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Article

Chemical constituents and pharmacological profile of *Gunnera manicata* L. extracts

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Gunnera perpensa L. (Gunneraceae) is a native South African plant widely used in traditional medicine as an antibacterial and antifungal. In southern Brazil there is the native species called *Gunnera manicata* L. that also belongs to the Gunneraceae. Nevertheless, there is no information about chemical and pharmacological properties of South American *Gunnera* species. Therefore this study aimed at assessing the phytochemical and pharmacological profiles of aqueous and methanol Brazilian *G. manicata* extracts. The results showed that antimicrobial activity in an agar diffusion assay was effective against *Staphylococcus aureus* and *Candida albicans*. Phenolic compounds were investigated by liquid chromatography coupled with a tandem mass spectrometer (LC-MS/MS) and all extracts presented gallic acid and only the methanol extract obtained from the leaves exhibited hyperoside. Rutin, quercetin and chlorogenic acid were not found in the samples analysed. Total phenolic content was higher in methanol extract and total flavonoid content was low in all extracts. Antioxidant activity was evaluated by the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical test, and all samples presented good to moderate antioxidant activity. These results encourage complementary studies on the chemical composition of the plant extracts focusing on isolation and structure elucidation of their active compounds.

Uniterms: *Gunnera manicata* L./phytochemistry. *Gunnera manicata* L./pharmacognosy. Gunneraceae/pharmacognosy. *Gunnera manicata* L./extracts/antimicrobial effect. *Gunnera manicata* L./extracts/antioxidant activity. *Gunnera manicata* L./extracts/antimicrobial assay.

Gunnera perpensa L. (Gunneraceae) é uma planta nativa do sul da África utilizada na medicina tradicional como antibiótico e antifúngico. *Gunnera manicata* L. é uma planta nativa do sul do Brasil também da família Gunneraceae e, apesar disso, não há informações sobre suas propriedades químicas e farmacológicas. Assim, o objetivo deste estudo foi avaliar o perfil fitoquímico e farmacológico dos extratos aquoso e metanólico de *G. manicata.* Os resultados do ensaio microbiológico de difusão em ágar demonstraram que os extratos testados foram ativos contra *Staphylococcus aureus* e *Candida albicans.* A presença de compostos fenólicos foi investigada pela técnica de Cromatografia Líquida acoplada a espectrômetro de massas em Tandem (CL-EM/EM). Em todas as amostras analisadas verificou-se a presença de ácido gálico e somente o extrato metanólico das folhas apresentou hiperosídeo. Rutina, quercetina e ácido clorogênico não foram encontrados. O conteúdo total de compostos fenólicos foi maior nos extratos metanólicos e o conteúdo de flavonóides totais foi baixo em todos os extratos. A atividade antioxidante foi avaliada pelo

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teste da atividade do radical 2,2-diphenyl-1-picril-hidrazil (DPPH) e todas as amostras apresentaram boa a moderada atividade antioxidante. Esses resultados encorajam estudos complementares da composição química dos extratos com foco no isolamento e na elucidação estrutural dos compostos ativos.

Unitermos: *Gunnera manicata* L./fitoquímica. *Gunnera manicata* L./farmacognosia. Gunneraceae/farmacognosia. *Gunnera manicata* L./extratos/efeito antimicrobiano. *Gunnera manicata* L./extratos/atividade antioxidante. *Gunnera manicata* L./extratos/ensaio antimicrobiano.

INTRODUCTION

Gunnera (Gunneraceae) species are mainly found in the Southern hemisphere. In Southern Africa, *Gunnera perpensa* L. is a medicinal plant used by traditional healers for the treatment of venereal diseases. These medicinal properties are due to the presence of antibacterial and antifungal compounds (Buwa, 2005). Rural inhabitants of the Eastern Cape Province use leaves of this plant as wound dressing (Drewes *et al.*, 2005). It is also a traditional herbal medicine used by many South African women as antenatal medication or to induce or augment labour, to treat female infertility, expel the placenta and/or prevent post-partum haemorrhage (Kaido *et al.,* 1997; Kahn *et al.,* 2004). The *Gunnera manicata* L. species is a native plant from Southern Brazil, it grows at high altitudes (above 900m) on the border between the states of Rio Grande do Sul and Santa Catarina, in a region locally known as "Aparados da Serra". Despite being appreciated as an ornamental plant and belonging to the same family (Gunneraceae) as *G. perpensa* (an extensively studied species), little data is found on *G. manicata* in scientific literature, and this includes the absence of anti-estrogenic activity (Mariotti *et al*., 2011a) and the acute toxicity of aqueous extracts of *G. manicata* against Wistar rats, that showed a high margin of safety (Mariotti *et al*., 2011b).

Considering these data, new approaches to this species are necessary. Therefore, this work aimed at evaluating pharmacological and phytochemical profiles of *G. manicata*. For this, the antimicrobial properties, antioxidant potential, total phenol content (TPC), total flavonoid content (TFC) and the presence of phenolic compounds by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) were investigated. The results obtained represent the first analysis of pharmacological and phytochemical proprieties of *G. manicata*.

MATERIAL AND METHODS

Plant material

Roots and leaves of *G. manicata* were collected in the "Aparados da Serra" region, Southern Brazil, and identified by Rodrigo B. Singer. A voucher specimen (R.B. Singer s. n., Serra da Rocinha, January 15th, 2009) is deposited at the ICN - Department of Botany - UFRGS herbarium in Porto Alegre, Brazil.

Preparation of extracts

The aqueous extracts were made with dried roots or leaves extracted in a water bath at 50 °C during 3 hours (ARE50/ALE50 Aqueous Roots Extracts and Aqueous Leaves Extracts, respectively) or at 100 °C during 1 hour (ARE 100/ALE 100 Aqueous Roots Extracts and Aqueous Leaves Extracts, respectively). The methanol extract was obtained by static maceration during 24 hours at room temperature (MRE/MLE – Methanol Roots Extracts and Methanol Leaves Extract, respectively). These extracts were collected, filtered, concentrated to dryness under reduced pressure and the residue was maintained at 4±2 °C until the experiments were performed.

Antimicrobial activity assay

The antimicrobial activities of *G. manicata* extracts were determined by the agar diffusion method. The dried plant extracts were dissolved in water or methanol to a final concentration of 25.0 mg/ml and filtered through 0.22 µm Millipore filters for sterilization. The antibacterial activities were screened with the extracts against Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Streptococcus pyogenes* ATCC 8668, *Bacillus subtilis* ATCC 19659), Gram-negative bacteria (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 25933) and yeast (*Candida albicans* ATCC 10231). By means of a sterile cork borer, 9 mm wells were punctured in plates containing Muller-Hinton agar previously seeded with one of the test microorganisms (the final inoculum size was $1x10^8$ CFU/mL, 0.5 MacFarland), then 100 µL of extract were added in each well. The Petri dishes were incubated at 8-10 °C for 16 h for the diffusion of the bioactive compounds. After this period, the incubation continued at 28-37 °C for 24 h. Then, the inhibition zones were measured. The diameters of inhibition zones were

used as a measure of antimicrobial activity. Assays were carried out in triplicate. A Gentamicin 10 µg disk was used as positive control for bacteria and a Nystatin 100 µg disk for yeast.

Spectrophotometric DPPH assay

Antioxidant activity against the stable radical DPPH was evaluated quantitatively by spectrophotometric measures of the consumption of this radical in the presence of antioxidants (Brand-Williams *et al*., 1995). Briefly, aliquots of aqueous or methanol solution of test samples at different concentrations were added to a DPPH ethanolic solution (molar absorption coefficient 517 nm: 11,500 M cm^{-1}). The DPPH solution absorbance began to be measured immediately after mixing the samples. The decrease in absorbance was monitored at λ=517 nm during 600 seconds with measurements every 1 second, at 25 ºC. For the evaluation of the antioxidant potential, experimental data (kinetic profiles of DPPH decay) were adjusted in terms of the percentage reduction of DPPH and Q, sometimes referred to as "inhibition" or "quenching", which is defined by:

$$Q = 100\ (Ao - Ac)/Ao$$

where Ao is the initial absorbance and Ac is the value of the absorbance of the added sample at concentration c. This value of Ac should be that in the cuvette in the absence of any DPPH, and should take into account the dilution of the original sample solution by the added DPPH solution (Molyneux, 2004). The experiments were performed in triplicate and results were expressed as the average of A/Ao and the IC_{50} (concentration of 50% DPPH radical bleaching) was calculated for each sample. Ethanol was used as a blank in the spectrophotometer. Quercetin was used as reference.

Phytochemical profile

Determination of total phenolic content

Total phenolic content (TPC) was assayed using the Folin-Ciocalteu (Singleton, Rossi, 1965) modified method. Appropriate dilutions of the samples were oxidized with 0.2 N Folin-Ciocalteu reagent (Merck Darmstadt, Germany; 2 N diluted 10-fold) and after 5 min the reaction was neutralized with saturated sodium carbonate (75 gL^{-1}). After 30 min of reaction at room temperature the absorbance of the resulting blue color was measured at 765 nm wavelength with an ultraviolet-visible Biospectro SP-220 spectrophotometer. TPC was expressed as milligrams of quercetin equivalent per 100 mg of dry extract (QE (100 $mgDE)^{-1}$) through a calibration curve for quercetin (Ivanova *et al*., 2005), with concentrations ranging from 50 to 500 mg mL^{-1} (r^2 >0.99).

Determination of total flavonoid content (TFC)

The total flavonoid content in aqueous and methanol extracts was determined according to the German Pharmacopoeia method (DAB 9) general method, using $AlCl_3$ 1% (m/v).

Each plant extract (1.0 mL of 1:100g mL^{-1}) was separately mixed with 7.0 mL of methanol prior to 2.0 mL of 1% aluminum chloride. The calibration curve was performed with methanol quercetin solutions at concentrations of 1.0 to 8.0 µg mL^{-1} (y=0.078x + 0.0065, r^2=0.998). Absorption of these sample solutions was measured after 10 min at 420 nm wavelength with an ultraviolet-visible Biospectro SP-220 spectrophotometer. The TFC was calculated from the equation:

$$C = \frac{A\ Fd}{m\ E^{1\%}_{1cm}}$$

where "C" is the TFC content expressed as grams of quercetin per 100 g of sample, "A" is the measured absorption (A.U.), "Fd" is a dilution factor, "m" is the sample weight (g) and "$E^{1\%}_{1cm}$" is the specific absorption of quercetin - $AlCl_3$ complex. Each analysis was performed in triplicate.

LC-MS/MS analysis

The analysis was performed by direct infusion in a Sciex API 5000 triple stage quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA). The mobile phase used in the infusion procedure was mixed with solvent A (0.1% formic acid) and solvent B (acetonitrile). The flow rate of the syringe was 10µl min^{-1}. The ions were monitored in full scan in positive and negative mode, nitrogen was used for both nebulizer and collision gas. The optimized parameters were: declustering potential 100 eV, entrance potential 10 eV, collision energy 35 eV, and collision cell exit potential 10 eV. The fragmentation profile was obtained from the selection of a pattern ion and monitored in MS2 mode. The target ions monitored were 169 for gallic acid; 301 for quercetin; 353 for chlorogenic acid; 463 for hyperoside and 609 for rutin (Tomás-Barberán, 2001; Clifford, 2003).

Data analysis

The IC_{50} values were determined by linear regression

TABLE I - Antimicrobial activity of *G. manicata* extracts 25 mg mL^{-1} (inhibition zones in diameter (mm))

Microorganism species	ARE100	ARE100	ARE50	ALE50	MLE	MRE	GEN	NYS
***Bacillus subtilis* ATCC 19659**	-	-	-	-	-	17	26	-
***Staphylococcus aureus* ATCC 25923a**	-	-	-	20	-	-	21	-
***Streptococcus pyogenes* ATCC 8668**	-	-	-	16	-	-	24	-
***Escherichia coli* ATCC 25922**[a]	-	-	-	-	-	-	23	-
***Pseudomonas aeruginosa* ATCC 25933**	-	-	-	-	-	-	20	-
***Candida albicans* ATCC 10231a**	-	-	-	-	18	16	-	20

*A dash (–) indicates no antimicrobial activity. The diameter of the inhibition zone is expressed in millimeters (mL). Gentamicin 10 μg and nystatin 100 μg served as controls for bacteria and fungi, respectively.

between the DPPH radical bleaching and sample concentration. All results were obtained from three independent experiments and given as mean ± standard deviation (SD). The correlation between the antioxidant activity and total phenolic content was also determined using the linear regression equation.

RESULTS AND DISCUSSION

G. manicata is a known and appreciated ornamental plant native to Southern Brazil. This study was performed taking these facts into account and the absence of scientific information about pharmacological and phytochemical aspects. For both, different extracts were tested against specific target microorganisms utilized as taxonomical representatives in an initial screening. The results of antimicrobial activity were assessed qualitatively by the presence or absence of inhibition zones. Different extracts at 25 mg mL^{-1} (w/v) obtained from *G. manicata* demonstrated these activities. As can be seen in Table I, ALE50 extracts of *G. manicata* showed activity against *S. pyogenes* and *S. aureus*, one of the most persistent infectious microorganisms commonly found in nosocomial infections (Steenkamp, 2004). MRE and MLE exhibited activity against *C. albicans*, a significant cause of infection in humans. Several of the more commonly isolated microorganisms among these species are less susceptible to commonly used azole antifungal drugs, which makes it very difficult to treat them effectively (Coleman *et al*., 1998).

None of the extracts tested exhibited activity against gram-negative bacteria. This result may be explained because these microorganisms have an outer membrane consisting of lipoproteins and lipopolysaccharides, which is selectively permeable and thus regulates access to the underlying structures. This renders the Gram-negative bacteria generally less susceptible to plant extracts than the Gram-positive bacteria (Samec *et al*., 2009).

In the assessment of antibacterial and antifungal (yeast) activities, G. *perpensa*, a native plant of South Africa, showed higher activity for the leaves compared with the stems and roots (Drewes *et al*., 2005), and the same occurred with *G. manicata*. While the activities recorded for the leaf extract were not impressive, *G. perpensa* inhibition of *S. aureus* was good (Drewes *et al*., 2005), corroborating the results of *G. manicata* for this Gram-positive bacteria.

The results of the antioxidant activity analysis, TPC and TFC are shown in Table II.

TABLE II - Total Flavonoid Content (TFC) (g% of extract), Total Phenolic Content (TPC) (QE(100 mg $DE)^{-1}$) and IC_{50} values (μg mL^{-1}) from aqueous and methanol *G. manicata* extracts

G. manicata extracts	TFC	TPC	IC_{50}
ALE50	1.69±0.0005	13.42±1.06	28.17±0.80
ARE50	1.59±0.002	19.47±1.53	15.68±0.36
ALE100	2.85±0.057	28.39±1.47	12.47±0.16
ARE100	0.31±0.002	28.11±1.61	15.92±0.38
MLE	0.53±0.004	11.70±1.38	39.89±0.38
MRE	3.85±0.002	30.13±1.24	33.93±0.61

In the analysis of antioxidant potential, the ability of samples to scavenge the DPPH radical was determined based on their concentrations providing 50% inhibition (IC_{50}). ALE100, ARE100 and ARE50 exhibited strong antioxidant activity. MRE and MLE showed moderate antioxidant potential, good antimicrobial and antifungal

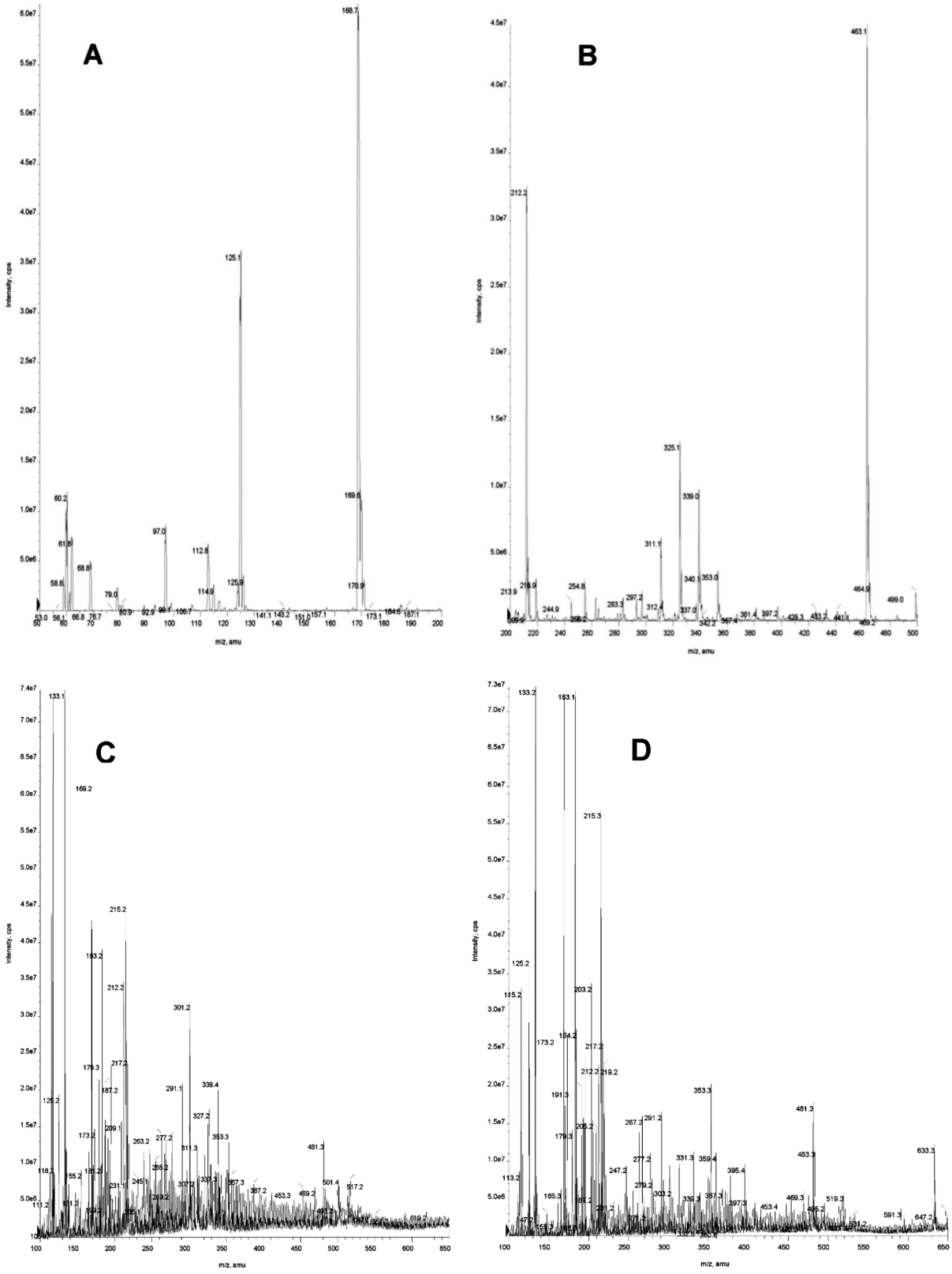

FIGURE 1 - Phenolic compounds found in the investigation by LC-MS/MS (A. Gallic acid analysis – target ion 169; B. Hyperoside analysis – target ion 463; C. ALE100 analysis, an example of an extract containing gallic acid; D. MLE analysis, the only extract that contained hyperoside).

activities and a high TPC. These data may indicate a correlation between antimicrobial activities and the amount of phenolic compounds.

G. manicata antioxidant activity results were similar to those obtained with *G. perpensa*, which showed possible scavenging activity in a concentration dependent manner, while aqueous extracts demonstrated higher activity than methanol extracts (Steenkamp, 2004).

The TPC of plant extracts was determined using a colorimetric assay method. Results were expressed as quercetin equivalent per 100 mg of dry extract. The amounts of TPC were higher in MRE, followed by ARE100 and ALE100. In statistical analysis, there was no linear correlation between TPC and antioxidant activity (r^2=0.2316).

In the analysis of TFC, all samples demonstrated low flavonoid content contrasting with the values of TPC.

In this work, no linear correlation was found between TPC and antioxidant potential. This relationship reported in the literature is somewhat confusing. Some investigators have proposed the existence of a close correlation between antioxidant activity and phenolic compound content of the extracts obtained from various natural sources. Others did not correlate them merely to each other and propose and suggest that a wide range of compounds, such as phenolics, peptides, organic acids and other components contribute to the antioxidant activity (Ebrahimabadi *et al.,* 2010).

The potential antioxidant capability of the other Gunneraceae species, *G. Perpensa*, has been established (Simelane *et al.*, 2010). It seems that *G. perpensa* contains compounds that might improve the action of natural dietary antioxidants. The relatively high total polyphenol content and the significant total antioxidant capacity could possibly contribute to *G. perpensa* antioxidant properties (Simelane *et al.*, 2010).

In the investigation of five different phenolic compounds by LC-MS/MS, all extracts present gallic acid and only methanol leaves extract exhibited hyperoside. Rutin, quercetin and chlorogenic acid were not found in the samples analyzed.

In Figure 1.C the ALE100 analysis is shown as an example of a gallic acid positive extract, since all the extracts presented this compound. MLE analysis can be seen in Figure 1.D as the only *G. manicata* extract composed by hyperoside.

Flavonoids constitute the largest group of plant phenolics (Balasundram *et al.,* 2006) and their antimicrobial effects have been extensively researched (Samec *et al*., 2009). Considering these data, the presence of flavonoids was investigated. As result, low TFC was found despite the large amount of TPC in the sample analyzed. These data suggest that another class of phenolic compounds, such as hydrolyzed tannins, for example, may be responsible for the amount of TPC. This fact may be partially confirmed by the LC-MS/MS analysis, which showed the presence of gallic acid in all extracts.

CONCLUSION

These findings leave open the possibility of further studies using *G. manicata* as a source for the discovery of novel leader structures to be used in drug production, and also for the development of active plant extracts that can be used for treatment of various diseases. The results found in this work encourage complementary studies on the chemical composition of the plant extracts focusing on isolation and structure elucidation of their active components and evaluation of the biological activity of each compound separately.

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