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Potential antioxidant and toxicological activity of the essential oil of *Rhaphiodon echinus* (Nees & Mart) Schauer (Lamiaceae): morphoanatomy and polyphenolic composition of its extracts

Potencial antioxidante y actividad toxicológica del aceite esencial de *Rhaphiodon echinus* (Nees & Mart) Schauer (Lamiaceae): morfoanatomía y composición polifenólica de sus extractos

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Abstract. The species *Rhaphiodon echinus*, known as betonica or creeping mint, is considered an infesting plant species, typical of the caatinga biome. Morphoanatomy is a very important tool to study the structure of plants, both its external and internal morphology, opening the knowledge about the production of secondary metabolites. These compounds are of great importance for scientific research, which may present themselves as the best antioxidants. Taking into account a lack of data in the literature on the essential oil of *R. echinus*, this study aimed to demonstrate its antioxidant activity and toxicity. In order to test this hypothesis, tests were carried out by the DPPH method, its toxicological activity on *Artemia salina* microcrustacean, as well as studies involving cuts of plant anatomy of leaf structures of the species. The vegetal material was collected at URCA and the essential oil was obtained by hydrodistillation, in a type of *Clevenger*. The HPLC profile of the extract revealed caffeic acid (62.45 mg/g), gallic acid (15.36 mg/g), Quercetin (9.02 mg/g) as the major compounds in the morning, while quercetin (4.15 mg/g) and caffeic acid (2.03 mg/g) were the major compounds in afternoon. DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical was used to assess the radical scavenging ability of the oil. The results demonstrated that the oil exhibited IC_{50} of 297.7 µg/mL in emulsions with ascorbic acid 73.07 µg/mL. The *A. salina* lethality assay showed that the oil was toxic at concentrations above 50 µg/mL, with an LC_{50} of 2.4 µg/mL compared to the positive control, LC_{50} was 11.50 µg/mL. Regarding the results of the research in Plant Anatomy, some peculiarities for *R. echinus* are described. Up to date, studies on the toxicity of this plant have not been published.

Keywords: Free radicals; *Rhaphiodon echinus*; DPPH; Lethality; Morphoanatomy.

Resumen. LLa especie *Rhaphiodon echinus*, conocida como la "betónica", se considera una especie plaga típica del bioma de la caatinga. La morfoanatomía es una herramienta muy importante para estudiar la estructura de las plantas, tanto la morfología exterior como interior, que posibilita también el conocimiento sobre la producción de metabolitos secundarios. Estos compuestos son de gran importancia para la investigación científica, que se pueden presentar como los mejores antioxidantes. Teniendo en cuenta la falta de datos en la literatura sobre el aceite esencial de *R. echinus*, este estudio tuvo como objetivo demostrar su actividad antioxidante y su toxicidad. Para probar esta hipótesis, se realizaron pruebas por el método DPPH, de su actividad toxicológica en el microcrustáceo *Artemia salina*, así como estudios implicando cortes de anatomía vegetal de las estructuras foliares de la especie. El vegetal material fue recogido en URCA y el aceite esencial fue obtenido por medio de hidrodestillación, en un tipo de *Clevenger*. El perfil HPLC del extracto reveló aácido carfeico (62,45 mg/g), ácido gálico (15,36 mg/g), y quercetina (9,02 mg/g) como los compuestos más importantes en la mañana, mientras que quercetina (4,15 mg/g) y ácido cafeico (2,03 mg/g) fueron los compuestos más importantes en la tarde. El radical libre de DPPH (1,1-difenil-2-picrilhidrazilo) se usó para evaluar la capacidad de eliminación de radicales del aceite. Los resultados demostraron que el aceite exhibió IC_{50} de 297,7 µg/mL, en emulsiones con ácido ascórbico 73,07 µg/ml. El análisis de letalidad *A. salina* constató que el aceite es tóxico a concentraciones superiores a 50 µg/mL, con un CL_{50} de 2,4 µg/mL comparado con el control positivo, CL_{50} fue 11,50 µg/mL. Considerando los resultados de la investigación en anatomía vegetal, se describen algunas peculiaridades de *R. echinus*. No se ha publicado ningún estudio sobre la toxicidad de esta planta.

Palabras clave: Radicales libres; *Rhaphiodon echinus*; DPPH; Letalidad; Morfoanatomía.

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INTRODUCTION

The imbalance caused by excessive production of reactive oxygen species and reactive nitrogen species can cause the so-called oxidative stress, leaving the defense system immune, generating serious oxidative damage at the cellular level, for example to nucleic acids, proteins, lipids and especially to the lipid peroxidation (Cheung et al., 2003; Lee et al., 2012; Fadda et al., 2014). Antioxidants inhibit and eliminate the free radicals produced by the oxidation of chemical substances, which occur naturally in metabolic reactions as in energy production, or by exogenous factors (Viswanad et al., 2011).

On the other hand, toxicological investigations on the species are restricted, limiting itself to a study carried out by Duarte et al. (2016b). In order to establish and assure the popular use, studies that evaluate the toxicity of natural products are relevant, so that many assays can be used, for example, lethality tests with the microcrustacean *Artemia salina* Leach, a model arthropod widely used for toxicological studies that are feasible, inexpensive and free from ethics committee (Meyer et al., 1982; Ruiz et al., 2005; Silva & Bernardi, 2007). The scarcity of data on toxicity assessment reinforces the need for studies of this nature that contribute greatly to the safe use of these products by the population, in addition to detecting significant differences in the pharmacokinetic and toxic profile (Ouedraogo et al., 2012).

Plant anatomy is a tool of great importance to study the structure of plants, both its external and internal morphology, enhancing the knowledge about the production of secondary metabolites, which in turn are the objectives of many pharmacological studies involving the chemical compounds, as reported in the literature (Bakkali et al., 2008; Duarte et al., 2016a). However, there are no studies on the plant anatomy of *R. echinus.*

Rhaphiodon echinus (Nees & Mart) Schauer is a unique representative of this genus of plants, mainly for its special botanical characteristics, with a branched structure (Harley, 2000; Lima et al., 2012). This species occurs in Brazil (Harley, 2000; Dias & Kill, 2007), a country with a large plant biodiversity in the world, whose abundance of plants have some pharmacological properties (Maciel et al., 2002). There are innumerable vegetables with antioxidants potential (Viswanad et al., 2011; Kamdem et al., 2013, Fadda et al., 2014). In addition, the species is characterized by special anatomical and physiological characteristics that allow it to produce essential oil, belonging to the group of aromatic plants (Pereira, 2014). It is widely used by popular medicine in the form of decoction for the treatment of cough, diabetes and inflammation (Harley, 1988; Albuquerque & Almeida, 2002, Menezes & Kaplan, 2006). There is record of pharmacological activity, as microbial activity and modulator effect of essential oil of this species, allied to its iron chelating properties (Sousa & Rodrigues, 2012; Duarte et al., 2016a; Costa et al., 2017); antiinflammatory and analgesic potential (Menezes et al., 1998), cytotoxic and antioxidant activity of extracts (Duarte, et al., 2016b) and its chemical composition (Menezes & Kaplan 2006; Dias & Kiill 2007; Harley et al., 2015). These are the few works published on this plant species.

Considering the scarcity of data in the literature on the essential oil of *R. echinus*, this study aimed to demonstrate its antioxidant activity and low toxicity. In order to test this hypothesis, our tests were carried out by the DPPH method, about its toxicological activity in *Artemia salina* microcrustacean, as well as studies involving plant anatomy cuts of leaf structures of the species.

MATERIALS AND METHODS

Collection, herborization, identification and cultivation of botanical specimens. The botanical material, leaves of *R. echinus*, was collected in the medicinal herb garden of the Regional University of Cariri-URCA. The plant material was identified and deposited in the Herbarium Caririense Dárdano de Andrade Lima (URCA), under the number 7347.

Extraction of essential oil. The essential oil of *R. echinus* was extracted from dried leaves, exposed to hydrodistillation in a *Clevenger* apparatus. After the collection, the leaves were crushed into small pieces and filled into a 1 L volumetric flask, where 300 ml of distilled water were added. The flask was coupled to the *Clevenger* apparatus under a heating mantle when the temperature was adjusted to the boiling point of the water. After boiling, the 2-hour time of the extraction cycle was started. At the end of each extractive cycle, the oil contained in the apparatus was collected with the help of a pipette, and stored in amber bottles and then refrigerated. After extraction, sodium sulfate was used to remove the aqueous phase present in the essential oil.

Chemical, apparatus and general procedures for HPLC of leaf extracts. All chemical were of analytical grade. Acetonitrile, formic acid, gallic acid, ellagic acid, *p*-coumaric acid, caffeic acid and chlorogenic acid were purchased from Merck (Darmstadt, Germany). Quercetin, apigenin, catechin and rutin were acquired from Sigma Chemical Co. (St. Louis, MO, USA). High performance liquid chromatography (HPLC-DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan). It was equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software (Boligon et al., 2014; R Core Team, 2014).

Quantification of compounds by HPLC-DAD of leaf extracts. Reverse phase chromatographic analyses were carried out under gradient conditions using C_{18} column (4.6 mm

× 250 mm) packed with 5 µm diameter particles; the mobile phase was water containing 2% acetic acid (A) and methanol (B), and the composition gradient was: 5% (B) for 2 min; 25% (B) until 10 min; 40, 50, 60, 70 and 80% (B) every 10 min. All the samples and mobile phase were filtered through 0.45 µm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.031 – 0.250 mg/mL quercetin and rutin, and 0.006 – 0.250 mg/mL for gallic, caffeic and chlorogenic acids. Quantification was carried out by integration of the peaks using the external standard method, at 257 nm for gallic acid, 325 nm for chlorogenic acid and 365 nm for quercetin and rutin. The flow rate was 0.8 mL/min and the injection volume was 40 µL. The chromatography peaks were confirmed by comparing their retention time and Diode-Array-UV spectra with those of the reference standards. All chromatography operations were carried out at ambient temperature and in triplicate. Calibration curves were: gallic acid: Y = 59851x - 1033.9 (r = 0.9992); chlorogenic acid: Y = 52462x - 1082.3 (r = 0.9951); caffeic acid: Y = 50723x - 1148.3 (r = 0.9989); rutin: Y = 49971x – 1235.7 (r = 0.9999) and quercetin: Y = 50337x – 1741.5 (r =0.9986).

The free radical scavenging ability of *R. echinus* essential oil. To evaluate the antioxidant potential, the free radical DPPH (1,1-diphenyl-2-picrylhydrazyl) was used as described by Kamdem et al. (2013), with some modifications. In general, 50 µL of the essential oil at different concentrations (4 - 1024 µg/mL) were mixed with 100 µL of freshly prepared DPPH solution (0.3 mM in ethanol). Thereafter, the plate was held in the dark at room temperature for 30 min. Reduction of the DPPH radical was measured by monitoring the absorption drop at 517 nm using a microplate reader (SpectraMax, Sunnyvale, CA, USA). Ascorbic acid was used as the standard compound, positive control.

Lethality test with *Artemia salina*. The toxicity test was performed as described by Meyer et al. (1982) with some modifications. In an artificially prepared seawater, cysts of *A. salina* were added and submitted to constant aeration for 24 h, period necessary for incubation of larvae. Thereafter, the essential oil was prepared in different concentrations (1- 1000 µg/mL) and 10 microcrustacean larvae were subsequently transferred for each concentration. The test was monitored using a potassium dichromate ($K_2Cr_2O_7$) as positive control, prepared in DMSO. The reading was performed after 24 h in a stereomicroscope.

Anatomy of plant organs. Fixation was done with FAA + glutaraldehyde (Lersten & Curtis, 1988) for a period of 18-24 hours. After fixation, the samples were stored in 50% ethanol. For the microscopic analysis of the botanical material, transverse, longitudinal and free hand cuts were performed with a razor blade. Temporary slides were prepared using 50% glycerinated water (Kraus & Arduin, 1997). The sections were clarified in 5% sodium hypochlorite solution and stained with astra blue + basic fuchsin (Luque, et al., 1996). The semipermanent slides were prepared with the bleached sections in 33% sodium hypochlorite solution, washed with distilled water, stained with 1% safranin and 1% astra blue solution, prepared on slide and cover slip, with glycerol gelatin (1: 1) (Sass, 1951). The material was analyzed under light microscopy and photographed with a camera coupled to a Zeiss / Axioplan microscope with Kodak Gold 100 film.

Statistical analysis. The statistical analysis of the averages in triplicate (n = 3) ± SEM were performed using One way (ANOVA), with post-hoc of Tukey, level of significance ($P<0.05$). The IC_{50} was calculated for the DPPH assay results. The LC_{50} was calculated by linear regression. All analyzes were performed using the software program GraphPad Prism 6.

RESULTS

Chemical composition. By analyzing the chemical composition, it was possible to observe different results for different collections of the material, since the material was collected in the morning and afternoon. Samples contained the following compounds gallic acid (retention time-t_R 13.35 min), chlorogenic acid (t_R = 22.67 min), caffeic acid (t_R = 26.09 min), rutin (t_R = 38.24 min) and quercetin (t_R = 49.62 min) (Fig.1A and 1B).

The extract of dried leaves of *R. echinus* showed in this HPLC analysis that some constituents were identified as major phytochemicals, among them caffeic acid (62.45 mg/g), Gallic acid (15.36 mg/g), Quercetin (9.02 mg/g) in the morning. In the afternoon, the major compounds detected were Quercetin (4.15 mg/g) and caffeic acid (2.03 mg/g). Significant variations were observed between the constituents, as well as between collection times. Its composition percentage is shown in Table 1.

Antioxidant evaluation by DPPH assay. The results showed that the oil exhibited IC_{50} of 297.7 µg/mL compared to the ascorbic acid control, with 73.07 µg/mL, indicating a moderate antioxidant activity (Fig. 2).

Toxicity of essential oil of *R. echinus* in *Artemia salina*. Figure 3 shows increasing mortality of *A. salina* with increasing concentration of the essential oil of *R. echinus* until the concentration of 50 µg/mL, where its maximum toxicity was reached. It was possible to observe that *R. echinus* leaf essential oil at lower concentrations (1-10 µg/mL) exhibited higher toxicity than that of the positive control used (i.e., $K_2Cr_2O_7$), with LC_{50} of 2.4 µg/mL in comparison to LC_{50} of 11.50 µg/mL of the positive control.

Table 1. Components of *Rhaphiodon echinus* collected at different hours of the day.
Tabla 1. Componentes de *Rhaphiodon echinus* recolectados a diferentes horas del día.

Compounds	Collection hours: 9:30 am (mg/g)	%	Collection hours: 15:30 pm (mg/g)	%
Gallic acid	15.36 ± 0.02 b	1.54*	0.37 ± 0.02 cd	0.04*
Chlorogenic acid	2.13 ± 0.11 e	0.21 ns	0.52 ± 0.01 cd	0.05 ns
Caffeic acid	62.45 ± 0.09 a	6.25*	2.03 ± 0.03 bcd	0.20*
Rutin	4.83 ± 0.05 d	0.48*	0.46 ± 0.04 cd	0.05*
Quercetin	9.02 ± 0.08 c	0.90*	4.15 ± 0.07 ab	0.42*

Results are expressed as mean ± standard deviation (SD) of three determinations. One-way ANOVA. The averages followed by different letters differ between the constituents by the Tukey test at P<0.05. The averages followed by * differ between the collection time by the Tukey test at P<0.05; ns: not significant.
Los resultados se expresan como medias ± error estándar (SD) de tres determinaciones. ANOVA de uma vía. Las cifras seguidas por letras diferentes entre los constituyentes por el test de Tukey a P<0,05. Las mediciones seguidas por * difieren entre el momento de colección por el test de Tukey a P<0,05; ns: not significativo.

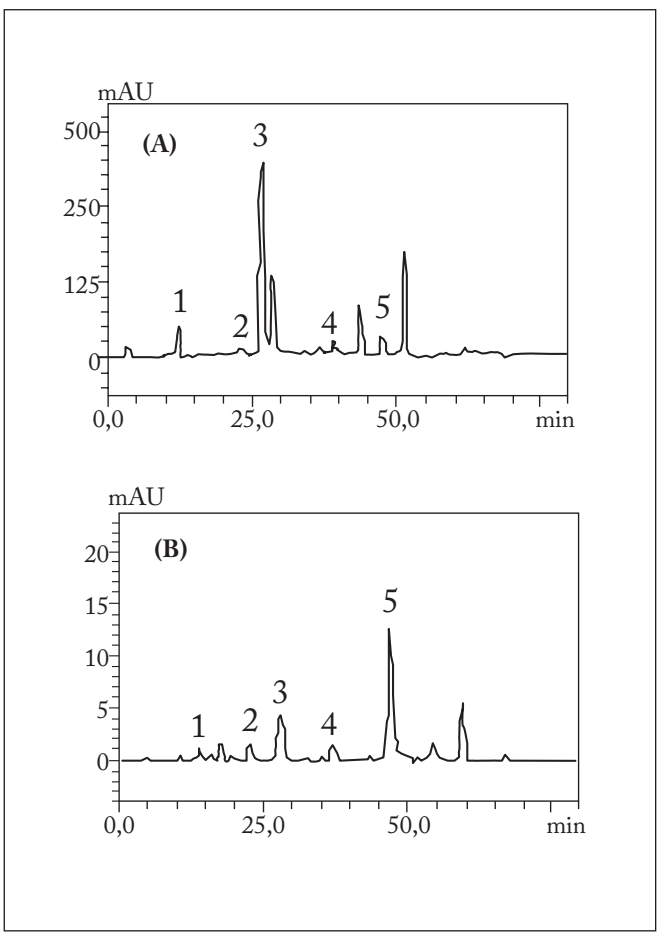

Fig. 1. A- Sample profile of *Rhaphiodon echinus* leaves collected in the morning (9:30 am), and B-profile harvested in the afternoon (3:30 pm). Gallic acid (peak 1), chlorogenic acid (peak 2), caffeic acid (peak 3), rutin (peak 4) and quercetin (peak 5).
Fig. 1. A- Muestra del perfil de las hojas de *Rhaphiodon echinus* recolectadas en la mañana (9:30 a.m.) y del perfil B cosechadas en la tarde (3:30 p.m.). Ácido gálico (pico 1), ácido clorogénico (pico 2), ácido cafeico (pico 3), rutina (pico 4) y quercetina (pico 5).

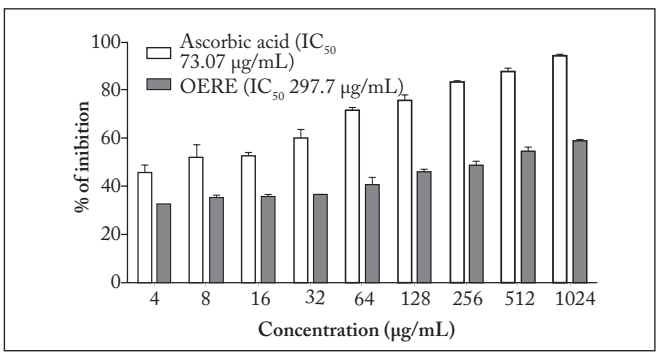

Fig. 2. Percentage of inhibition of DPPH radicals of *R. echinus* essential oil (OERE). Data are expressed as average SEM, n = 3 independent experiments.
Fig. 2. Porcentaje de inhibición de los radicales DPPH del aceite esencial de *R. echinus* (OERE). Los datos se expresan como SEM promedio, n = 3 experimentos independientes.

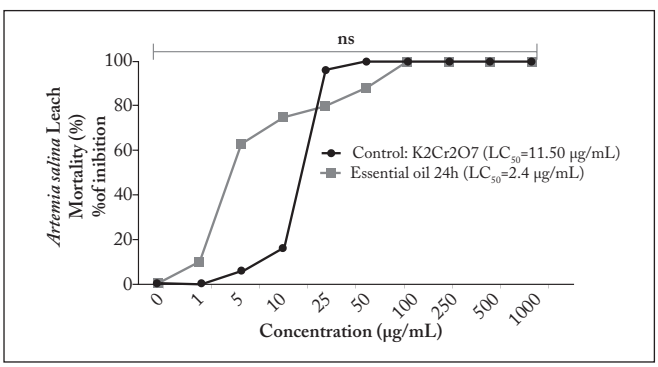

Fig. 3. Mortality rate of crustacean *Artemia salina* Leach, exposed to the essential oil of *R. echinus*. Control: Potassium Dichromate ($K_2Cr_2O_7$). The values were calculated as a percentage of the control, N = 3. One-way ANOVA with Tukey post hoc test (P<0.05); ns: not significant.
Fig. 3. Tasa de mortalidad de crustáceos *Artemia salina* lixiviada, expuesta al aceite esencial de *R. echinus*. Control: dicromato de potasio ($K_2Cr_2O_7$). Los valores se calcularon como un porcentaje del control, N = 3. ANOVA de una vía con la prueba post hoc de Tukey (P<0,05); ns: no significativo.

Anatomy of plant organs. With respect to the results presented by the investigation in Plant Anatomy, some peculiarities for *R. echinus* were described. The central vein in the leaves of this species presents uniseriate epidermis, in the subepidermal colenquimous position of the annular type and isodiametric parenchyma cells (Figs. 4A-B). The mesophyll is dorsiventral with 1 layer of palisade parenchyma and 3 to 5 layers of well spaced lacunal parenchyma (Fig. 4D). Vascular bundles are collateral, presenting isodiametric parenchyma cells (Figs 4C, 6A, 6B and 7B). Rich in starch grains (Figs 7A-C). Phytochemicals can be excreted by well-represented unicellular and multicellular trichomes (Fig. 5A-C). These cells in turn are rich in flavonoids that can confer pharmacological action of this species. Papillae are present on the abaxial face and unicellular and multicellular acicular tector trichomes are present on both faces of the epidermis.

DISCUSSION

Considering that *R. echinus* has been widely used in folk medicine, as shown in other studies for its anti-inflammatory (Melo et al., 1998; Falcão et al., 2005), antimicrobial (Sousa & Rodrigues, 2012; Duarte et al., 2016a, 2016b) and antitussive (Pereira, 2014) properties, this study was fundamentally designed to evaluate the toxicological potential of *R. echinus* leaf essential oil (EO). In fact, preliminary studies were carried out focusing on the LC_{50} of EO in *A. salina* (Meyer et al., 1982; Van Stappen, 1996; Mclaughlin, 1998; Lima et al., 2014) in order to verify signs of toxicity induced by *R. echinus* in this microcrustacean. All concentrations of EO showed significant toxicity within 24 hours of administration (Fig. 3), indicating that in this range, EO is toxic in the assay, since LC_{50} values were less than 100 µg/mL. In the evaluation of the toxicity of natural products from plants by the mentioned bioassay, an LC_{50} less than 1000 µg/mL is considered bioactive (Meyer et al., 1982). In the present study, the oil exhibited LC_{50} <1000 µg/mL, indicating that it has biological activity. *R. echinus* leaf EO showed toxic effect at relatively lower concentration with a lethal concentration (LC_{50}) of 2.4 µg/mL. Similar findings were found for natural products, which showed cytotoxic effects at the lethal concentration (LC_{50}) of 17.1 µg/mL (Alluri et al., 2005).

In relation to the chemical composition, there are reports in the literature of the presence of germacrene D and α-guaiene, cited as major compounds of this essential oil, whose composition is endowed with mono- and sesquiterpenes, mainly bicyclogermacrene and trans-caryophyllene (Torres et al., 2009; Duarte et al., 2016a). In the same study by Torres et al. (2009), the main compounds found were: bicyclogermacrene (28.13%), caryophyllene (23.07%), caryophyllene oxide (5.40%) as major compounds. In addition, it was also reported the presence of ellagic acid, caffeic acid and chlorogenic acid as major compounds of the aqueous and ethanolic extracts of this species (Duarte et al., 2016b; Costa et al., 2017). The variation of the chemical composition between schedules can be due to the physical, chemical and biological factors to which

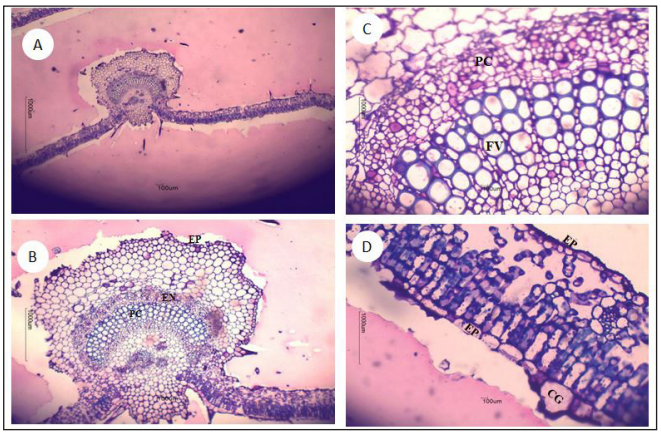

Fig. 4. (A-C) *Rhaphiodon echinus* (Nees & Mart) Schauer (LAMIACEAE). Leaflet in cross-section: A, Central vein; B, Detail of the central vein; C, Detail vascular bundle. (D) Mesofilo. Abbreviations: EP: epidermis, EN: endoderm, PC: pericycle, FV. Vascular bundles, CG. Glandular cells. Bar = 1000 µm and 100 µm.

Fig. 4. (A-C) *Rhaphiodon echinus* (Nees & Mart) Schauer (LAMIACEAE). Folleto en corte transversal: A, Vena central; B, Detalle de la vena central; C, Detalle del haz vascular. (D) Mesofilo; Abreviaturas: EP: epidermis, EN: endoderm, PC: periciclo, FV. Paquetes vasculares, CG. Células glandulares. Bar = 1000 µm y 100 µm.

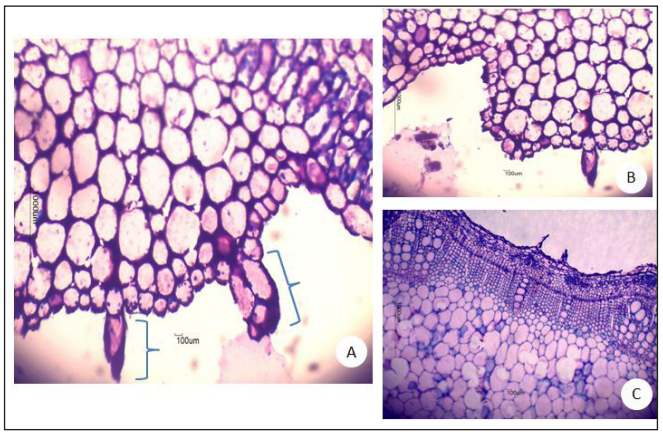

Fig. 5. *Rhaphiodon echinus* (Nees & Mart) Schauer (Lamiaceae). Leaflet, in cross-section: (A-B) Multicellular glandular trichoma in the central vein, showing a secretion being exuded; B, Unicellular glandular trichoma; C, Glandular trichoma in the epidermis and leaf cortex. Bar = 1000 µm and 100 µm.

Fig. 5. *Rhaphiodon echinus* (Nees & Mart) Schauer (Lamiaceae). Folleto, en corte transversal: (A-B) Tricoma glandular multicelular en la vena central, que muestra una secreción que se exuda; B, tricoma glandular unicelular; C, Trichoma glandular en la epidermis y la corteza de la hoja. Bar = 1000 µm y 100 µm.

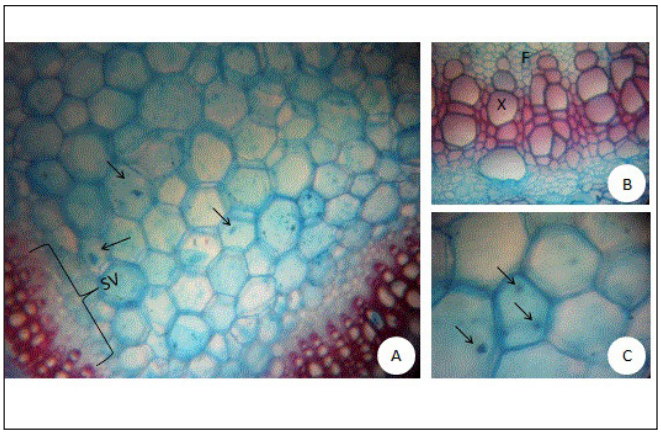

Fig. 6. *Rhaphiodon echinus* (Nees & Mart) Schauer (Lamiaceae). Cross-sectional leaf morphoanatomy: A, SV- Vascular system and representation of starch grains inside parenchyma cells (arrows); B, Details of xylem (X) and phloem (F); C, starch grain in parenchyma cells.

Fig. 6. *Rhaphiodon echinus* (Nees & Mart) Schauer (Lamiaceae). Morfoanatomía transversal de la hoja: A, SV- Sistema vascular y representación de los granos de almidón dentro de las células del parénquima (flechas); B, Detalles de xilema (X) y floema (F); C, grano de almidón en células de parénquima.

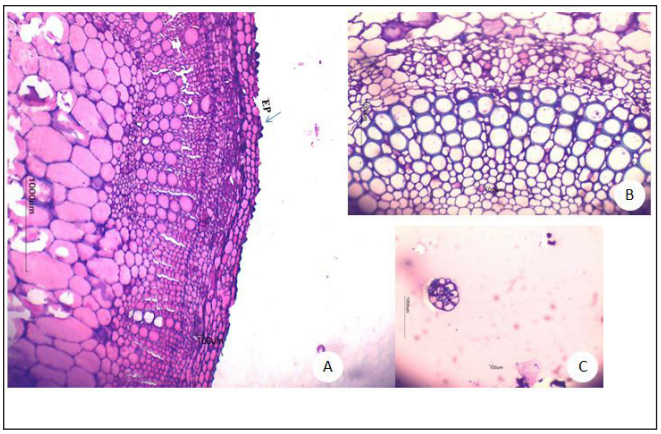

Fig. 7. *Rhaphiodon echinus* (Nees & Mart) Schauer (Lamiaceae). Leaflet, in cross section: A, Papillose Epidermis (arrow); B, more detailed cortex with the representation of the vascular bundles; C, Cluster of isolated cells. Abbreviations: EP: epidermis. Bar = 1000 µm and 100 µm.

Fig. 7. *Rhaphiodon echinus* (Nees & Mart) Schauer (Lamiaceae). Folíolo, en la sección transversal: A, Epidermis de la papilosa (flecha); B, Corteza más detallada con la representación de los haces vasculares. C, Racimo de células aisladas. Abreviaturas: EP: epidermis. Bar = 1000 µm y 100 µm.

the species is found. Factors such as the rate of sunshine, light, water stress, and heat can alter the phenolic compounds present in the volatile oils of the vegetable.

Essential oils are produced by plant structures called trichomes, which are present in both leaf blade and stalk (Alonço et al., 2015). They are known as secondary metabolites, which act to defend the plant and confer characteristic of aromas. These oils are rich in polyphenolic compounds, which in literary reports behave as excellent natural antioxidants (Morais de Souza et al., 2008).

Parenchyma cells contain phenolic contents that can be considered as secretory cells or taniniferous idioblasts, giving the plant the function of internally secreting these substances (Esau, 1974). It is in these cells that the production of secondary compounds occurs, that in pharmacology and other sciences can contribute to the action against pathogens and healing and anti-inflammatory action (Castro et al., 2004). The presence of phenolic compounds in leaf parenchyma cells was confirmed by the phytochemical analyzes performed in the studies of Duarte et al. (2016a). Other studies of this nature have already been published by other authors (Menezes et al., 2005; Menezes & Kaplan, 2006; Torres et al., 2009).

According to the work of Duarte et al. (2016b), which tested the ability of the aqueous and ethanolic extracts of *R. echinus* to eliminate or scavenge the DPPH radical, it was observed that they present a significant IC_{50}, aqueous (IC_{50}: 227.9 µg/mL) and ethanolic (IC_{50}: 111.9 µg/mL). If we compare these results with the DPPH radical scavenging activity of the EO obtained from *R. echinus*, it is possible to state that the EO exhibited lower antioxidant activity with IC_{50} = 297.7 µg/mL compared to that of the aqueous (227.9 µg/mL) and ethanolic extract (111.9 µg/mL) of *R. echinus*. There is little data available on the antioxidant activity for *R. echinus*, limiting the antioxidant evaluation of its extracts, and no reports for essential oil.

The biological and pharmacological activity of natural products such as toxicological and antioxidant effects may be due to the combination of active components present in vegetable oils and extracts, supporting the use by the population or even scientific research (Romeilah et al., 2010; Lalisan et al., 2014; Mentor et al., 2014).

Studies with *R. echinus* have been developed by several researchers, such as: microbiological activity (Sousa & Rodrigues, 2012), anti-inflammatory and analgesic activity (Menezes et al., 1998), pollination ecology (Dias & Kill, 2007), and cytotoxic and antioxidant activity (Duarte et al., 2016b). In relation to the chemical composition, there are reports in the literature about the presence of germacrene D and α-guaiene, cited as the major compounds of this essential oil, whose composition is endowed with mono and sesquiterpenes, mainly bicyclogermacrene and trans-caryophyllene (Torres et al., 2009; Duarte et al., 2016a). In this study, the main compounds found were: bicyclogermacrene (28.13%), caryophyllene (23.07%), caryophyllene oxide (5.40%), as the major compounds since they presented higher percentages.

In the analysis of the chemical composition detected in the sample of the aqueous and ethanolic extract of *R. echinus* we detected the presence of chlorogenic acid (t_R = 18.65 min, peak 2), caffeic acid (t_R = 22.49 min, peak 3), ellagic acid (t_R = 31.67 min, peak 4). In other works, the presence of germacrene D

and α-guaiene is reported, with a higher percentage of essential oil, stating that the essential oil of leaves of this species is rich in mono- and sesquiterpenes, mainly bicyclogermacrene and trans-caryophyllene (Torres et al., 2009). In the aqueous and ethanolic extracts the presence of gallic acid, chlorogenic acid, caffeic acid and elagenic acid (Duarte et al., 2016b)

Sesquiterpenes constitute a class of substances with a variety of natural organic structures. For instance, germacrene D has the ability to eliminate free radicals, and consequently, it has the ability to protect the cell against damaging action caused by reactive oxygen and nitrogen species. About α-guaiene, there are no reports on its activity. Studies on essential oils and their isolated components may show different results, since some metabolites behave better when tested alone, while others may present more significant results when combined with other compounds.

CONCLUSION

The toxic effects of *R. echinus* leaf essential oil evaluated on *Artemia salina* showed that the oil is toxic at relatively lower concentration and exhibited its maximum toxicity at 50 μg/mL. In addition, the species is popularly used to treat cough and throat infections, which served as a stimulus for our toxicology research. Our findings evidenced the moderate antioxidant activity of the oil by the DPPH radical scavenging activity, as evidenced by IC_{50} of 297.7 μg/mL. As for anatomy, there are no reports on the morphoanatomy of this species describing its production, storage and secretion of any toxicological and pharmacological substances. This is the first study to investigate the toxicity of this plant species. Nevertheless, new studies involving chronic treatments and the antioxidant defense system should be conducted in order to establish clear criteria for their safe medicinal use.

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