Pontificia Universidad JAVERIANA Bogotá

Univ. Sci. 28(2): 217–229, 2023
doi: 10.11144/Javeriana.SC282.besv

ORIGINAL ARTICLE

Black-eyed Susan vine (*Thunbergia alata*): chemical and antifungal potential evaluation of an invasive plant species in Colombia

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Received: 28-11-2022
Accepted: 11-05-2023
Published online: 03-07-2023

Citation: Vanegas-Romero MA, Chitiva-Chitiva LC, Bravo-Chaucanés CP, Hernández AX, Parra-Giraldo CM, Costa GM. Black-eyed Susan vine (Thunbergia alata): chemical and antifungal potential evaluation of an invasive plant species in Colombia, *Universitas Scientiarum*, 28(2): 217–227, 2023.
doi: 10.11144/Javeriana.SC282.besv

Funding: n.a.

Electronic supplementary material: n.a.

Abstract

Thunbergia alata (Black-eyed Susan) is a plant species from East Africa and grows in tropical regions worldwide, including Colombia, being considered an invasive species in some countries. Even though it is used by local communities to treat several illnesses, including malaria, there are few biological and chemical studies on *T. alata*. Therefore, this study aimed to explore the chemical composition and *in vitro* antimycotic (against *Candida* strains) activity of *T. alata* crude ethanolic stem and leaf extracts. This was achieved via phytochemical analyses and chromatographic profiling (HPTLC, UPLC-DAD, and LC-MS). A two-fold serial microdilution method was used to determine the minimum inhibitory concentration (MIC) against selected Candida strains. Results showed the presence of saponins, terpenes, and flavonoids in the *T. alata* crude ethanolic stem and leaf extracts, although rutin and chlorogenic acid were the main components of stem and leaf extracts, respectively. Concerning biological assays, *T. alata* leaf extracts demonstrated moderate antimycotic activity, with MIC values between 5.00 mg mL^{-1} and 1.25 mg mL^{-1} against *C. albicans* and *C. auris* strains. The results indicate that *T. alata* extracts, including some of their major identified compounds, possess promising antifungal properties against two significant microorganisms.

Keywords: Antifungal activity; *Candida albicans*; *Candida auris*; HPTLC, LC-MS, *Thunbergia alata*, UPLC.

1. Introduction

Human societies have used plants for the treatment of many diseases. According to the World Health Organization (WHO), over 80 % of the world's population depends on traditional medicine or herbs for their primary healthcare needs [1]. To date, searching for natural products obtained from plants is one of the strategies used for developing new drug leads due to the unmatched chemical diversity within the plant kingdom [1, 2, 3]. Plant extracts boast a broad array of secondary metabolites, such as flavonoids, tannins, alkaloids, and terpenoids, which are anti-infective agents and generate fewer side effects than those of synthetic antimicrobial drugs [1, 4, 5, 6].

Antifungal activity is one of the different features of these plant extracts. One of the fungal species generally used to assess these extracts is *Candida albicans*, a dimorphic commensal yeast and frequently benign, that commonly colonizes the human skin and the gastrointestinal and genitourinary tract. However, in vulnerable patients, *C. albicans* causes life-threatening bloodstream infections. Candidemia incidence has increased over the past three decades, escalating morbidity and mortality in immunocompromised patients, with mortality rates between 30 % and 50 % [7]. In Colombia, especially in patients admitted to the intensive care unit, the incidence rate of candidemia accounts for 88 % of all fungal infections [8].

Similarly, the antifungal potential of plant extracts has been studied due to the problem of multidrug-resistant microbial strains and the extent of microorganisms with reduced susceptibility to antifungals [9]. A drastic increase in resistance to antifungal treatment has been reported in *Candida auris* in more than 40 countries on six continents [10], and it is an emerging multidrug-resistant human pathogen for which the current MIC breakpoints were first reported in 2018 [11, 12]. In Colombia, a national alert on the circulation of this emerging resistant fungus was issued in 2016, with mortality rates varying significantly across geographic regions globally from 32.5 % to 72 % [13, 14].

Scientists have shifted their attention to natural products derived from plants with antifungal activities when designing new antimycotic medications because of a handful of reasons: (i) New resistant strains are constantly emerging; (ii) the number of antifungal drug classes available is limited [15, 16]. Also, (iii) there are adverse side effects for humans, such as toxicity. Lastly, (iv) it is also cumbersome to develop new antifungals [1, 17, 18].

The plant species *Thunbergia alata* (Acanthaceae), widely known as Black-eyed Susan and in Colombia called "ojo de poeta" [19], has been evaluated for its antifungal, antiviral, and antitumor properties [20, 21]. Furthermore, Black-eyed Susan is traditionally used within communities to treat inflammation, fever, and malaria. *T. alata* is a plant species native to East Tropical Africa, widely cultivated and naturalized in tropical regions. This plant has wavy margins, a winged 2-6.5 cm long petiole, solitary flowers on long peduncles, orange corolla, and sagittate leaves [22, 23, 24]. In particular, *T. alata* is an aggressive invasive plant prized for its fast-climbing habits. It forms a dense blanket over large areas and has naturalized mainly in disturbed areas in tropical, subtropical, and warmer temperate regions [25, 26]. For instance, in Colombia, it is considered one of the ten most problematic invasive species [27].

However, *T. alata* has been scarcely studied chemically, with previous work having isolated and identified some iridoid glycosides, such as 6-epi-stilbericoside, alatoside, and thunaloside [28]. Likewise, with High-Performance Liquid Chromatography (HPLC), phenolic compounds such as L-malic, feruloylamic, and *O*-coumaroylamic acids were determined; and by using Thin Layer Chromatography (TLC), glycerols, such as diacylglycerol and triacylglycerol [29] were detected. Related to its antifungal properties, there are no studies evaluating its potential antifungal effect on the reference strain C. albicans SC5314 or other clinical isolates. Vlietinck and Cols (1995) reported an in vitro assay of 267 plant extracts, including *T. alata*. This extract is effective against dermatophytes and against *S. aureus* and *P. aeruginos*a but not for yeasts at an initial concentration of 500 mg/mL by disk diffusion technique [21].

Given its widespread occurrence in various countries, expert concern about its invasive behavior, and the still limited chemical and biological studies of *T. alata*, more studies are necessary to broaden the knowledge of the species, with a possible subsequent therapeutic use. In this context, the present study aimed to evaluate the chemical composition and the in vitro antifungal activity of leaves and stem extracts of *T. alata* against *C. albicans* and *C. auris* yeasts.

2. Materials and Methods

2.1. General material

All the solvents used for extraction and HPTLC analysis were purchased in analytical grade from Merck® (Darmstadt, Germany). High-Performance Thin Layer Chromatography (HPTLC) analyses were conducted using HPTLC silica gel $60F_{254}$ chromatographic plates purchased from Merck® (Darmstadt, Germany). The reference standards for ultra-performance liquid chromatography (UPLC) coupled with photodiode array detection (PDA) and liquid chromatography-mass spectrometry (LC-MS), water (H_2O), methanol (MeOH), acetonitrile (ACN), and formic acid (FA) were purchased in LCMS grade from Merck®. Fluconazole was obtained from Sigma–Aldrich.

2.2. Preparation of crude extracts

The leaves and stems of adult *Thunbergia alata* individuals were collected in the locality of Pacho (Cundinamarca), Colombia. The dried and ground material (80 g for leaves and stem) was extracted by percolation using 96 % ethanol (EtOH) in a 1:10 w/v ratio, performing four extraction cycles every 24 hours with solvent replacement. The extract was dried by rotary evaporation and stored for further analysis.

2.3. Phytochemical Analysis

The initial phytochemical analysis was carried out following the methodology described by Sanabria, 1997 [30]. This analysis was performed using chemical tube tests to qualitatively determine the presence of different groups of secondary metabolites.

The analysis of the chemical profile was performed in a CAMAG® HPTLC chromatogram equipped with an autosampler (ATS 4), developer (ADC), derivatizer (DV), visualization camera, and VisionCATS software. The sample was prepared by weighing 10 mg of crude extract from leaves and stems dissolved in 1 mL of MeOH. For plate seeding, 10 µL of each extract were banded, eluted, and developed with different mobile phases and derivatizing agents for each metabolite group. The mobile phases (MF) and derivatizing agents (DA) used corresponded to: Terpenes, MF: Toluene:Chloroform:MeOH (4:4:1, v/v/v/v), DA: sulfuric anisaldehyde, a *Tillandsia usneoides* extract was used because it contains triterpenes among its major compounds; Saponins, MF: BuOH:H_2O:acetic acid (84:14:7, v/v/v), DA: sulfuric anisaldehyde and heat to visible, a *Passiflora edulis* extract was used because of its enriched saponin content; Tannins, MF: Toluene:EtOAc:FA (3:4:1, v/v/v/v), DA: $FeCl_3$ 1 %; Glycoside flavonoids, MF: EtOAc:Acetone:Acetic acid:H_2O (6:2:1:1:1, v/v/v/v/v/v), DA: Natural Reagent at UV 366 nm.

An Acquity H Class UPLC Waters® system was used for the UPLC-PDA analysis. All data were preprocessed in the Empower® 3 software. The analysis was performed on a Phenomenex® Kinetex EVO C18 column (100 × 2.1 mm, 2.6 µm, 100 Å) at 30 °C using ACN (solvent B) and H_2O in 0.1 % FA (solvent A) as mobile phases using the following run gradient: 3 % B from 0 to 3 min, 3 % to 95 % B from 3 to 30 min, 95 % B from 30 to 32 min and 95 % to 3 % B from 32 to 35 min, finally 3 % B from 35 to 40 min for conditioning with a flow rate of 400 µL mL^{-1}. Detection was performed at wavelengths 320 nm and 350 nm. For the LC-MS analysis, a QTOF 8060 Nexera X2 LC-MS instrument (Shimadzu, Duisburg, Germany) was used. The chromatographic conditions were the same as described above. The ionization method was ESI in negative mode. Samples were analyzed at a concentration of 1000 ppm in MeOH grade LC-MS.

2.4. Fungal Strains and Drugs

In this study, the *C. albicans* strain SC5314 along with a *C. albican's* fluconazole (FLC)-resistant isolate (CAAL256), and two clinical isolates of *C. auris* sensitive to FLC (CAAU435), and one resistant to FLC and amphotericin b (AMB) (CAAU537) were identified by MALDI-TOF MS and used in this work [24]. Subsequently, strains were grown on Sabouraud dextrose agar (SDA; Difco) plates and incubated overnight at 37 °C and stored at 4 °C for further use. Crude extract stocks were prepared in Dimethyl sulfoxide (< 1 %) (DMSO) Panreac Applichem®) and stored at −20 °C.

2.5. *In vitro* Antifungal Susceptibility Test

T. alata leaves and stem crude extracts' *in vitro* minimum inhibitory concentrations (MIC) were determined according to the CLSI M27-A3 guidelines using the broth microdilution method with slight modifications [31]. Briefly, the test was performed on 96-well microplates containing 100 µL of liquid RPMI 1640 medium (Roswell Park Memorial Institute (RPMI), Grand Island, NY, USA) with 2.1 mM L-glutamine and buffered with 165 mM MOPS (3-(N-morpholino) propane sulfonic acid) (pH 7.0) (Sigma-Aldrich). Yeast suspensions were adjusted at 15×10^{-6} cells mL^{-1} (0.5 McFarland standard). The final concentrations of the crude extracts studied ranged from 10.0 mg mL^{-1} to 0.781 mg mL^{-1}. FCL was used as a control (0.125 µg mL^{-1} to 128 µg mL^{-1}). The visual reading and densitometry (595 nm) were performed after 48 h of incubation as previously determined. The obtained data is reported as the lowest concentration of *T. alata* crude extracts capable of inhibiting 80 % of yeast growth. The *in vitro* fungicidal concentration (MFC) was determined as previously described [32]. Briefly, after 48 h, 10 µL was subcultured to plates containing SDA, from each well from the susceptibility testing assay. The plates were incubated at 37 °C for 24 h. The highest dilution with complete inhibition on the agar plate was considered as MFC (99 % to 99.5 % killing activity) [33].

3. Results and discussion

3.1. Phytochemical analysis

The initial phytochemical screening of the ethanolic extracts of *T. alata* leaves and stems revealed the presence of secondary metabolites consisting of terpenoids, saponins, flavonoids, and tannins (Table 1.)

The secondary metabolites detected with screening were also evaluated by HPTLC analysis, which allowed identifying *T. alata'*s fingerprint for these metabolites. The selection of different mobile phases was made according to the polarity of each group of compounds following literature reports [34]. Steroids, terpenoids (Figure 1A), saponins (Figure 1B), and flavonoids (Figure 1C) were analyzed. Results confirmed the presence of terpenoids and saponins in both *T. alata* extracts. Interestingly, leaf and stem extracts revealed distinct phenolic profiles (Figure 1c). The presence of glycosylated flavonoids was observed mainly in the stem extract; in particular, a correlation between the standard flavonoid rutin and a band in the stem extract with the same Rf 0.4. was detected. On the other hand, the dominance of fluorescent blue color, observed in leaves extract, indicated the presence of phenolic carboxylic acids (*e.g.*, phenylpropanoid acids), which can also be observed under the same chromatographic conditions, according to Wagner and Bladt (1996) [34].

Table 1. Group of metabolites in *T. alata* ethanolic extracts analyzed by phytochemical screening.

Group of metabolites	Assay	Positive control	Leaves	Stems
Alkaloids	Draggendorf	Caffeine	-	-
Cardiotonic	Molisch	*Digitalis purpurea extract*	-	-
Carotenoids	Salkowski	β-carotene	-	-
Coumarins	Fluorescence	Umbelliforme	-	-
Flavonoids	Shinoda	Rutin	NC	+
Saponins	Foam	*Passiflora edulis extract*	++	++
Tannins	FeCl3	Tannic acid	++	++
	Gelatin-salt	Tannic acid	++	++
Terpenoids	Liebermann-Burchard	*Tillandsia usneoides extract*	+	+

Note: (-): negative result; (+): positive result; (++): abundant positive result; NC: Not conclusive.

UPLC-PDA and LC-MS assays were performed to identify some of the main compounds in the extracts (Figure 2 and Figure 3). The chromatogram of the leaf extract revealed three prominent peaks that eluted between 6 and 11 minutes. Their UV-Vis spectra, with maximum absorption around 325 nm with a left shoulder, indicated phenylpropanoid-type compounds. Co-injection with a reference standard allowed the identification of peak 1 as chlorogenic acid based on its retention time (6.80 min), UV-Vis spectra (244, 325sh λ_{max}), and MS spectra ($[M\text{-}H]^-$ 353.0 *m/z*).

The chromatogram of the stem extract showed major peaks accumulated between 8 and 13 minutes. In contrast to the leaf extract, the major peak revealed UV-Vis spectra related to flavonoid compounds, in agreement with the preceding HPTLC analysis. The major peak was identified as the flavonoid rutin when co-injection of the extract with a reference standard was performed (Rt 9.45 min; UV-Vis spectra 254, 353 λ_{max}; MS spectra $[M\text{-}H]^-$ 609.5 *m/z*).

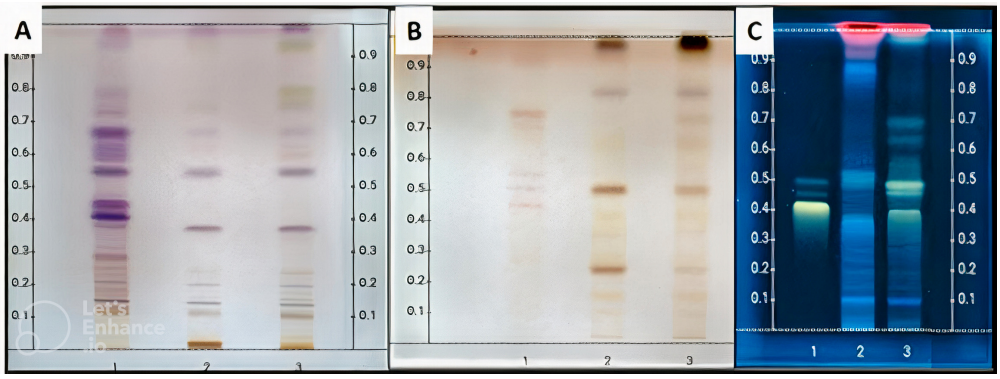

Figure 1. *T. alata* HPTLC chromatograms by HPTLC. (A) For steroids and terpenoids. Lane 1, extract of *T. usneoides*; lane 2. *T. alata* leaf extract; lane 3, *T. alata* stem extract. Mobile phase toluene:chloroform:methanol (4:4:1). (B) For Saponins: lane 1, extract of *P. edulis*; lane 2, *T. alata* leaf extract; lane 3, *T. alata* stem extract. Mobile phase: butanol: water: acetic acid (84:14:7). (C). For glycosylated flavonoids: lane 1, Rutin standard; lane 2, *T. alata* leaf extract; lane 3, *T. alata* stem extract. Mobile phase: ethyl acetate: acetone: acetic acid: water (6:2:1:1).

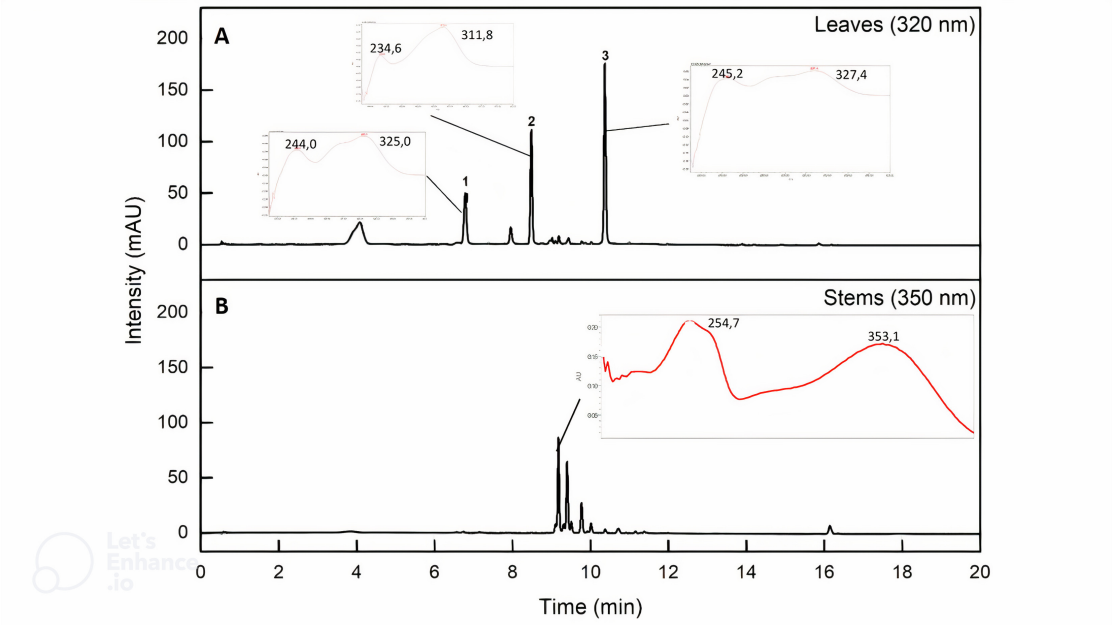

Figure 2. Chromatogram of *T. alata* by UPLC. (a) Leaf extract, obtained at 320 nm. (b) Stem extract, obtained at 350 nm. Marked peaks: 1, Chlorogenic acid; 2, Unknown phenylpropanoid derivative; 3, Unknown phenylpropanoid derivative.

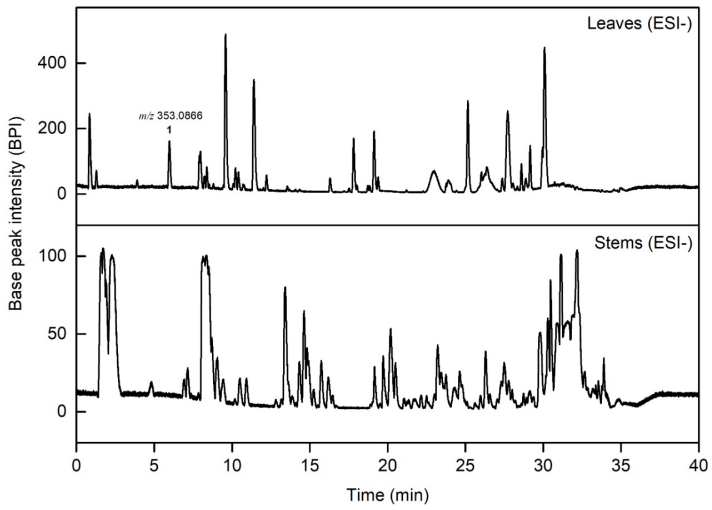

Figure 3. TIC chromatogram of T. *alata* by LC-MS. (a) Leaf extract. (b) Stem extract. Marked peak 1: Chlorogenic acid.

3.2. *T. alata* extract activity on *C. albicans* and *C. auris* growth

In this study, the effects of *T. alata* crude leaf and stem extracts were determined against the growth of *C. albicans* and *C. auris.* The assays revealed MIC values of the *T. alata* leaf extract of 5.0 mg mL^{-1} and 2.5 mg mL^{-1} on the SC5314 and CAAL256 strains, respectively. The MFC

value of this extract was 10 mg mL^{-1} for both strains. In addition, in *C. auris,* the MIC values of the *T. alata* leaf extract were 2.5 mg mL^{-1} and 1.25 mg mL^{-1} for the CAAU435 and CAAU537 strains, respectively. The results indicated that the CAAU537 strain is twice as susceptible to the *T. alata* leaf extract as *C. albicans*. Conversely, the *T. alata* stem extract on screening was not active against *C. albicans* and *C. auris* strains (Figure 4).

The ethanolic *T. alata* leaf extract tested on *C. albicans* and *C. auris* strains showed a dose-dependent antifungal activity. This agrees with previous reports in which similar observations were made for leaf extracts showing activity against *Salmonella typhi, P. aeruginosa, Vibrio Cholera,* and *Aspergillus fumigatus* [20, 35, 36, 37].

One of the main compounds identified in the active leaf extract was chlorogenic acid. According to Sung and Lee (2010) [38], chlorogenic acid exerts its antifungal activity by damaging the membrane's permeability barrier and disturbing its lipid bilayers. This alteration leads to the leakage of ions and other compounds, which form pores in the membrane, and the dissipation of the electrical potential between the interior and exterior of the cell. Additionally, chlorogenic acid induces caspase activation and DNA fragmentation, which are considered apoptotic markers [39]. Nevertheless, potential synergic effects between the different extract components, commonly observed in plant extracts, could also account for the antimicrobial activity observed.

In addition, the leaf ethanol extract exhibited potent antifungal activity against the CAAU537 strain, with the MIC for this strain being half that of the wild-type *C. albicans* strain. This reduced resistance may be due to multiple causes, such as a mutation in 14-sterol demethylase affecting resistance or overexpression of the ERG11 gene and ABC1 efflux pumps, mechanisms associated with antifungal resistance [40]. The fungicidal effect of *T. alata* on *Candida spp*. may be due to the lysis of the fungal cell wall and cytoplasmic membrane due to the release of antifungal products [41]. However, this hypothesis still needs confirmation.

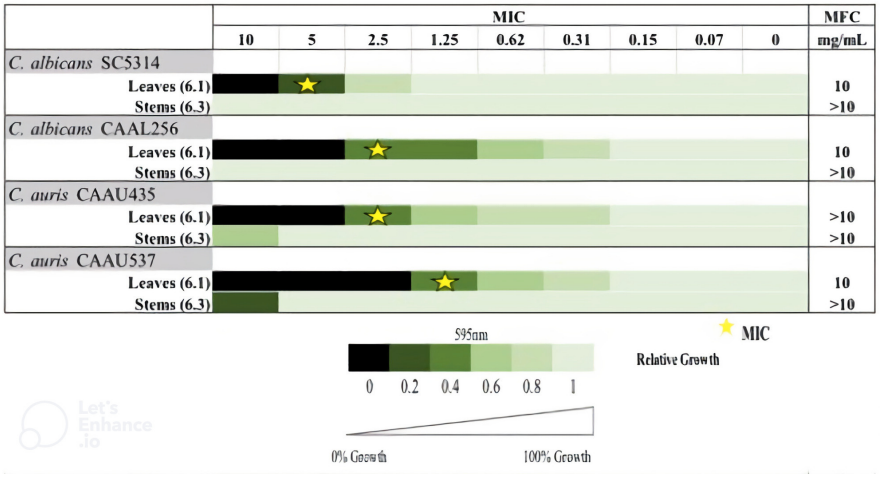

Figure 4. *In vitro* antifungal susceptibility test results. Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC) of *T. alata* (leaf and stem) extracts against *C. albicans* and *C. auris* strains. The green color bar indicates relative fold growth.

4. Conclusions

Among the metabolites in *T. alata* extracts, tannins, saponins, flavonoids, and terpenes stand out. HPTLC allowed the identification of the differences between the profiles of leaf and stem phenolic extracts, revealing the presence of glycosylated flavonoids in the stem extract and abundant phenolic acids in the leaf extract. UPLC-PDA and LC-MS allowed the identification of chlorogenic acid in the leaf extract and rutin in the stem extract as some of their main compounds. In addition, this work reports, for the first time, the antifungal activity of the ethanolic extract obtained from the leaves, which showed activity against *C. albicans* and *C. auris*.

5. Acknowledgements

The authors are thankful to Pontificia Universidad Javeriana, al Ministerio de Ciencia, Tecnología e Innovación, al Ministerio de Educación Nacional, al Ministerio de Industria, Comercio y Turismo e ICETEX, 2a Convocatoria Ecosistema Científico—Colombia Científica 792-2017, Programa "*Generación de alternativas terapéuticas en cáncer a partir de plantas a través de procesos de investigación y desarrollo traslacional, articulados en sistemas de valor sostenibles ambiental y económicamente*" (Contrato no. FP44842-221-2018) and the Ministerio de Ambiente y Desarrollo Sostenible for allowing the use of genetic resources and products derived (Contract number 212/2018; Resolution 210/2020).

6. Conflict of interests

The authors declare no conflict of interest.

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Ojo de poeta (*Thunbergia alata*): evaluación del potencial químico y antifúngico de una especie vegetal invasora en Colombia

Resumen: *Thunbergia alata* (ojo de poeta) es una especie vegetal originaria de África Oriental que crece en regiones tropicales de todo el mundo, incluyendo Colombia, siendo considerada una especie invasora en algunos países. Aunque es utilizada por comunidades locales para tratar diversas enfermedades, incluyendo la malaria, existen pocos estudios biológicos y químicos sobre *T. alata*. Por lo tanto, este estudio tuvo como objetivo explorar la composición química y la actividad antimicótica *in vitro* (contra cepas de *Candida*) de los extractos crudos etanólicos del tallo y la hoja de *T. alata*. Esto se logró mediante análisis fitoquímicos y perfilado cromatográfico (HPTLC, UPLC-DAD y LC-MS). Se utilizó un método de microdilución serial en dos etapas para determinar la concentración mínima inhibitoria (MIC) contra cepas seleccionadas de *Candida*. Los resultados mostraron la presencia de saponinas, terpenos y flavonoides en los extractos crudos etanólicos del tallo y la hoja de *T. alata*, aunque la rutina y el ácido clorogénico fueron los principales componentes de los extractos de tallo y hoja, respectivamente. En cuanto a los ensayos biológicos, los extractos de hoja de *T. alata* demostraron una actividad antimicótica moderada, con valores de MIC entre 5 y 1.25 mg/mL contra las cepas de *C. albicans* y *C. auris*. Los resultados indican que los extractos de *T. alata*, incluyendo algunos de los principales compuestos identificados, poseen propiedades antifúngicas prometedoras contra dos microorganismos significativos.

Palabras Clave: actividad antifúngica; *Candida albicans*; *Candida auris*; HPTLC; LC-MS; *Thunbergia alata*; UPLC.

Amarelinha (Thunbergia alata): avaliação do potencial químico e antifúngico de uma espécie de planta invasora na Colômbia

Resumo: *Thunbergia alata* (Amarelinha) é uma espécie vegetal originária da África Oriental que cresce em regiões tropicais ao redor do mundo, incluindo a Colômbia, sendo considerada uma espécie invasora em alguns países. Embora seja utilizada por comunidades locais no tratamento de várias doenças, incluindo a malária, existem poucos estudos biológicos e químicos sobre *T. alata*. Portanto, este estudo teve como objetivo explorar a composição química e a atividade antimicótica *in vitro* (contra cepas de *Candida*) dos extratos brutos etanólicos do caule e da folha de *T. alata*. Isso foi alcançado por meio de análises fitoquímicas e perfil cromatográfico (HPTLC, UPLC-DAD e LC-MS). Foi utilizado um método de microdiluição serial em duas etapas para determinar a concentração inibitória mínima (MIC) contra cepas selecionadas de *Candida*. Os resultados mostraram a presença de saponinas, terpenos e flavonoides nos extratos brutos etanólicos do caule e da folha de *T. alata*, embora a rutina e o ácido clorogênico tenham sido os principais componentes dos extratos de caule e folha, respectivamente. Em relação aos ensaios biológicos, os extratos de folha de *T. alata* demonstraram uma atividade antimicótica moderada, com valores de MIC entre 5 e 1.25 mg/mL contra as cepas de *C. albicans* e *C. auris*. Os resultados indicam que os extratos de *T. alata*, incluindo alguns dos principais compostos identificados, possuem propriedades antifúngicas promissoras contra dois microorganismos significativos.

Palavras-chave: atividade antifúngica; *Candida albicans*; *Candida auris*; HPTLC; LC-MS; *Thunbergia alata*; UPLC.

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