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ORIGINAL ARTICLE

Phytochemical study and antifungal activity of leaf and flower extracts of the Asteraceae species *Chromolaena scabra* (LF) R. King & H. Rob.

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Abstract

The *Chromolaena* genus is composed by about 170 plant species widely distributed in tropical areas. Several kinds of secondary metabolites and different biological activities have been described for species belonging to *Chromolaena*. This work described, for the first time, the phytochemical analysis of different polarity leaf and flower extracts of the Colombian species *Chromolaena scabra* (LF) R. King & H. Rob. Conducted assays resulted in the purification and identification of fatty acids derivatives, steroids, a diterpene, and flavonoid metabolites. The preliminary phytochemical analysis revealed the presence of the main groups of secondary metabolites: steroids, triterpenes, carotenoids, alkaloids, tannins, and flavonoids. In parallel, the antifungal activity of these *C. scabra* leaf and flower extracts against the fungus *Fusarium oxysporum* f. sp. *lycopersici,* was determined.

Keywords: asteraceae; eupatorium; *Fusarium oxysporum*; secondary metabolites.

1. Introduction

Fusarium is a genus of fungi with a worldwide distribution and is particularly abundant in tropical and temperate zones. Fusarium entails phytopathogenic fungi, that damage crops and ornamental plants [1] and is responsible for a broad spectrum of diseases, such as root rot, stem base, fruit rot, descending death, leaf spots, and vascular wilting. *Fusarium oxysporum* is one the species well known for causing wilting [2] and having documented resistance to chemical treatments. This fungal species possesses multiple strategies to overcome plant defenses thus, affecting different economically important crops and threatening food safety [3].

The Asteraceae is a plant family comprising 25 000 species within, approximately, 1000 genera. It is one of the families with the broadest global distribution and with the largest number of described species [4]. Around 10 % of the Asteraceae species have been classified within the Eupatorium tribe, and they occur chiefly in neotropical areas. King & Robinson [5] grouped several species of this tribe in the *Chromolaena* genus. Species of this genus are characterized by being invasive and cosmopolitan, and by presenting a high morphological diversity linked to their adaptation to different environments [6]. The genus is composed of 170 species scattered in northern Mexico, southeastern United States, western India, and South America [7]. In Colombia, 27 species have been reported at altitudes ranging from sea level to 3900 m.

Efforts to identify predominant secondary metabolites in *Chromolaena* have been undertaken. As a result, a large number of such metabolites has been reported, including sesquiterpenes, triterpenes [8], and cadinenes, which have been identified in almost a third of the species of this genus, thus being regarded as genus-specific metabolites [9]. Furthermore, polyhydroxylated and polymethoxylated flavone and flavanones, chalcone-type metabolites, phenolic acids, and coumarins are metabolites with high occurrence in the genus [10].

These studies show that the genus *Chromolaena* is a source of characteristic phenolic secondary metabolites with interesting molecular structures [11]. Moreover, different types of biological activities such as antibacterial, antifungal, analgesic, anti-inflammatory, and antioxidant have been reported in several *Chromolaena* species [12, 13, 14]. These biological properties are probably related to the terpenes, flavonoids and sesquiterpene lactones derivatives, which are often identified in phytochemical analysis.

Recently, the antioxidant activity of ethanol and dichloromethane extracts of leaves and flowers of *Chromolaena scabra* was determined using the ABTS and DPPH assays, revealing that the extracts of medium-high polarity have significant potential as natural antioxidant agents [15]. Similarly, the same authors reported the antibacterial and antifungal activity of *C. scabra* extracts against *Staphylococcus aureus, Escherichia coli, Aspergillus niger,* and *Penicillium digitatum*. In addition, a high antibacterial effect of the petroleum ether extracts of *C. scabra* leaves and flowers against *S. aureus*, *A, niger,* and *P. digitatum* was reported [16]. However, to the best of our knowledge, there are no reports of the phytochemistry of *C. scabra*.

This work presents a preliminary phytochemical analysis of the extracts of different polarity of leaves and flowers of *C. scabra*. The isolation and identification of the major secondary metabolites using chromatographic, spectroscopic, and spectrometric techniques are also presented. Additionally, the determination of the antifungal activity of the extracts and isolated compounds against *F. oxysporum* f. sp. *lycopersici* is discussed.

2. Materials and Methods

2.1. Plant material

C. scabra material was collected in the northeastern sector of the city of Bogota-Colombia, the samples consisted of leaves (650 g) and flowers (200 g) of plants at a foliation stage. A sample was sent to the National Herbarium (Institute of Natural Sciences at the National University of Colombia), where it was identification as C. *scabra* (LF) R. King & H. Rob was confirmed with voucher number 517195.

2.2. Extract preparation and isolation

Following published protocols for natural products [17], the plant material was dried at room temperature, resulting in 140 g of leaves and 42 g of flowers. Once dried the materials were crushed in a mill and subjected to successive extractions in a Soxhlet equipment, yielding petroleum ether extract (A) and ethyl acetate extract (B) from leaves. Then, extracts were subjected to solid-liquid fractioning and the obtained fractions were processed using column chromatography and preparative thin layer chromatography. Once the major constituents were isolated, their melting points were determined and their spectroscopic and spectrometric analysis were conducted.

Simultaneously, successive macerations allowed the preparation of petroleum ether extract (C) and dichloromethane extract (D) from flowers, which were directly fractionated by column chromatography.

For column chromatography, silica gel 60 from 0.040 mm to 0.063 mm was used; for liquid vacuum chromatography (LCV), silica gel 60 was used; and for thin layer chromatography, silica gel HF254 chromatoplate was used. All solvents used were of analytical grade. Gas chromatography-mass spectrometry (GC-MS) analyzes were performed on an Agilent 6890 5973 chromatograph coupled to a HP 5975B VL MSD mass detector, equipped with an Agilent $30\,\text{m} \times 0.32\,\text{mm} \times 1\,\text{mm}$ HP-5MS column. The assay proceeded with an injector temperature of 150 °C and an initial column temperature of 80 °C, using a ramp of $30\,^{\circ}\text{C}\,\text{min}^{-1}$, reaching a final column temperature of 300 °C. For compound identification, the spectra stored in the Wiley 7.0 database was used were employed as reference. All the compounds detected presented values of coincidence higher than 90 %.

All of the 1H NMR, 13C NMR, COSY, HMQC, and HMBC nuclear magnetic resonance spectra were taken on a BRUKER Advance III HD Ascend 400 (400 MHz) equipment. Chemical shifts (δ) and coupling constants (J) were expressed in ppm and Hertz, respectively. TMS was used as an internal standard. Melting points were determined on a Mel-temp 1101D equipment.

2.3. Mycelium inhibition growth assay

The activity of C. *scabra* extracts and isolated compounds against *F. oxysporum f. sp. lycopersici*, was determined by a mycelium inhibition growth assay [18, 19]. The fungal pathogen *F. oxysporum f. sp. lycopersici* was isolated from tomato plants with symptoms of fusarium wilt. The percentage of fungal growth inhibition was calculated by comparing the diameter of the treated colonies with of the control (Ketoconazol), calculated with the formula:

$$\%\ \text{inhibition} = \left(\frac{\text{growth in control} - \text{growth in extract}}{\text{growth in control}}\right) \times 100. \tag{1}$$

3. Results and Discussion

3.1. Preliminary phytochemical analysis

The ethanolic extracts of *C. scabra* leaves and flowers were obtained by maceration with 96 % ethanol for seven days, once the extracts were concentrated, tests for metabolites as lactones, cardiac glycosides, steroids, triterpenoids, carotenoids, tannins, alkaloids, flavonoids, and quinones were performed in duplicate using pure compounds as positive controls.

The preliminary phytochemical analysis of *C. scabra* extracts resulted in the identification of the following secondary metabolite groups: steroids, triterpenes, carotenoids, alkaloids, tannins, and flavonoids. This analysis also revealed that some secondary metabolites, such as sesquiterpenes lactones, cardiotonic glycosides, alkaloids, and quinones were only present in the leaves. This finding was consistent with previous studies with other species of this genus [10].

Table 1. Compounds detected by gas chromatography – mass spectrometry (GC-MS) in the petroleum ether extract of leaves (A) from *C. scabra.*

	Type of Compound	Name
1	Fatty acid esters	Methyl hexadecanoate
2		Ethyl lineolate
3		Ethyl octadecanoate
4		Ethyl 9,12,15 Octadecatrienoate
5	Diterpene	Phytol
6	Steroids	Stigmasterol
7		γ-sitosterol

3.2. Petroleum ether leaf extract

The leaf petroleum ether extract (A) (6.65 g) was fractionated by liquid vacuum chromatography using, as mobile phase, petroleum ether: dichloromethane 0 % to 100 %. **Table 1** presents the compounds detected by GC-MS in the petroleum ether extract of leaves (A) from *C. scabra*. Three fractions were obtained: A1 (1.79 g), A2 (2.40 g) and A3 (2.16 g).

Fraction A1 was subjected to chromatographic fractionation on a silica gel column and eluted with petroleum ether: dichloromethane (95 : 05) to dichloromethane (100 %), yielding 20 fractions. The combined fractions 8, 9, and 10 were analyzed by gas chromatography – mass spectrometry (GC-MS), up to four methyl and ethyl esters. Methyl hexadecanoate, ethyl lineolate, ethyl octadecanoate, ethyl 9,12,15 Octadecatrienoate were identified in these combined fractions.

Fraction A2 yielded further 15 fractions after being subjected to column chromatography on a silica gel column and eluted with petroleum ether: dichloromethane (80 : 20) to dichloromethane (100 %). The resulting fractions 3 and 4 were jointly analyzed by GC-MS, revealing the presence of the acyclic diterpene alcohol phytol (5).

Fraction A3 was also subjected to silica gel column chromatography and eluted with petroleum ether: dichloromethane (70 : 30) to dichloromethane (100 %) to obtain 19 fractions. The resulting fraction 6 was analyzed by GC-MS, revealing the presence of stigmasterol and gamma sitosterol in this mixture.

3.3. Ethyl acetate leaf extract

The ethyl acetate extract from leaves (B) (18.04 g) was fractionated by vacuum liquid chromatography with solvents of increasing polarity obtaining three fractions: B1 (2.98 g) eluted with dichlorometane, B2 (8.44 g) eluted with chloroform, and B3 (5.95 g) eluted with ethyl acetate.

Fraction B2 was separated through successive column chromatography, with silica gel 60 as the stationary phase and mobile phase chloroform: acetone (90 : 10), obtaining 36 fractions (B2/1 to B2/36). Combined fractions B2/16 to B2/20 (208 mg) were purified in column chromatography using dichloromethane: acetone (30 : 70) as eluent, to obtain compound 8 (20 mg). Fraction B2/28 (128 mg) was subjected to column chromatography with silica gel 60 as stationary phase and a mixture of dichloromethane: methanol (98 : 2) as mobile phase, to obtain compounds 9 (17 mg) and 10 (12 mg).

Compound 8 had a melting point of 313 °C to 315 °C and tested positive for the ferric chloride reagent ($FeCl_3$), indicating the presence of at least one phenolic-type substituent. The ^{1}H NMR spectrum (400 MHz, CD_3OD), showed aromatic signals at $\delta = 7.77$ (d, $J = 2.2$ Hz, 1H), $\delta = 7.68$ (dd, $J = 8.6$ and 2, 2 Hz, 1H) and $\delta = 6.92$ (d, $J = 8.6$ Hz, 1H) corresponding to signals from a disubstituted aromatic ring, characteristics of the B ring of a flavonoid, as well as signals at $\delta = 6.43$ (d, $J = 2.1$ Hz, 1H) and $\delta = 6.22$ (d, $J = 2.1$ Hz, 1H) corresponding to a *meta*-disubstituted benzene, characteristics of the A ring of a flavonoid. Compound 8 corresponded to the flavonoid quercetin [20]. **Figure 1** shows the 1H RMN spectrum of compound 8.

Compound 9 had a melting point of 248 °C to 250 °C and tested positive against the $FeCl_3$ reagent, indicating the presence of a phenolic group. Its ^{1}H NMR spectrum (400 MHz, CD_3OD) showed aromatic signals at $\delta = 7.32$ (d, $J = 8.4$ Hz, 2H) and $\delta = 6.77$ (d, $J = 8.4$ Hz, 2H) corresponding to the disubstituted ring B of a flavonoid. The signals corresponding to ring A of the structure of a flavonoid were also observed at $\delta = 5.89$ (d, $J = 2.0$ Hz, 1H) and $\delta = 5.86$ (d, $J = 2.0$ Hz, 1H), along with three signals present at $\delta = 5.29$ (dd, $J = 12.9$ Hz and 2.8 Hz, 1H), $\delta = 3.14$ (dd, $J = 17.0$ Hz and 12.9 Hz, 1H), and $\delta = 2.72$ (dd, $J = 17.0$ Hz and 2.8 Hz, 1H), which in conjunction with the signal at $\delta = 41$ in the carbon spectrum, suggested the presence of a phenolic compound of the flavanone type. When comparing the spectroscopic data and the physical constants obtained for compound 9, it could be identified as the flavanone naringenin [21]. **Figure 2** shows the 1H RMN spectrum of compound 9.

Compound 10 had a melting point of 276 °C to 278 °C and tested positive against the $FeCl_3$ reagent, indicating, as it was done for compounds 8 and 9, the presence of a phenolic OH group. Its ^{1}H NMR spectrum (400 MHz, CD_3OD) showed a signal profile similar to that of compound

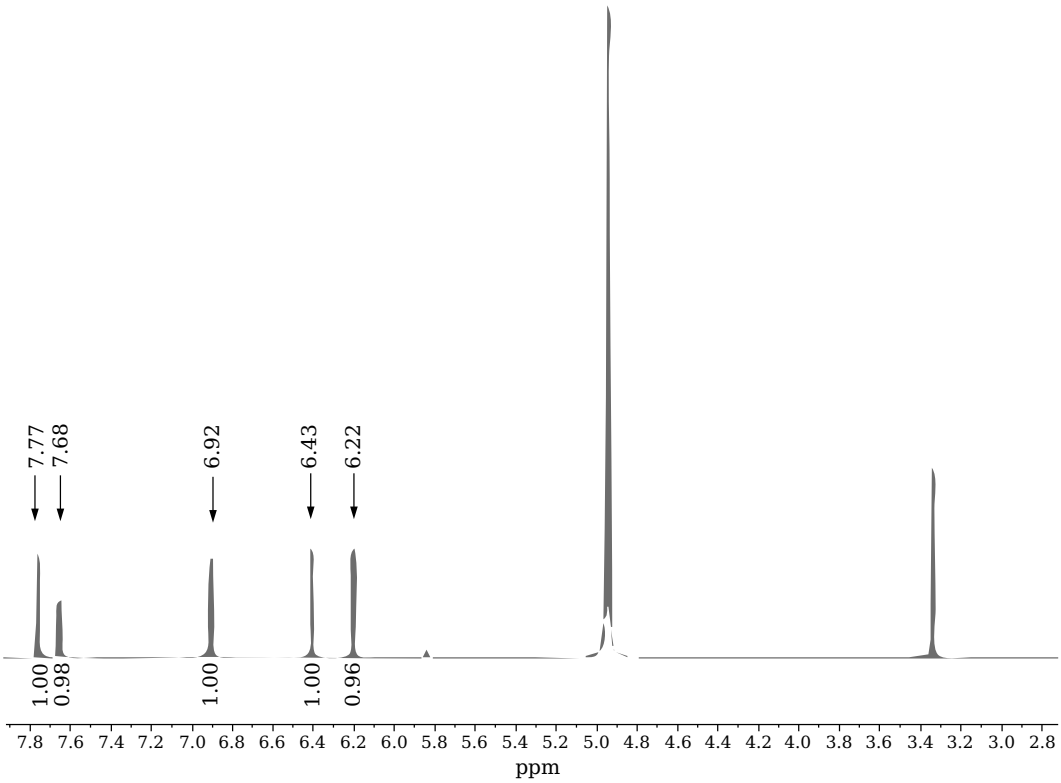

Figure 1. H_1 Nuclear Magnetic Resonance spectrum (400 MHz, CD_3OD) for compound 8: Quercetin.

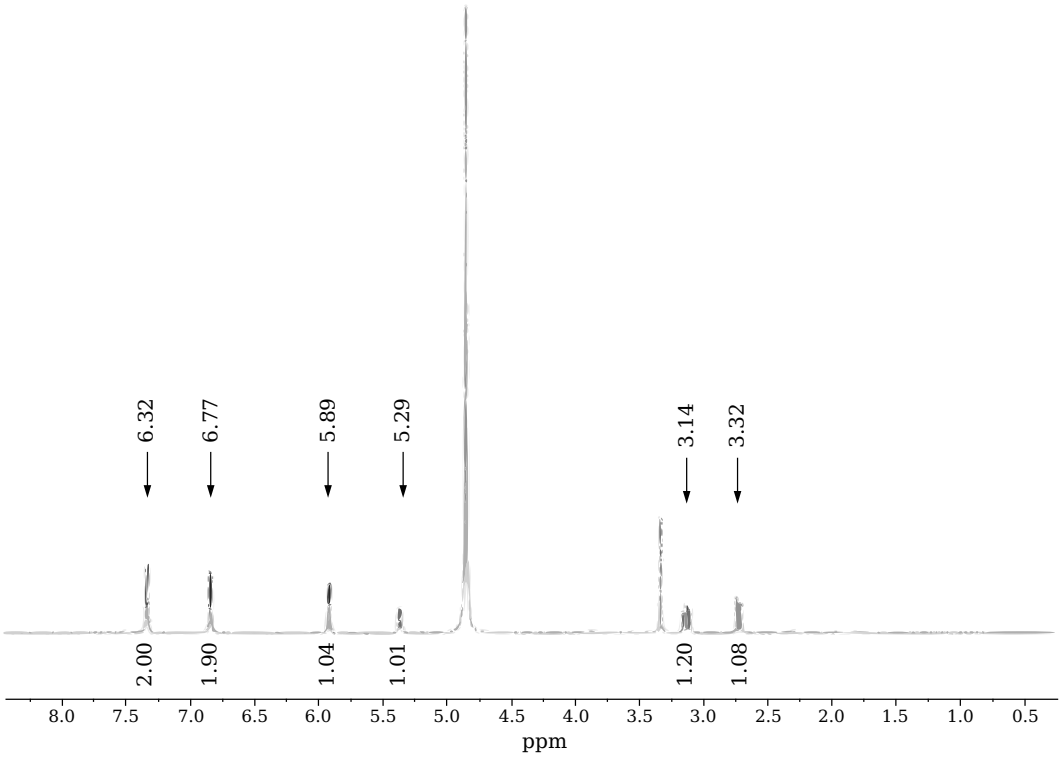

Figure 2. H_1 Nuclear Magnetic Resonance spectrum (400 MHz, CD_3OD) for compound 9: Naringenin.

9, presenting aromatic signals at $\delta = 8.04$ (d, $J = 9.0$ Hz, 2H) and $\delta = 6.91$ (d, $J = 9.0$ Hz, 2H) corresponding to the B ring for a substituted flavonoid, as well as, signals at $\delta = 6.43$ (d, $J = 2.0$ Hz, 1H) and $\delta = 6.16$ (d, $J = 2.0$ Hz, 1H) characteristics of the *meta*-substituted A ring of the same kind of natural product. When comparing the spectroscopic data and the physical constants of compound 10 with those reported in the literature, it was possible to establish that this compound corresponds to a flavonoid kaempferol [20]. **Figure 3** shows the 1H RMN spectrum of compound 10, and the molecular structures of all leaf-isolated flavonoids are shown in **Figure 4**.

3.4. Dichloromethane flower extract.

The dichloromethane extract of flowers (D) (4.88 g), was fractionated by column chromatography, using silica gel 60 as stationary phase and as mobile phase a mixture of dichloromethane: ethyl acetate (90 : 10 to 0 : 100) in gradient to obtain 29 fractions (D1 to D29). Fraction D17 (59 mg) was purified by column chromatography using silica gel 60, with a 94 : 6 dichloromethane: methanol mixture as mobile phase, obtaining the compound 10 (10 mg), which was previously obtained from the ethyl acetate extract from leaves and characterized as flavonoid kaempferol.

Our findings are in accordance with previous studies for the genus *Chromolaena*, which is recognized by a high content of phenolic compounds, being flavonoids the most important [22, 23, 24]. In the last decade, the interest in phenolic compounds has been renewed because of their antiviral, antioxidant, anti-mutagenic, and anti-carcinogenic effects *in vitro* and *in vivo*. The hydroxyl groups of flavonoids are of paramount importance for their broad biological activity. This kind of compounds protect human cells from the damage caused by free radicals and can reduce the oxidative damage of radicals generated by cellular stress [25]. Flavonoids, such as quercetin, kaempferol, and naringenin are abundant in fruits and vegetables with antioxidant, anticancer,

antibacterial, and anti-inflammatory properties. These flavonoids have a preventive effect on neurodegenerative and cardiovascular disorders and possess gastroprotective properties [26, 27]. *Chromolaena scabra* is a promising source of different flavonoids and bioactive molecules.

3.5. Determination of Antifungal Activity

In order to determine the antifungal potential of the extracts and flavonoids obtained from *C. scabra* against phytopathogenic fungi *F. oxysporum* f. sp. *lycopersici*, the agar diffusion method was implemented. In this essay ketoconazole, at a concentration of 50 µg mL^{-1}, was employed as positive control and 1 % DMSO as a negative control. The evaluation of the extracts and compounds was carried out in triplicate, at concentrations of 1 mg mL^{-1} of medium for extracts and 0.1 mg mL^{-1} of medium for compounds. Incubation with the phytopathogenic fungi was carried out at 20 °C, with 60 % to 70 % relative humidity and for a period of 8 days. To determine the radial growth, the measurement of the diameter of the colony were carried out on day 8, and to obtain the percentage of growth inhibition, these results were compared with the negative control (DMSO 1 %) [28].

The results of the antifungal activity of the extracts (A-D) and the isolated flavonoids are shown in **Table 2**. None of the different polarity extracts exerted significant inhibitory activity against the fungus *F. oxysporum* f. sp. *lycopersici*. Similarly, the flavonoids isolated from the leaves and flowers: quercetin (**8**), naringenin (**9**) and kaempferol (**10**) had a low activity against that phytopathogenic fungus. However, it is worth mentioning that other flavonoids structurally related to those reported here have shown significant activity against fungi of the genus *Aspergillus* (*repens*, *amstelodami*, *chevalieri*, *flavus* and *petrakii*) [29].

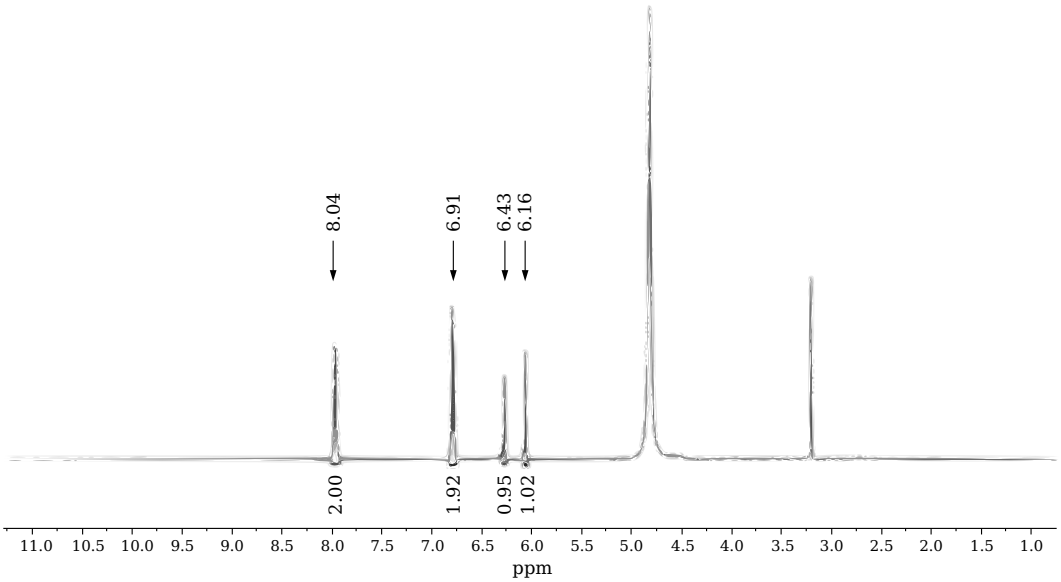

Figure 3. H_1 Nuclear Magnetic Resonance spectrum (400 MHz, CD_3OD) for compound 10: Kaempferol.

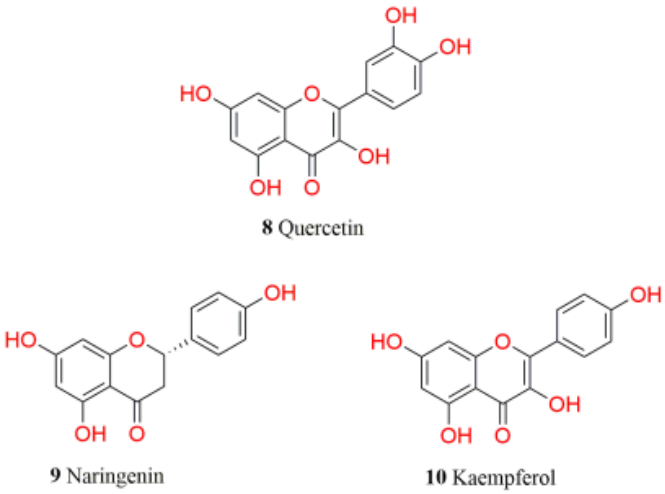

Figure 4. Molecular structures of flavonoids isolated from *C. scabra* leaves.

4. Conclusions

In this work we have deepened the current knowledge of the chemical potential of the Asteraceae plant family. Our work represents the very first determination of the major secondary metabolites of leaves and flowers of the Asteraceae species *Chromolaena scabra* (LF) R. King & H. Rob. Our study allowed the isolation and identification of several types of secondary metabolites such as sterols, fatty acids derivatives, diterpenes, and flavonoids from extracts of different polarity.

The isolation and determination of the molecular structure of several types of secondary metabolites, and particularly three polyhydroxylated flavonoids: quercetin, naringenin, and kaempferol may be of chemotaxonomic significance, given the high occurrence of the latter kind of metabolites in species of the genus *Chromolaena*. The evaluation of the antifungal activity of the obtained extracts did not show significant activity against the fungus *Fusarium oxysporum* f. sp. *lycopersici.*

Table 2. Antifungal activity of extracts and isolated flavonoids of *C. scabra* against *F. oxysporum* f. sp. *lycopersici.*

Extract/compund	*F. oxyporum f. sp. lycopersici*	
	Growth diameter (mm)	**Growth inhibition percentage**
Petroleum Ether from leaves (A)	53.5 ± 0.5	1.44
Ethyl acetate from leaves (B)	53.6 ± 1.0	0.88
Petroleum ether from flowers (C)	53.0 ± 0.8	2.03
Dichloromethane from flowers (D)	53.2 ± 0.5	1.62
Quercetin (8)	51.7 ± 0.7	4.54
Naringenin (9)	52.4 ± 0.8	3.25
Kaempferol (10)	52.3 ± 1.0	3.59
(+) Ketoconazol	2.10 ± 0.30	90.6
(−) DMSO	54.1 ± 0.5	-

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6. Conflict of interest

The authors declare no conflict of interest.

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Estudio fitoquímico y actividad antifúngica de extractos florales y foliares de la especie Asteraceae *Chromolaena scabra* (LF) R. King & H. Rob.

Resumen: El género *Chromolaena* está compuesto por cerca de 170 especies de plantas ampliamente distribuidas en áreas tropicales. Se han descrito algunos tipos de metabolitos secundarios y diferentes actividades biológicas para especies pertenecientes a *Chromolaena*. Este trabajo describió, por primera vez, el análisis fitoquímico de extractos florales y foliares de diferente polaridad de la especie colombiana *Chromolaena scabra* (LF) R. King & H. Rob. Los ensayos llevados a cabo resultaron en la purificación e identificación de derivados de ácidos grasos, esteroides, un diterpeno y metabolitos flavonoideos. El análisis fitoquímico preliminar reveló la presencia de los grupos principales de metabolitos secundarios: esteroides, triterpenos, carotenoides, alcaloides, taninos y flavonoides. Paralelamente, se determinó la actividad antifúngica de estos extractos florales y foliares de *C. scabra* contra el hongo *Fusarium oxysporum* f. sp. *Lycopersici*.

Palabras Clave: Asteraceae; Eupatorium; *Fusarium oxysporum*; metabolitos secundarios.

Estudo fitoquímico e atividade antifúngica de estratos de folhas e flores da espécie de Asteraceae *Chromolaena scabra* (LF) R. King & H. Rob.

Resumo: O gênero *Chromolaena* é composto por aproximadamente 170 espécies de plantas amplamente distribuídas em áreas tropicais. Vários tipos de metabólitos secundários e diferentes atividades biológicas foram descritos em espécies pertencentes a *Chromolaena*. Este trabalho descreveu, pela primeira vez, a análise fitoquímica de extratos de folhas de diferente polaridade e flores da espécie colombiana *Chromolaena scabra* (LF) R. King & H. Rob. Os ensaios conduzidos resultaram na purificação e identificação de derivados de ácidos graxos, esteroides, um diterpeno, e metabólitos flavonoides. A análise fitoquímica preliminar revelou a presença dos principais grupos de metabólitos secundários: esteroides, triterpenos, carotenoides, alcaloides, taninos e flavonoides. Paralelamente, foi determinada a atividade antifúngica dos estratos de folhas e flores de *C. scabra* contra o fungo *Fusarium oxysporum* f. sp. *Lycopersici*.

Palavras-chave: Asteraceae; Eupatorium; *Fusarium oxysporum*; metabólitos secundários.

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