material, the entire sample was subjected to air drying at room temperature for 10 days. Subsequently, all the material was triturated using an electric grinder until it was completely pulverized, aiming to facilitate the preparation of the desired extract. For the extraction process, 500 milliliters of a hydroalcoholic mixture composed of 65% ethanol and distilled water were used. The mixture was concentrated to a 1:1 ratio using maceration at room temperature as the extraction technique. The obtained extract was then subjected to rotary evaporation until a dry extract was obtained (Figure 1).


(a)


(b)

(c)

Figure 1. Photographic record of methodology: **(a)** Comparison between: fresh sample of floral parts collected from *Thunbergia alata*, dried sample of floral parts collected from *Thunbergia alata and* pulverized sample of floral parts collected from *Thunbergia alata*. **(b)** Extract obtained through filtration. **(c)** Solid residue of the extract obtained after filtration.

After obtaining the extract, preliminary phytochemical screening tests were conducted using a porcelain plate [9]. In this plate, 3 drops of extract were applied, followed by the addition of 4 drops of each corresponding reagent for the proposed tests.

2.1. Alkaloid test

The Mayer's reagent was used for the alkaloid test. The alkaloid test with Mayer's reagent is based on the formation of a yellow or white precipitate when alkaloids react with the reagent. The underlying phytochemical principle involves the formation of insoluble complexes between alkaloids and the reagents present in the solution [10].

2.2. Phenol test

For the phenol test, ferric chloride was used. The phytochemical principle behind this test lies in the ability of phenols to react with ferric ions (Fe^{3+}) present in the reagent, forming colored coordination complexes. [10].

2.3. Saponin test

The foam test was employed for the saponin test. The foam test for saponins is based on the ability of saponins to form stable foam when agitated in the presence of water. The phytochemical principle behind this test lies in the chemical structure of saponins. When a solution containing saponins is vigorously agitated in water, saponins form micelles due to their amphiphilic structure [10].

2.4. Tannin test

The gelatin-salt test and potassium dichromate test were used for the tannin test. The phytochemical principle behind the gelatin-salt test involves the ability of tannins to form precipitated complexes with proteins like gelatin in the presence of metal salts, indicating that the obtained result was a product of the union of cross-links of a biopolymer such as gelatin. Similarly, the tannin test with potassium dichromate reagent is based on the ability of tannins to reduce dichromate ions (VI) to chromium (III) in an acidic medium. The phytochemical principle in this test is the oxidation-reduction reaction between tannins and potassium dichromate [10].

2.5. Phenolic antioxidant test

Additionally, the Folin-Ciocalteu reagent was used for the phenolic antioxidant test. This reagent contains phosphomolybdic and phosphotungstic acids in an aqueous solution. When added to a sample containing phenolic antioxidants, the phenolic groups of the antioxidants can reduce the molybdovanadate (VI) ions present in the reagent to molybdenum (V) ions. This process leads to the formation of a colored complex. The intensity of the formed color is related to the amount of phenolic antioxidants present in the sample [10].

2.6. Flavone test

For the flavone test, sulfuric acid was used. The flavone test with sulfuric acid reagent is based on the ability of flavones to form heterocyclic compounds and the consequent production of characteristic coloration. [10].

2.7. Flavonoid test

Likewise, the Shinoda reagent was used for the flavonoid test. The flavonoid test with Shinoda reagent is based on the formation of intensely colored complexes between flavonoids and Shinoda reagent, which contains a solution of aluminum trichloride and hydrochloric acid. The phytochemical principle behind this test lies in the reaction of hydroxyl groups of flavonoids with aluminum trichloride in an acidic medium [10].

2.8. Anthraquinone test

Finally, the anthraquinone test used hydrochloric acid in conjunction with ammonium hydroxide. Anthraquinones are organic compounds containing a structure consisting of aromatic rings with specific functional groups such as hydroxyl and ketone groups. When anthraquinones are treated with hydrochloric acid and ammonium hydroxide, various reaction products can be produced, including chlorides, condensates, and other derivatives [10].

2.9. Solvent election

For the solvent selection, various aspects were considered that led to the conclusion that hydroalcoholic solutions allow for easier extraction of secondary metabolites and are the most efficient option for extracting active principles from plants. This is reflected in the favorable recovery percentages obtained when using such mixtures, contributing to the establishment of standardized methods for monitoring potential biological activities in plant extracts. Ethanol was highlighted due to its low toxicity compared to methanol, and its environmentally friendly properties. Additionally, it has been found that the best extraction yields are obtained with different concentrations of ethanol (35-90%) in aqueous solution. The concentration percentage of ethanol in the extraction medium significantly affects the recovery yield. Furthermore, the presence of water enhances mass transfer between solid and liquid phases, increasing the permeability of the plant matrix and thereby improving heating efficiency [11]. Hydroalcoholic mixtures are used in plant extraction because they combine the solvent properties of both water and alcohol, thereby enhancing extraction efficiency. Water is effective for polar compounds, similar to ethanol. By combining these solvents in a hydroalcoholic mixture, a wide variety of compounds with different polarities can be extracted, expanding the solvent's utility. Moreover, ethanol in hydroalcoholic mixtures increases the solubility of certain phytochemicals by forming hydrogen bonds with hydroxyl groups present in compounds such as

phenolic compounds. Additionally, the ethanol concentration used in the solvent has antimicrobial effects, which contribute to preserving the extract.

2.10. *Artemia salina* toxicity test

For the hatching of *Artemia* cysts, a container was prepared with 12.5 grams of sea salt in a 500 mL beaker, filled with distilled water to simulate seawater concentration. The water was oxygenated using a pump and maintained under continuous light for 48 hours. After hatching, the nauplii were used, as this is the stage where they mature to an optimal naupliar larval state (Figure 2).

Figure 2. Dark container suitable for incubation of *A. salina* nauplii.

After incubation, a light source was positioned at one side of the container to attract second-stage larvae and separate them from the unhatched eggs. Figure 3. Subsequently, the *Artemia* were transferred to test vials using a Pasteur pipette. The number of second-stage larvae collected and added to each plate was determined using a stereoscopic microscope. Figure 4.

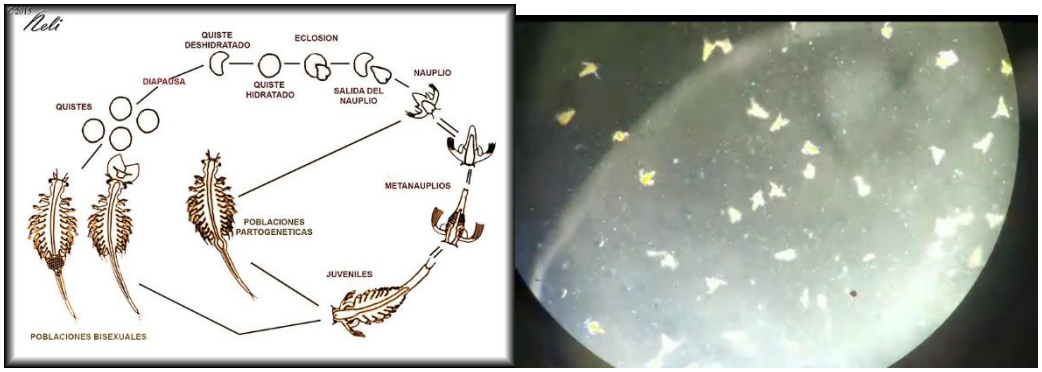


Figure 3. Naupliar stages [2].

The assay involved exposing only nauplius larvae to five increasing concentrations (5, 10, 50, 100, and 500 μg/mL) of the extract from *Thunbergia alata* species for 24 hours at room temperature under continuous light conditions. These five concentrations were chosen to cover a range from moderately to extremely toxic based on the toxicity table reported by CYTED [3]. Figure 5. Three replicates were used for each concentration. Ten nauplius larvae were added to individual vials, and the corresponding amount of concentrated extract was applied to each vial, which was then filled with seawater.

The stock solution of the extract was prepared by adding 50 grams of pulverized plant material to 500 mL of ethanol (at 65% concentration) and concentrating it to achieve a dry extract. From this extract, volumes of 0.5, 0.1, 0.05, 0.01, and 0.005 mL were taken to obtain concentrations of 500, 100, 50, 10, and 5 µg/mL, respectively.

After 24 hours of exposure, the number of dead organisms was counted, and the mortality percentage was calculated. Larvae were considered dead if they did not exhibit movement for 20 seconds of observation. Additionally, strychnine was used as a reference standard, and therefore, the results obtained for the ethanol extract of *Thunbergia alata* were compared with those reported in the literature [12] for strychnine. This comparison aimed to provide a reference framework, verify the sensitivity and reliability of the assay, ensure consistent results, and facilitate interpretation of the findings.

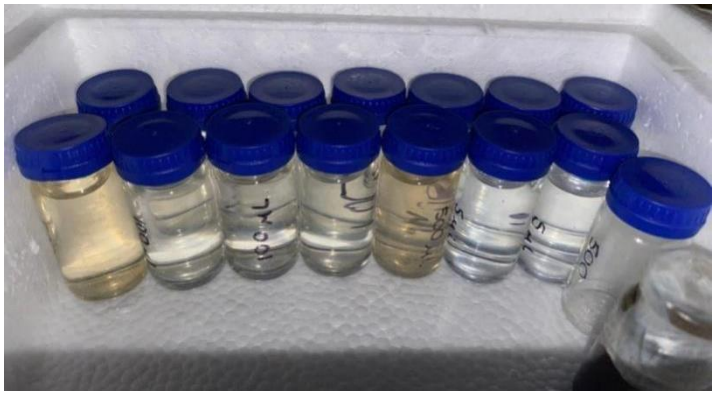


Figure 4. Glass vials used for the lethality bioassay in *Artemia salina.*

The selection of the appropriate method for lethality bioassay in *Artemia salina* was chosen because it has been used in multiple research studies with valid results for over 40 years. *Artemia spp*. larvae have been employed in toxicological and ecotoxicological investigations, and their biology and potential applications in various fields have been extensively studied. It is a cost-effective and practical method for assessing the bioactivity and preliminary toxicity of synthetic compounds and natural products [13]. Additionally, the use of *Artemia* is beneficial for predicting pharmacological and pesticide activities since it responds to a wide range of chemical and pharmacological compounds. This is crucial in research and development programs for new drugs and natural origin pesticides [12]. Furthermore, the potential application of the *Artemia* assay is linked to the particular advantages that bioassays provide in the standardization and quality control of various botanical products. These products may be considered "heterogeneous" due to the presence of combinations of bioactive compounds from the same botanical source or intentionally prepared mixtures.

On the other hand, there are advantages such as not needing to maintain a live colony permanently. Tests can be conducted anywhere and at any time as needed. There is always an adequate number of individuals of the same age and physiological condition available, overcoming ethical issues related to the use of larger animals [14].

Finally, for the execution of the Probit method, the logarithm base 10 was calculated for each of the concentrations used, and subsequently, the Probit values were calculated based on the mortality percentages obtained for each concentration using the table shown below [15]. Table 1.

Table 1. Transformation of mortality percentage into Probit values [15]

%	0	1	2	3	4	5	6	7	8	9
0	-	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.25	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
-	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

Finally, the calculated Probit values were plotted together with the logarithms calculated for the concentrations used. For the purpose of calculating the LC_{50}, it was necessary to determine the value corresponding to the intersection of the graph with the trend line on the Y-axis (Probit) at the assigned value of 5 as this corresponds to 50% lethality.

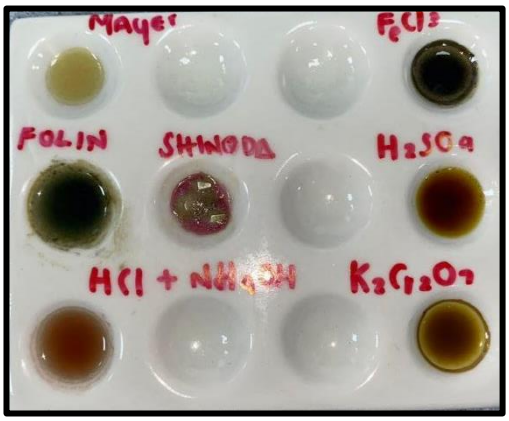
3. RESULTS

3.1. Preliminary phytochemical analysis of floral parts of *Thunbergia alata*

The results of the phytochemical analysis were interpreted as positive or negative based on variations in coloration, precipitation, turbidity, and other indicators in the phytochemical tests for different metabolites as are summarized in Table 2 and can be observed through photographic documentation in Figure 5.

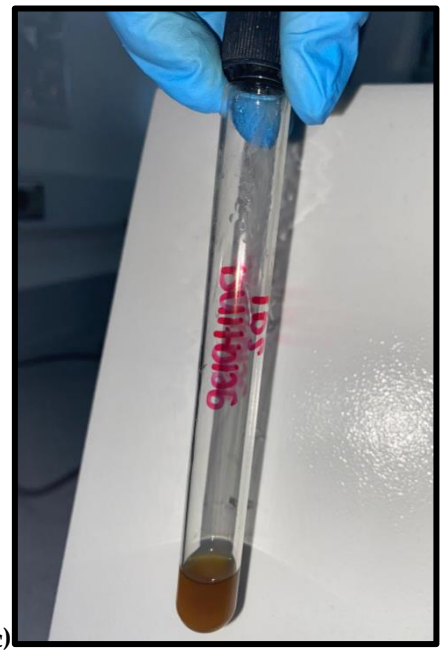
Table 2. Results of the preliminary phytochemical analysis of the floral parts of *Thunbergia alata*

Metabolite	**Test**	**Result**
Alkaloids	Mayer	Negative (-)
Phenols	Ferric chloride	Positive (+)
Saponins	Foam	Positive (+)
Tannins	Gelatin-salt	Positive (+)
Phenolic antioxidants	Potassium dichromate	Positive (+)
Flavones	Folin-Ciocalteou	Positive (+)
Flavonoids	Sulfuric acid	Positive (+)
Anthraquinones	Shinoda	Positive (+)
	Hydrochloric acid + Ammonium hydroxide	



(a)

(b)



(c)

Figure 5. Results of the preliminary phytochemical analysis of the floral parts of *Thunbergia alata*: **(a)** Porcelain plate showing the color changes observed when applying the respective reagents from the tests described earlier to the ethanolic extract of *Thunbergia alata* floral parts. **(b)** Test tube where the saponin test was performed, showing a positive result with the appearance of foam. **(c)** Test tube where the gelatin-salt test was performed, showing a positive result with the formation of a light brown precipitate.

3.2. Lethality bioassay in *Artemia Salina*

The results of the Lethality Bioassay in *Artemia Salina* were interpreted based on the movement of the nauplii to confirm their survival after the addition of the extract. Each assay used 10 nauplii. It is important to note that the results are expressed as a percentage of mortality, calculated using the following formula:

$$Percentage\ of\ mortality = (Number\ of\ deaths\ /\ Total\ population)\ x\ 100$$

3.2.1. Concentration of 5 µg/mL

At a concentration of 5 µg/mL, 3 live nauplii were observed across all 3 replicates, resulting in a mortality rate of 70%.

3.2.2. Concentration of 10 µg/mL

At a concentration of 10 µg/mL, 2 live nauplii were observed across all 3 replicates, resulting in a mortality rate of 80%.

3.2.3. Concentration of 50 µg/mL

Furthermore, at a concentration of 50 µg/mL, the first replicate resulted in 1 live nauplius, corresponding to a mortality rate of 90%, while the remaining 2 replicates showed 0 live nauplii, resulting in an average mortality rate of 100%.

3.2.4. Concentration of 100 µg/mL

For the concentration of 100 µg/mL, 0 live nauplii were observed across all 3 replicates, resulting in a mortality rate of 100%.

3.2.5. Concentration of 500 µg/mL

Lastly, at a concentration of 500 µg/mL, 0 live nauplii were observed across all 3 replicates, resulting in a mortality rate of 100%.

Similarly, the average percentage of mortality was calculated for the 3 replicates of each concentration, yielding 70% for 5 µg/mL, 80% for 10 µg/mL, 97% for 50 µg/mL, and 100% for both 100 µg/mL and 500 µg/mL. Additionally, standard deviation was calculated, resulting in values of 0 for all replicates of all concentrations except for the 50 µg/mL concentration, where one of the 3 replicates showed a different result compared to the other 2 replicates. The aforementioned results are documented in Table 3.

It is noteworthy that these results do not allow for the calculation of the LD_{50} because the graphical representation of the dose-response relationship, p (Percentage of mortality) vs. d (Concentration of the substance), often shows a parabolic curve that can be difficult to linearly model (as observed in Figure 6). To address this issue, the dose variable, d, is transformed to a logarithmic scale, $X = \log_{10}(d)$. This transformation produces a dose-response relationship with an S-shaped or sigmoidal form, resulting in a distribution of points p vs. X that resembles a normal distribution. Subsequently, using Probit tables, the percentage effect, p, is converted into Probit units. Tables 4 and 5. This involves looking up in a normal distribution table the z-value corresponding to a cumulative probability equal to p. This process generates a distribution of points in a bivariate system that can be analyzed using conventional regression analysis through the method of least squares [16].

The Probit method provides maximum likelihood estimates of regression parameters and natural rates, such as mortality rates, in biological assays. This is achieved by analyzing the percentage effects against the doses within the context of regression, thereby calculating the LD_{50} [16]. All of this is reflected in Figure 7, where the results are graphed and the linear equation is obtained: $y = 1.438X + 4.556$

Table 3. Results of the Lethality Bioassay in *Artemia Salina* for the ethanolic extract of *Thunbergia alata*

Concentration [µg /mL]	Number of nauplii per replicate	Number of live nauplii			Mortality percentage (%)			Average mortality percentage	Standard deviation
		R1	R2	R3	R1	R2	R3		
Blank	10	10	10	10	0	0	0	0	0
5	10	3	3	3	70	70	70	70	0
10	10	2	2	2	80	80	80	80	0
50	10	1	0	0	90	100	100	97	5.8
100	10	0	0	0	100	100	100	100	0
500	10	0	0	0	100	100	100	100	0

Table 4. Data corresponding to the graph Concentration vs. Average Percentage of Mortality.

Concentration	Average mortality percentage
0	0
5	70
10	80
50	97
100	100
500	100

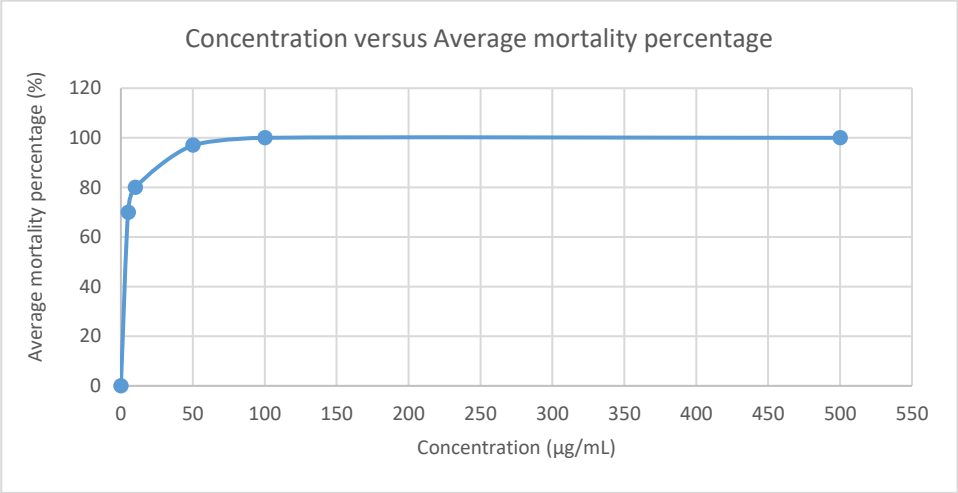


Figure 6. Concentration vs. Average Percentage of Mortality Graph.

Table 5. Data corresponding to the Probit vs. Logarithm10 of Concentration graph.

Logarithm of Concentration	Probit
0	0
0.69897	5.52
1.00000	5.84
1.69897	6.88
2.00000	8.09
2.69897	80.9

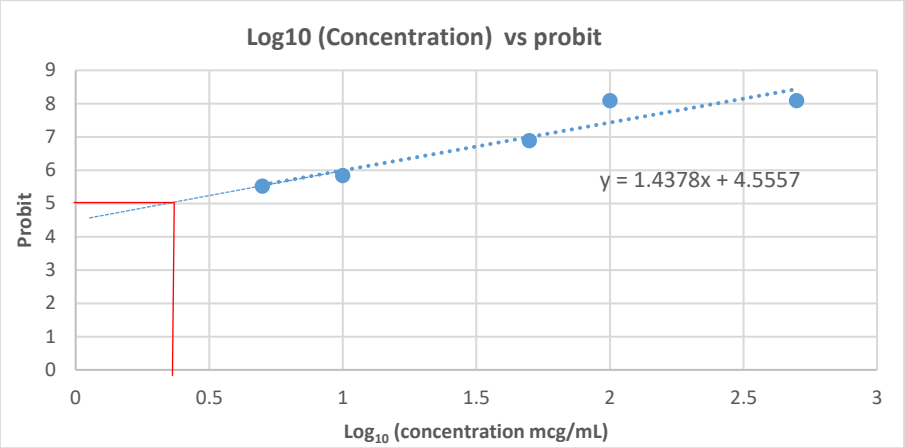


Figure 7. Probit vs. Logarithm10 of Concentration Graph

3.2.6. Calculation of the LD50

$$y = 1.438X + 4.556$$
$$5 = 1.438X + 4.556$$
$$X = (5 - 4.556)/1.438$$
$$X = 0.30876$$
$$LD50 = 10^{x}$$
$$LD50 = 10^{0.30876}$$
$$LD50 = 2.036\ \mu g/mL$$

4. DISCUSSION

Thunbergia alata belongs to the *Acanthaceae* family, within which various biological activities are present, such as antifungal activity [8], anti-inflammatory, antihypertensive, and antioxidant effects [17]. Additionally, several studies indicate the presence of smooth muscle relaxant and calming effects, as well as analgesic and anti-inflammatory properties [18].

It is noteworthy that *Thunbergia alata (Acanthaceae)* has been used in traditional medicine to alleviate various inflammatory conditions, such as fever, cough, and diarrhea, in East African nations like Uganda and Kenya [17]. In India, *Thunbergia alata* has been employed medicinally to treat patients suffering from back and joint pain, ocular inflammation, hemorrhoids, rectal cancer, and gall disease. Some animal studies have demonstrated beneficial biological activities, including improvements in cognitive and emotional deficits in mice using aqueous extracts [19].

Furthermore, the secondary metabolites found in the flowers of *Thunbergia alata* exhibit significant pharmacological interest due to their therapeutic activities. Specifically regarding phenols, Hernandez et al. (2022) suggest that antioxidant activity is closely related to phenolic compounds present in plant extracts [20]. Similarly, concerning saponins, these exhibit a wide range of pharmacological effects from expectorant action to diuretic, antiedematous, and anti-inflammatory effects. The expectorant action is attributed to the stimulation of tracheobronchial secretion through an autonomic reflex originating from gastric mucosa, while the diuretic effect enhances renal blood circulation and glomerular filtration [20].

Regarding tannins, plants containing these metabolites possess astringent, hemostatic, antiseptic, and toning properties. Tannins coagulate tissue and mucosal proteins, forming an isolating and protective layer that relieves skin irritation and pain. Tannin-containing plant products are used to stop minor local bleeding, treat mouth inflammations, bronchitis, burns, skin ulcers, wounds, and other conditions, and also manage diarrhea. However, caution is advised

for individuals with digestive issues due to potential intolerance, as prolonged consumption may inhibit the absorption of certain vitamins and minerals [21].

Additionally, phenolic antioxidants exhibit a wide array of therapeutic activities such as anti-diabetic, anticancer, anti-inflammatory, analgesic, vasodilatory, antidepressant, antihypertensive, antithrombotic, anticoagulant, antimicrobial, anti-aging, anti-allergic, and osteoporosis-fighting effects [22]. Among phenolic antioxidants, flavones are notable for their phytoestrogenic and antimicrobial activity [23]. Many flavonoids have demonstrated antifungal and antiviral activity in addition to their antibiotic, anticancer, antidiarrheal, anti-inflammatory, estrogenic, anti-hepatotoxic, and antispasmodic effects [24]. Finally, another group of metabolites present in *Thunbergia alata*, namely anthraquinones, exhibit interesting antimicrobial and antiviral activities reported through in vitro and/or in vivo studies, making them ideal candidates for exploring new treatments [25].

It is important to note that *Thunbergia alata* (*Acanthaceae*) has been used in traditional medicine to alleviate various inflammatory conditions such as fever, cough, and diarrhea in East African countries like Uganda and Kenya [17]. In India, *Thunbergia alata* has been used medicinally to treat patients with back and joint pain, ocular inflammation, hemorrhoids, rectal cancer, and gall disease. Some animal studies have shown beneficial biological activities, including improvements in cognitive and emotional deficits in mice using aqueous extracts [19].

Furthermore, the secondary metabolites found in the flowers of *Thunbergia alata* are of significant pharmacological interest due to their therapeutic activities. Specifically, phenols are associated with antioxidant activity in plant extracts [20]. Saponins exhibit a wide range of pharmacological effects from expectorant and diuretic properties to anti-inflammatory effects [26]. Tannins found in plants have astringent, hemostatic, antiseptic, and toning properties, while phenolic antioxidants have various therapeutic activities such as anti-diabetic, anticancer, anti-inflammatory, analgesic, vasodilatory, antidepressant, antihypertensive, antithrombotic, anticoagulant, antimicrobial, anti-aging, anti-allergic, and osteoporosis-fighting effects [22].

Justicia secunda Vahl, another member of the *Acanthaceae* family, has been studied extensively, including phytochemical screening and toxicity testing of its extracts. This family is known for its diverse range of herbaceous or erect plants, with a few shrubs or small trees, and includes approximately 230 genera and 4000 species distributed in tropical regions. *Justicia*, the largest genus within this family, comprises about 600 species found in tropical and subtropical regions worldwide. *Justicia secunda* Vahl, also known as "singamochila," "cascajera," or "sanguinaria," is a perennial shrub reaching 0.5 to 1 m in height, with red-violet flowers grouped in corollas of 5 petals. It is common in tropical and temperate regions and widely distributed in several countries [25].

Populations in Latin America have traditionally used *Justicia secunda* Vahl to treat various conditions such as anemia, pain, hypoglycemia, and as an energizer, although its efficacy has not been fully established. It is used in different regions for kidney stones, as an antipyretic, for glucose-related disorders, infections, and even in ethnoveterinary applications for snakebite injuries and dysentery in hunting dogs [25]. Studies have identified tannins, steroids, saponins, anthraquinones, and flavonoids, particularly derivatives of luteolin, in the leaves of *Justicia secunda* Vahl collected from different regions. Sanabria *et al.* [10] have highlighted the presence of these secondary metabolites in plant extracts and their correlation with lethality in *Artemia salina* larvae. This observation could explain the low toxicity observed in the ethanolic leaf extract of *J. secunda* Vahl in this study, as certain secondary metabolites like cumarins were absent [25].

Comparing these findings with *Thunbergia alata*, both species share similar types of secondary metabolites, therapeutic activities, and measured toxicological potential via *Artemia salina* lethality bioassay. Toxicity in both extracts is attributed to saponins and tannins, validating the reliability of the results and their interpretation [25].

To analyze the *Artemia salina* lethality bioassay, it is necessary to clarify the definition of toxicity. A toxin can be defined as any substance capable of causing harmful effects in a biological system [24]. Various studies have evaluated the toxicity of synthetic organic chemicals and plant extracts against *Artemia nauplii* to determine the median lethal concentration (LC_{50}) [27].

The findings on the median lethal concentration are presented in Figure 7, showing the cytotoxic percentage of the plant extract on *Artemia salina*. It was concluded that the toxicity value was 2.036 μg/mL. Figure 7 illustrates the survival and mortality of crustaceans relative to concentration; it is evident that as the concentration in μg/mL increases, so does mortality. This result, consistent with toxicity criteria established by CYTED [3], classifies the plant's toxicity as "extremely toxic," suggesting *Thunbergia alata* may have pharmacological potential. Table 6.

Table 6. Toxicity classification according to CYTED reported in the literature [3]

Toxicity classification according to CYTED	
I Extremely toxic	1 – 10 μg/mL
II Highly toxic	10 – 100 μg/mL
III Moderately toxic	100 – 500 μg/mL
IV Slightly toxic	500 – 1000 μg/mL
V Practically non-toxic	1000 – 1500 μg/mL
VI Relatively harmless	> 1500 μg/mL

It should be noted that when compared with the toxicity standard, which in this case corresponds to Strychnine, which reported a LC_{50} of 50 μg/mL in the reviewed literature [12]. Table 7. This allowed confirming that this organism is highly sensitive to the active principles present in these samples. This supports the utility of the assay in preliminary studies of fraction activity based on toxicity. It is possible to mention that there are several studies indicating that this test is comparable to tests in mammals. It is worth noting that in 1984, a team of scientists from the *Artemia* Reference Center in Belgium developed an acute toxicity test (short-term) with *Artemia salina*. This assay, known as the ARC-TEST, is based on determining the concentration that causes the death of 50% of *Artemia* larvae within 24 hours. The assay was tested in collaboration between research centers in Europe and America. The results were compared with those of other organisms used in toxicology tests, such as *Daphnia* spp. and Brachydanio spp. It was found that the ARC-TEST is as accurate and reproducible as tests with Daphnia spp. and in some cases similar to those with *Brachydanio spp*. Despite the relatively low sensitivity of the assay in the ARC-TEST study conducted in Belgium, the authors concluded that it is a valuable tool for the initial assessment of chemical toxicity [14].

Table 7. Toxicity results obtained for the *Artemia salina* Lethality Bioassay of Strychnine as reported in the literature [12].

Strychnine standard						
[c] μg/mL	Dead	Alive	Lethality	Category	LC_{50} μg/mL Experimental	LC_{50} μg/mL Literature
80	6	4	60%	Highly toxic	50	77.2
70	6	4	60%	Highly toxic		
60	6	4	60%	Highly toxic		
50	5	5	50%	Highly toxic		
Ethanol standard						
80	1	9	10%			

To provide context, it is possible to clarify that the Meyer's toxicity index is a quantitative measure of a substance's lipid solubility, often calculated by dividing the lethal dose 50 (LD_{50}) by the octanol-water partition coefficient (Log *P*) of the substance. The use of this index is related to predicting the potency and toxicity of various chemical compounds based on their ability to dissolve in lipids. In other words, the higher the Meyer's toxicity index, the greater the substance's ability to penetrate the cell membrane of an organism and consequently affect its cellular function, thereby increasing its toxicity. This index allows framing the obtained result within a toxicological classification, as follows [28].

Similarly, upon reviewing Meyer's toxicity index as a theoretical model and contrasting it with the obtained result, it can be stated that the extract is considered toxic since the LC_{50} result is less than 1000 μg/mL (Table 8).

Table 8. Classification of the ethanol extract of *Thunbergia alata* based on Meyer's toxicity index

Extract	**Lethal Concentration 50 (LC_{50})**	**Classification**
Thunbergia alata	2.036 μg/mL	Toxic

[1] Toxicity categories according to Meyer's toxicity index: toxic (LCL_{50} < 1000 μg/mL) and non-toxic (LC_{50} > 1000 μg/mL) [27].

Similar to the previously mentioned Meyer's toxicity index, Clarkson's toxicity index is a measure of acute toxicity that originated from the need to calculate the concentration of multiple heavy metals in blood, determining that higher concentrations of these substances in the human body pose greater toxicity risks. Currently, it is used to quantify and analyze the toxic capacity of various chemical substances when exposed to living organisms. Like Meyer's index, Clarkson's index allows results to be classified according to established guidelines [29]. Additionally, based on Clarkson's toxicity index (Table 9), the result obtained from the *Thunbergia alata* extract is considered highly toxic.

Table 9. Classification of the ethanolic extract of *Thunbergia alata* based on the Clarkson toxicity index

Extract	**Lethal Concentration 50 (LC_{50})**	**Classification**
Thunbergia alata	2.036 μg/mL	Highly toxic

[1] Toxicity categories according to the Clarkson toxicity index: highly toxic (CL_{50} from 0 to 100 μg/mL), moderately toxic (CL_{50} from 100 to 500 μg/mL), slightly toxic (CL_{50} from 500 to 1000 μg/mL), and non-toxic (CL_{50} > 1000 μg/mL) [29].

It is important to consult various sources reporting toxicity indices to conclusively verify the accuracy of the classification of the obtained result, which is why the result was compared with the Clarkson [29], Meyer [28], and CYTED [3] index.

Upon obtaining a result indicating a high degree of toxicity, it is possible to relate this toxicity to the presence of secondary metabolites in *Thunbergia alata*. Specifically, it could be said that saponins play a fundamental role in the toxic nature attributed to the studied extract, as saponins are known to possess toxic properties attributed to their ability to form complexes with sterols. This could interfere with the assimilation of these compounds by the digestive system or lead to the disruption of cell membranes once absorbed into the bloodstream [30].

It is pertinent to mention that there are various toxicity mechanisms shared by the secondary metabolites found in the extract, including interference with cellular signaling pathways, inhibition of metabolic enzymes, oxidative stress, mitochondrial dysfunction, inhibition of cell wall synthesis, activation of enzymes that degrade the cell wall, increased cell membrane permeability, promotion of uncontrolled cell proliferation, and disruption of redox balance. These mechanisms may be related to the toxicological activity of the studied extract, although this was a biological assay and direct confirmation is not feasible [31].

Additionally, it is noteworthy that when secondary metabolites are present together in an extract, they can exhibit synergistic effects. Specifically, tannins and flavonoids have shown synergistic effects in inhibiting fungi such as *R. solani* and *S. rolfsii*, meaning their antifungal capacity is enhanced when these two groups of metabolites are present in the same extract [32]. Similarly, Einhelling and Rasmussen, as well as Williams and Harland, have documented additive or synergistic effects among different phenolic compounds in allelopathic interactions, even at low concentrations. For instance, Quayyum *et al.* (1999) examined how aqueous phenolic extracts of *Myriophyllum verticillatum* Linnaeus, applied at a concentration of 200 mg per 100 g fresh weight, affected germination and root development of *Lactuca sativa*. The results showed significant inhibition, with a 75% reduction in *L. sativa* root length. This underscores the potential impact of phenolic compounds on plant interactions and how even small amounts can have significant effects on the growth and development of other plant species [33-35].

As a recommendation for future phytochemical studies, thorough investigation of the non-polar secondary metabolite components present in *Thunbergia alata* through extractive fractionation using various non-polar solvents is suggested. Additionally, conducting analysis via High-Performance Liquid Chromatography (HPLC) to determine the chromatographic profiles of the secondary metabolites is recommended. Experimental tests such as the MTT assay (cell viability and metabolic activity assay) are advised to elucidate the toxicity mechanisms exerted by the major secondary metabolites present in the extract, thereby expanding the scope and depth of this research. Furthermore, conducting a bioassay-guided study for the isolation of active principles from the species and thorough investigation of their pharmacological activities is recommended.

5. CONCLUSIONS

The toxicological potential detected in the ethanolic extract of the floral parts of *Thunbergia alata* correlates with the presence of secondary metabolites such as saponins and tannins, based on the toxicity mechanisms reported in the literature for these compounds. Additionally, preliminary identification of the secondary metabolites present in the ethanolic extract of *Thunbergia alata* floral parts revealed the presence of generic groups such as phenols, flavonoids,

phenolic antioxidants, tannins, anthraquinones, flavones, and saponins. This reflects the diverse pharmacological potential of the studied extract due to the various therapeutic activities associated with these metabolite groups.

In conclusion, the capacity of the general lethality bioassay in *Artemia salina* was confirmed for evaluating the toxicity of the plant species *Thunbergia alata*, correlating the established concentrations with the responses obtained from the used nauplii. The toxicological potential was categorized as extreme under these conditions, suggesting that this study serves as a crucial starting point for future research endeavors.

CONFLICTS OF INTEREST

The authors declare no conflict of interest. This paper is based on the undergraduate thesis carried out at the Universidad El Bosque (Bogotá-Colombia) as a requirement to qualify for the title of pharmaceutical chemist: (2024-04-30) "Evaluación toxicológica del extracto etanólico de la especie *Thunbergia alata* mediante el bioensayo general de letalidad en *Artemia salina*". Available in: https://hdl.handle.net/20.500.12495/12132 [36].

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