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Preliminary investigation on phytochemical composition and biological activity of *Cunila microcephala* Benth.

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Ethnobotanical studies have investigated the use of leaves of *Cunila microcephala* for respiratory, stomach and gastrointestinal disorders. The essential oil of this plant is mostly composed of menthofuran, which has a hepatotoxic effect. Due to the popular use of this plant in southern Brazil, this study aimed to perform phytochemical profile of *C. microcephala* to detect and determine the amount of secondary metabolites and to evaluate their microbiological activity *in vitro* and genetic damage *in vivo*, indicative of genotoxicity, to ensure safe use of the plant. The leaf extract of *C. microcephala* was investigated for the presence of phenolic compounds, such as tannins, coumarins and flavonoids. This extract contained 193.23 mg/ml of phenolic compounds. In our *in vitro* analysis of microbiological activity, the crude leaf extract of *C. microcephala* showed 6 mm zone of inhibition against *Staphylococcus aureus*; fractions of dichloromethane and ethyl acetate showed 4 mm zones. In the genotoxic analysis, using the comet assay, no genotoxic effects were observed in blood samples and the liver at 125, 250 and 500 mg/kg. According to the results, *C. microcephala* presents interesting secondary compounds with biological activity, showing antimicrobial effects *in vitro* and no genotoxic effects *in vivo*.

Key words: Antimicrobial activity, damage to DNA, medicinal plant, toxicity.

INTRODUCTION

Data showed an increase in the marketing of herbal medicines in the Brazilian pharmaceutical market (Suzuki,

2002; Ogava et al., 2003; Silva et al., 2006). Plants are used worldwide for the treatment of diseases and novel

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drugs continue to be developed through research from plants (Berg, 1993; Revene et al., 2008; Heywood, 2011). Brazil is one of the few megadiversity countries in the world but investigations of its flora have barely revealed the present phytochemical diversity (Newman et al., 2000; Sa and Elisabetsky, 2012).

In this context, we mention the native Brazilian species *Cunila microcephala* Benth, which belongs to the family Lamiaceae and is known popularly as Poejinho. *C. microcephala* is native to southern Brazil, Argentina and Uruguay and is widely used in folk medicine in southern Brazil. *C. microcephala* has the same popular designation and therapeutic indication as *Mentha pulegium* (Bordignon et al., 1997). The latter plant, of European origin, is present in the National Medicinal Plants of Interest to SUS (Ministério da Saúde, 2009) and there are possibly more scientific studies reporting its use compared with *C. microcephala*.

Ethnobotanical surveys have shown that *C. microcephala* is one of the most widely used medicinal plants in the region of Porto Alegre, Rio Grande do Sul (RS), Brazil (Vendruscolo and Mentz, 2006; Simões, 2003). In Santa Catarina, more precisely in Criciúma and region, the community is represented by 'Pastoral da Saúde Regional Sul IV' through the Project Rational Phytotherapy, developed at the University do Extremo Sul Catarinense (UNESC), which highlighted the use of tea made from *C. microcephala* leaves for respiratory diseases (chronic cough) and stomach disorders, among others. The essential oil of *C. microcephala* has a diverse chemical composition (Bordignon et al., 1997; Apel et al., 2009). Bordignon et al. (1997) found the presence of mainly menthofuran (82.3 to 85.1%), limonene (2.1 to 3.8%), β -caryophyllene (3.3 to 3.9%), α -pinene, camphene, β -pinene, sabinene, β -myrcene, 1-8-cineole, β -ocimene, γ -terpinene, linalool, pulegone, α -terpineol, and germacrene D. There are no reports about the use of this plant as a poison. However, due to the presence of a high content of menthofuran, caution in its use is suggested (Bordignon et al., 1997). This is because menthofuran is a hepatotoxic substance which, when in contact with the organism, leads to the electrolytic formation of a metabolite that binds to cellular proteins via a covalent connection, which prolongs its stay in the body (Madyastha and Raj, 1994).

In a study in which mice were given menthofuran once daily orally, there was a decrease in the levels of cytochrome P450, a key enzyme involved in the metabolism of xenobiotics (Thomassen et al., 1992). For *C. microcephala*, few references were found on its chemical constituents (Apel et al., 2009; Bordignon et al., 2003; Agostini et al., 2009); pharmacological and microbiological data were not found, highlighting the lack of studies on this native species. This is due to the hepatotoxic effect of menthofuran, coupled with its high

concentration in the essential oil of *C. microcephala* and the use of this medicinal plant by the population of the southern region of Santa Catarina. This study aimed to carry forward the evaluation of phytochemical study of leaves of *C. microcephala* to detect and determine the amount of secondary metabolites present in the plant, evaluate their antibacterial activity and analyse the occurrence of genetic damage, indicative of possible genotoxic relapses.

MATERIALS AND METHODS

Plant

The leaves of *C. microcephala* were identified by Dra. Vanilde Citadini-Zanette and were collected in February, 2009 in Grão Pará, SC, Brazil (28° 13' 58,7" S e 49° 17' 58,9" W). The voucher specimen was deposited in the CRI Herbarium Pe. Dr. Raulino Reitz of the UNESC-SC, Brazil, number CRI 7620. The leaves were dried by a drying oven system at approximately 50 to 60°C (Melo et al., 2004) and cut into small pieces.

Phytochemical analysis

Phytochemical analysis (flavonoids, tannins, anthraquinones, alkaloids, saponins, coumarins, and cardiotoxic glycosides) with the leaves of *C. microcephala* was carried out according to the methods described by Costa (2002) and Xorge (1976). Analysis by thin layer chromatography was performed following the systems and revealing indicated by Wagner and Bladt (1996) for the analysis of coumarins, and following the methods of British Pharmacopoeia (1997) for cardiotoxic glycosides. The analyses were realized between March and August, 2012.

Preparation of the infusion

The aqueous extract was prepared by infusion at a ratio of 1:10 (w/v) (Anvisa, 2010).

Total phenolic content (TPC)

The concentration of phenolics in plant extracts was determined by spectrophotometric method using Folin-Ciocalteu reagent. 0.5 ml extract (infused) *C. microcephala* was diluted to 5 ml Folin-Ciocalteu reagent (1:10 dilution made with water). 4 ml of 1 M sodium carbonate solution was added after 3 min and they were stirred in a vortex until diluted and kept for incubation at 45°C temperature. Absorbance was measured at 765 nm, after 45 min against blank. Gallic acid was used as standard and calibration curve was plotted using various concentrations (36.25 to 500 μ g/ml). Total phenolic content was expressed as mg/Gallic acid equivalents (GAE) per gram of air dried sample. Measurements were done in triplicate (n = 3) (Shahidi and Naczka, 1995).

Total flavonoids content (TFC)

Total flavonoid content was estimated by colourimetric method using aluminium chloride. To 0.5 ml extract (infused) *C. microcephala* was distilled to 1.5 ml methanol, 0.1 ml of 1 M potassium acetate and 2.8

ml of water, mixed and allowed to stand for 3 min and 0.1 ml 10% aluminium chloride solution was added. Absorbance was measured at 415 nm after 30 min against blank. Quercetin was used as standard and calibration curve was plotted using various concentrations (12.5 to 200 µg/ml). Total flavonoid content was expressed as mg/Quercetin equivalents per gram of air dried sample. Measurements were done in triplicate (n = 3) (Chang et al., 2002).

Evaluation of antibacterial activity

Preparation of extracts and fractionation

The crude leaf extract of *C. microcephala* was prepared by maceration through hydroalcoholic solvent (ethanol 70%) at a ratio of 1:5 (w/v). The maceration remained for 15 days stored in the dark. After that, it was filtered and the solvent was removed by rotoevaporation (Anvisa, 2010; Kalluf, 2008). Some part of the crude leaf extract was stored for analysis. Other part was submitted to a process of liquid-liquid partition with solvents of increasing polarities: dichloromethane, ethyl acetate, and *n*-butanol. The partition methods were based according to a methodology adapted from Cechinel-Filho and Yunes (1998). The fractions were concentrated by rotoevaporation. The infusion also was prepared according the methodology by Anvisa (2010).

Experimental design

For the evaluation of antimicrobial activity, two procedures were performed. The first was the study of halo formation of growth inhibition (Silveira et al., 2011), for which the microorganisms *Staphylococcus aureus* (ATCC25923), *Escherichia coli* (ATCC25922), *Klebsiella pneumoniae* (ATCC700603) and *Salmonella typhimurium* (ATCC14028) were seeded on plate count agar (PCA) in petri dishes. In a hole of 1 cm diameter, the amount of three per plate were applied 100 µl of crude leaf extract, infusion, fractions (dichloromethane, butanol, and ethyl acetate) at a concentration of approximately 100 mg/ml. Negative control plates were also produced, using the solvent used for dilution of the extract and fractions. These plates were incubated for 24 h at 37°C and the efficiency of the compounds was observed in addition to the size of the growth inhibition zone around the hole (Sandri et al., 2007). In the second step, the minimum inhibitory concentration (MIC) of the substances that demonstrated growth inhibition in the first test was determined by testing serial dilutions in broth (Santos et al., 2011). The crude leaf extract and fractions were weighed (dichloromethane and ethyl acetate) at a concentration of 200 mg/ml (Sandri et al., 2007). The samples were then tested in triplicate at concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 mg/ml. Incubation was performed with Luria Bertani (LB) agar and *S. aureus* (ATCC25923). Only this bacterium was tested because it was the only one that showed sensitivity in the previous tests. All incubations were diluted 10⁻¹ to 10⁻⁵ and 10 µl aliquots were applied on PCA plates, where they remained for 24 h at 37°C in an oven. After this period, the number of colony forming units (CFUs) was counted and the results were plotted in the form of tables and graphs (Bussman et al., 2010).

Experiments on animals

Preparation of the crude leaf extract

Thirty grams of dried leaves of *C. microcephala* were crushed and

the aqueous extract was prepared by infusion (1 g: 10 ml) (Anvisa, 2011). After cooling and filtration, the extract was dried, stored in the dark in order to obtain (3.54 g, yield: 11.8% w/w) the crude leaf extract *C. microcephala*.

Animals

Male CF-1 mice were used, which were obtained from the vivarium of the Universidade do Extremo Sul Catarinense (UNESC). Six mice were housed per box with free access to food and water and kept on a cycle of 12 h of light and 12 h of darkness (the light was turned on at 7 am), with a controlled temperature of 22 ± 1°C. All experimental procedures were performed in accordance with the recommendations of the Brazilian College of Animal Experimentation (COBEA, 1991). The project had the approval of the CEUA UNESC, registered with Protocol No. 26/2010. All animal experiments were carried out also in accordance with the European Communities Council Directive of 24 November, 1986 (86/609/EEC) and/or other international institutions, and they were formally approved by the animal subjects review board of our institution. All efforts were made to minimize the number of animals used and their suffering.

Experimental design

For this study, 24 male CF-1 mice were used. They were divided into four groups and treated acutely by gavage. Each group received one treatment: group 1 (negative control); groups 2, 3, and 4 (crude leaves extract of *C. microcephala* at doses of 125, 250, and 500 mg/kg, respectively - diluted with water). After administration of the extracts, peripheral blood samples were collected (after 2, 6 and 24 h) in the caudal region of all animals to perform the comet assay, which followed established international protocols (Tice et al., 2000) with adaptations (Silva et al., 2000).

Comet assay

The protocol used to perform the test was based on the alkaline version developed by Singh et al. (1988), with modifications suggested by Tice et al. (2000). Each piece of liver was placed in 0.2 ml of phosphate buffered saline (PBS) and finally minced to obtain a cell suspension. Liver cell suspensions (20 µl) and peripheral blood (5 µl) were soaked in 80 and 95 µl, respectively agarose low melting point (0.75%). This mixture was placed on microscopic slides pre-coated with a coverage of 300 µl of normal agarose at a concentration of 1.5%. Then, it was covered with a coverslip. After solidification in the refrigerator for approximately 5 min, the coverslips were carefully removed and then were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0 to 10.5, with an additional hour using 1% Triton X-100 and 10% DMSO) for at least 1 h and up to 2 weeks at 4°C. Subsequently, the coverslips were incubated in alkaline buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 20 min to conduct the DNA. The electrophoretic run was performed for 15 min at 300 mA and 2.5 V. All these steps were conducted under indirect yellow light. Then, the coverslips were neutralised with 0.4 M Tris (pH 7.5). Finally, the DNA was stained with ethidium bromide. For observation under the microscope, 100 cells were analysed per individual (50 of each blade doubled). The cells were rated visually in five classes according to the size of the tail (0 = no tail to 4 = maximum length of tail). Thus, the Damage Index (DI) of each group studied ranged from zero (100 × 0 = 0; 100 completely undamaged cells observed) to 400 (100 × 4 = 400; 100

Table 1. Reactions indicative of substances in *Cunila microcephala*.

Class of substances	Result	Reactions used
Phenolic substances	+	Ferric chloride, potassium hydroxide
Tannins	+	Ferric chloride, gelatin solution
Flavonoids	+	Magnesium oxide, hydrochloric acid, spectrophotometric analysis
Anthraquinones	-	Potassium hydroxide
Coumarins	+	Reaction with potassium hydroxide and sodium hydroxide, viewing under ultraviolet light.
Saponins	-	Distilled water and shaking in a graduated cylinder, hydrochloric acid
Cardiotonic glycosides	-	Baljet Reaction, Keller-Kiliani Reaction, Salkowsky Reaction
Alkaloids	-	Mayer Reaction, Dragendorf Reaction, Bouchardat Reaction, Bertrand Reaction

+: Positive for reaction. -: Negative for reaction.

cells with maximum damage observed). The frequency of damage (FD) in each sample was calculated based on the number of cells versus the number of tail tailless cells (Collins, 2004).

Statistical analyses

The normality of variables was evaluated by the Kolmogorov-Smirnov test. Results are expressed as mean \pm standard deviation and the statistical significance of the DI and FD was determined using an analysis of variance (ANOVA). In all comparisons, $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS AND DISCUSSION

Phytochemical profile

Phytochemical analysis could be performed to identify and determine the content and existence of groups of secondary metabolites in *C. microcephala*. These substances are largely related of the ethno-medical properties of the crude extracts. For the pharmacological as well as pathological discovery of novel drugs, the essential information's regarding the chemical constituents are generally provided by the qualitative phytochemical screening of plant extracts (Barreiro and Bolzani, 2009; Mishra and Tiwari, 2011; Dhimi, 2013). In the present study, qualitative tests for all four extracts showed significant indication about the presence of metabolites. The colour and precipitation reactions performed in this study showed positive results for phenolic substances such as tannins, coumarins and flavonoids, and negative results for the presence of anthraquinones, saponins, alkaloids and cardiotonic glycosides, these results are presented in Table 1. Tests conducted with ferric chloride and potassium hydroxide showed positive results for the class of phenolic compounds for *C. microcephala* extract. Furthermore, the total phenolic content of *C. microcephala* extract, expressed

as GAE, was 193.23 mg/ml.

The spectrophotometric method was used to quantify total flavonoids, where an extract was prepared from aluminium chloride and the results are expressed as quercetin equivalent based on the quercetin calibration curve, resulting in a concentration of 151.7 mg/ml flavonoids in the leaf extract of *C. microcephala*. Previous studies (Toledo et al., 2004) detected and isolated flavonoids from *C. microcephala* extracts. The presence of 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone and 5,3',4'-trihydroxy-7-methoxyflavone was detected. According to some studies, flavonoids and diterpene compounds are the most frequently found compounds in *Cunila* species and are described as good chemotaxonomic markers of the family Lamiaceae (Bordignon et al., 2003). It is normally present as free aglycones and very rarely in a glycosylated form, which are characteristics of this family (Simoes, 2003).

Microchemical tests (Toledo et al., 2004) already revealed the presence of phenolic compounds in *C. microcephala*, which provided an indication of the presence of some secondary metabolites, such as tannins. This result was confirmed in the present study, which detected the presence of condensed tannins. In tests conducted under ultraviolet light of 365 nm wavelength, the presence of coumarins was observed, which was confirmed on thin layer chromatography (TLC) (Toledo et al., 2004). Since several studies in the literature showed the effect of tannins and flavonoids in combating inflammatory agent and as an antioxidant (Chakraborty et al., 2012; Marín and Máñez, 2013), mechanisms related to the diseases reported by the population.

Antibacterial assay

It was observed that with the fractions obtained with more polar solvents, such as butanol and water, it did not show results. The infusion had a slight inhibitory effect on

Table 2. Analysis of damage index (DI) (mean \pm standard deviation) of DNA in peripheral blood collected at 2, 6, and 24 h in mice treated with saline or one dose of crude extract of *C. microcephala* (125, 250, or 500 mg/kg).

DI	Saline	125 mg/kg	250 mg/kg	500 mg/kg
Blood 2 h	19.33 \pm 7.06	12 \pm 3.03	17.33 \pm 7.94	14.17 \pm 3.43
Blood 6 h	23.5 \pm 14.86	23 \pm 12.12	22 \pm 10.86	16.33 \pm 10.58
Blood 24 h	8.00 \pm 4.05	17.83 \pm 13.57	12.83 \pm 12.59	14.50 \pm 12.42

Table 3. Analysis of the frequency of damage (FD) (mean \pm standard deviation) of DNA in peripheral blood collected at 2, 6, and 24 h in mice treated with saline or one dose of crude extract of *C. microcephala* (125, 250, or 500 mg/kg).

FD	Saline	125 mg/kg	250 mg/kg	500 mg/kg
Blood 2 h	11.33 \pm 2.66	8.50 \pm 2.48	10.67 \pm 3.01	9.83 \pm 2.48
Blood 6 h	11.80 \pm 3.92	11.83 \pm 4.67	11.67 \pm 4.55	9.17 \pm 3.06
Blood 24 h	5.67 \pm 1.86	10.17 \pm 6.40	5.80 \pm 3.71	6.33 \pm 3.83

S. aureus and the butanol fraction did not inhibit the growth of any of the tested bacteria. The fractions obtained with a solvent with intermediate polarity and a nonpolar solvent, respectively represented by ethyl acetate and dichloromethane, formed small zones of inhibition (4 mm) against the growth of *S. aureus*. These results compared with the results of polar fractions obtained with butanol and infusion can be due to the lipophilic character of the compounds extracted as other studies have demonstrated (Pereira-Maia et al., 2010; Santos et al., 2010). Furthermore, it has been found that less polar solvents such as dichloromethane and ethyl acetate allow the extraction of lipids such as steroids, coumarins, terpenoids and sesquiterpene lactones (Carvalho and Carvalho, 2001; Simões, 2003), and it has been shown that terpenes are active against many microorganisms which could explain the formation of an inhibition zone in front of the bacterium *S. aureus*. These results are also consistent with other literature (Cechinel-Filho and Yunes, 1998; Yunes and Calixto, 2001).

The result of 6 mm of inhibition against *S. aureus* with the crude leaf extract of *C. microcephala* obtained with 19.87% yield which is bigger than the other results obtained with the other extracts suggest a synergistic activity of the compounds, once the hidroalcoholic extraction promotes bigger collection of molecules (Kalluf et al., 2008). Regarding the minimum inhibitory concentration (MIC), the extracts analysed that showed the best results in the agar diffusion test were the crude leaf extract and fractions of ethyl acetate and dichloromethane. The MIC was determined by detecting the lowest dilution that completely inhibited bacterial growth in a liquid medium (Andrews, 2001). Figure 1 shows the results. As shown in Figure 1, the extracts showed different inhibition values. The fractions of

dichloromethane and ethyl acetate showed MICs of 25 and 100 mg/ml, respectively. With the crude leaf extract, the MIC was 12.5 mg/ml. These results indicate that it may not be only one active compound that is responsible for the observed microbiological activity of *C. microcephala*. Once again, synergy may be the explanation for the result (Yunes and Cechinel-Filho, 2009; Williamson, 2001).

Genotoxicity test

Regarding genotoxicity, Tables 2 and 3 show the results of the evaluation of aqueous extract of *C. microcephala* by different parameters of the comet assay (DI and FD). This study showed no significant increase in the levels of damage to genetic material in the blood cells of mice treated orally with the infusion of this plant used at different concentrations and for different exposure times, compared with the saline group. Based on studies of plant phytochemicals, it is known that phenolic compounds, in general, can stimulate the DNA repair system, through transcriptional regulation of mRNA stabilisation (Franke et al., 2005). Coumarins also have antioxidant activity and are especially effective against the hydroxyl radical (Lo et al., 2004). It is important to note that the damage remained constant within each group, independently of exposure time. There was no statistically significant difference, indicating that the damage was not reversed by the DNA repair mechanism, after the 24 h of exposure. This is because the lesion was small, without harmful damage to the genetic material. Table 4 shows that the aqueous extract of *C. microcephala* for both parameters of the comet assay (DI and FD), did not show high levels of DNA damage in liver cells, despite studies in the literature reporting that *C. microcephala* can cause hepatotoxicity

Table 4. Analysis of damage index (DI) and frequency of damage (FD) (mean \pm standard deviation) of DNA in the liver collected after 24 h from mice treated with either saline or one dose of crude extract of *C. microcephala* (125, 250, or 500 mg/kg).

Liver	Saline	125 mg/kg	250 mg/kg	500 mg/kg
DI	42.00 \pm 11.16	43.83 \pm 16.75	54.00 \pm 29.08	43.83 \pm 7.41
FD	23.60 \pm 7.33	19.50 \pm 5.54	28.33 \pm 7.2	26.00 \pm 3.95

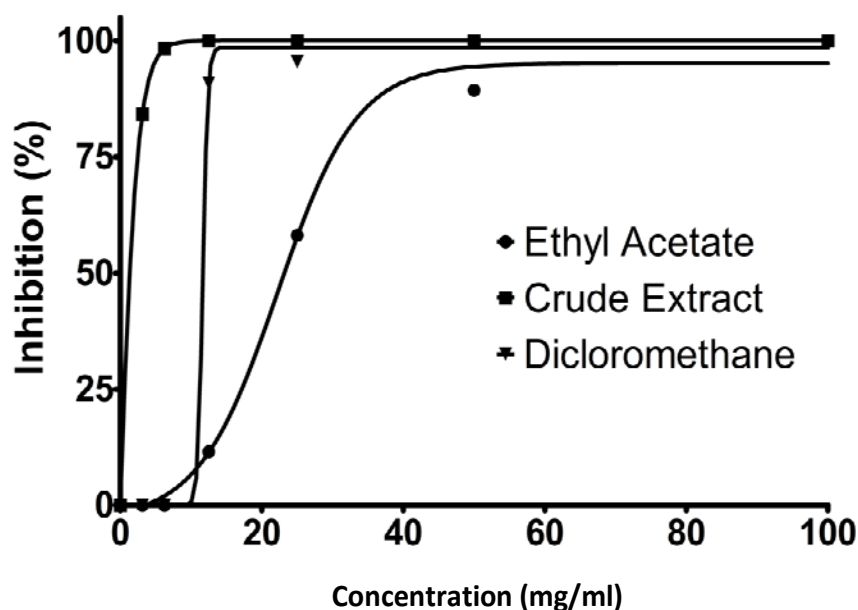


Figure 1. Growth inhibition curve of crude extract and fractions (dichloromethane and ethyl acetate) of *Cunila microcephala* Benth. against the bacterium *Staphylococcus aureus*.

due to the presence of menthofuran in its composition (Madyastha and Raj, 1994; Kramlinger et al., 2012). The lesion was not identified at the DNA level in this cell type during the period of this study, as there was no statistically significant difference between the different treatment groups compared with the control group.

Conclusion

Although *C. microcephala* has been a popular medicine mainly in the south of Santa Catarina, few pharmacologic studies have been published. In this preliminary phytochemical analysis of leaves of *C. microcephala*, phenolic compounds were detected: tannins, flavonoids and coumarins; the concentration of these compounds was 193.23 mg/ml for the crude leaf extract. These results indicate that these compounds may possibly be responsible for the biological activity described by the

population (treatment of respiratory problems). Some extracts of *C. microcephala* showed results in relation to the zone of inhibition against *S. aureus* in the agar diffusion test: crude leaf extract (6 mm), dichloromethane fraction (4 mm), and ethyl acetate fraction (4 mm). Further, the minimum concentration that inhibited *S. aureus* growth totally (MIC) was determined: crude leaf extract 12.5 mg/ml, dichloromethane fraction 25 mg/ml and ethyl acetate fraction 100 mg/ml. Regarding DNA damage, the data do not suggest genotoxic and hepatotoxic action of the aqueous extract *C. microcephala* at the doses tested in the *in vivo* experimental model, indicating that it is safe to use by the population.

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Conflict of interest

The authors report no declarations of interest.

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