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Original Article

Antioxidant and Anti-inflammatory Activity of *Mikania glomerata* and *Mikania laevigata* Extracts

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ABSTRACT

Background: *Mikania glomerata* and *Mikania laevigata (guaco)* extracts are popularly used for the treatment of asthma and cough as well as for their anti-inflammatory activity, indistinctly, despite their different chemical composition. Both species may present these activities however, the specific components and the cellular mechanisms are not fully identified. **Objectives:** To determine the activity of fractions obtained by countercurrent chromatography pooled based on their TLC and UHPLC-MS chromatographic profiles. **Materials and Methods:** Fractions with antioxidant activity in DPPH and ORAC tests were assayed in dystrophic primary muscle cell cultures from mdx mice, the experimental model of Duchenne muscular dystrophy (DMD), to evaluate their cellular anti-inflammatory and antioxidant activity. The inflammatory process was evaluated by determining the TNF-α, NF- κB and IL-1β content by immunoblotting; content of 4-hydroxynonenal, superoxide dismutase (SOD); catalase, glutathione peroxidase (GPx); glutathione reductase (GR) and glutathione (GSH) were determined to evaluate their antioxidant activity. **Results:** The crude *M. glomerata* and *M. laevigata* extracts, as well as 3 selected fractions presented antioxidant capacity in the ORAC assay and only Mlet Fr13 did not present activity by DPPH. Immunoblotting revealed no significant differences between the experimental groups, so no cellular anti-inflammatory effect was observed, however, reduced levels of anti-oxidant defence system components were observed for all fractions. **Conclusion:** Both species contain compounds that effectively reduced anti-oxidant defense system components, but none of these fractions significantly reduced inflammatory markers, suggesting that the reported anti-inflammatory activity of these species may be mediated by oxidative stress reduction.

Keywords: Countercurrent chromatography, DPPH, Dystrophic primary muscle cells, Guaco, ORAC, UHPLC-MS.

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INTRODUCTION

Two species of medicinal plants, popularly known as *guaco, Mikania glomerata* Spreng. and *Mikania laevigata* Sch. Bip ex Baker, are popularly used for the treatment of asthma, bronchitis and cough. They also present anti-inflammatory, antispasmodic, anti-hemorrhagic, antiophidic, antiviral and antimicrobial activity.[1-3]

Suyenaga *et al.*[4] evaluated the anti-inflammatory properties of *M. laevigata* decoction in an animal model with carrageenan. The Brazilian Phytotherapic Formulary indicates the use of leaves of both species interchangeably in both infusions and hydroalcoholic extracts.[5] This may be due to the fact that both species are creepers, grow in the same regions in Brazil and present similar foliage[6] possibly leading to misidentification of the plant material. However, studies performed by our group have repeatedly shown both species to present a different chemical composition[7,8] relentless of growth conditions.[9] Therefore, the chemical analysis of their extracts is necessary for the correct identification of these species.

Nevertheless, it is plausible that both species could present therapeutic potential, despite their difference in composition. Therefore, we have undertaken an evaluation of the biological activity of chemically characterized plant extracts, obtained from identified individuals of both species. Della Pasqua *et al.*[10] reported the anti-inflammatory activity of aqueous extracts of *M. glomerata* and *M. laevigata*, comparing their activity with

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that of coumarin, the compound reportedly responsible for their anti-inflammatory activity. This anti-inflammatory activity was evaluated via paw edema, pleurisy and mast cell methods. The best results were obtained with the *M. laevigata* extract, which contained a considerable amount of coumarin (13.46 mg/g). However, some effect was observed with the *M. glomerata* extract which contained much less coumarin (0.023mg/g), indicating that other components were also involved in the anti-inflammatory activity, according to the authors. Studies in other plant species have shown that kaurenoic acid and caffeoylquinic acids, also present in guaco extracts, present anti-inflammatory activity.[11-13] However, it is also known that many phenolic compounds, such as both mono- and dicaffeoylquinic acids, present antioxidant activity *in vivo* and *in vitro*[14] which may also contribute to reducing inflammation.

However, the specific components and the cellular mechanisms responsible for these activities are not fully identified. Complex plant matrixes with a large number of components having a wide variety of polarity and concentrations are not easily fractioned by classical (column or plate) chromatography techniques; countercurrent chromatography (CCC) was applied to enable the fractionation with minimal sample loss. CCC combines the benefits of continuous extraction and partition chromatography, which enables the isolation of large amounts of high purity compounds in a shorter time using biphasic solvent mixtures with different ratios and without prior purification of the crude extract.[15,16] Initially, enriched fractions of components present in the hydroethanolic extracts of *M. glomerata* and *M. laevigata* were obtained by CCC and their composition was determined by ultra-high- performance chromatography-mass spectrometry.

Selected fractions representing the main classes of compounds found in *guaco* extracts were tested to evaluate their antioxidant activity via DPPH and ORAC *in vitro* tests. These models were selected as they detect different types of antioxidant mechanisms: DPPH mainly for electron donors and ORAC for hydrogen transfer. Furthermore, the extracts and fractions were assayed in dystrophic primary muscle cell cultures from mdx mice, the experimental model of Duchenne muscular dystrophy (DMD).[17] The DMD physiopathogenesis involves specific mechanisms, such as an exacerbated inflammatory process and oxidative stress. It has been reported that inflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β), as well as the nuclear factor kappa-B (NF-κB) play a major role in the DMD phenotype.[18] Regarding oxidative stress, high levels of reactive oxygen species (ROS) and lipid peroxidation have been reported in DMD patients and mdx muscle cells.[19,20] In addition, elevated concentrations of antioxidant enzymes, including catalase, glutathione peroxidase (GPx) and superoxide dismutase (SOD), have been identified in dystrophic muscles, leading to the conclusion that these muscles are oxidatively stressed.[21] This model was selected as it could evaluate both the anti-inflammatory and antioxidant activity of the selected fractions, to better elucidate the cellular mechanism of the activity of the medicinal plant species.

MATERIALS AND METHODS

Plant Extracts

Adult specimens of identified *M. glomerata* and *M. laevigata* creepers were growing in the experimental field of the Institute of Biology (latitude -22.818439; longitude -47.064721), University of Campinas (UNICAMP); vouchers deposited at the UEC herbarium under number 189254 for *M. laevigata* and 189252 for *M. glomerata*, and SisGen registration number ABC 6041. Healthy leaves of these specimens were collected, frozen, ground under liquid nitrogen, then extracted. The hydroethanolic extraction (70% solution of ethanol/water) was performed using a proportion of 60g of ground leaves to 300 mL of solvent, in a sonic bath for 30 min then filtered. The leaves were re-extracted twice again in the same manner and the three solutions were pooled, then concentrated under vacuum (HEIDOLPH; Model: Rotary Evaporator Hei VAP; LABCONCO; CentriVap Acid-Resistant Concentrator Systems) resulting in dry hydroethanolic extracts of *M. glomerata* (Mget) and *M. laevigata* (Mlet).

CCC Fractionation of Crude Extract

Semi-purified fractions were obtained using a DE-Midi CCC instrument (Dynamic Extractions, Tredegar, UK), coupled to Agilent HP1200 HPLC system with two 100 mL capacity pumps for mobile phases and a multiwavelength (MWV) detector (Santa Clara, California, USA) with preparative UV cell, Knauer K-1800 preparative pump with 1000 mL capacity (Berlin, Germany) for stationary phase and automatic fraction collector Gilson FC202 (Villiers-le-Bel, France). The sample solution was injected through a Knauer K-6 injection valve (Berlin, Germany).

In each preparative CCC separation, two columns connected in series with a total volume of 912.5 mL, were first filled with the upper phase (UP) of the hexane-ethyl acetate-methanol- water (1/1/1/1) system (HEMWat 17) used as the stationary phase and set rotating at 1250 rpm and 30°C. Then the lower phase (LP) of the same HEMWat 17 used as the mobile phase was pumped into the column at a flow rate of 24 mL/min. After reaching a stable hydrodynamic equilibrium, 4 g of crude sample dissolved in 50mL of a proportion of 80% of the lower phase to 20% of the upper phase of the HEMWat 17 system was injected via a 50 mL sample loop heated to 40°C in a water bath. The 120 min linear gradient between 100% of the LP HEMWat 17 (1/1/1/1) to 100% of the LP HEMWat 27 (19/1/19/1) was started after the solvent front was eluted from the column. Fractions were collected every 2 min for 180 min starting from the injection point.

TLC Analysis

Since the complex crude extract contained both UV active and non-active compounds, the UV trace obtained during the CCC separation could not be used as an indication to pool fractions together. Therefore, the CCC fractions were analyzed by thin-layer chromatography (TLC) on silica gel plates, GF254 normal phase 60 (Merck Art. 05554, Darmstadt, Germany). The TLC plates were developed with hexane: acetone 4: 1 (v / v) mix and dried. After nebulizing with H_2SO_4 (1%, v / v) and vanillin (6% m / v) solution in ethanol, the plates were heated in an oven at 105°C. Fractions with similar compositions were pooled. Since CCC technology provides high reproducibility, only odd-numbered fractions were routinely analyzed by TLC (results not shown).

UHPLC-MS Analysis

Chromatographic analysis by UHPLC-MS of fractions and crude extracts was performed using a method validated by de Melo and Sawaya[7] for quantification of coumarin and chlorogenic acid. An Ultra High-Efficiency Liquid Chromatographer (UPLC® Acquity-Waters with a C_{18} BEH Acquity Waters column (1.7µm x 2.1mm × 50mm), oven temperature at 30°C was used. Gradient elution with a flow of 200 µL / min, using purified water (Milli-Q) with 0.1% formic acid as the mobile phase A and acetonitrile (HPLC grade) as mobile phase B, beginning with 10% Bm ramping to 25% B in 4 min and to 100% B in 8 min, held until 8.50 min, then returning to the initial condition and equilibrating until 10 min. Detection was performed by a triple-quadrupolar mass spectrometer (Waters TQD Acquity) with electrospray ionization source (ESI), scanning in positive and negative mode under the following conditions: capillary of ± 3000 V, ± 35 cone V, 1.0 V extractor, source temperature of 150°C and desolvation temperature of 300°C. Aliquots of 4µL of each sample were injected, diluted in 70% ethanol. The contents of: coumarin (Sigma-Aldrich, ≥ 99%), o- cumaric acid (Sigma-Aldrich, 97%), p-cumaric acid (Sigma-Aldrich, 97%), umbelliferone (Sigma -Aldrich, 99%), kaurenoic acid (Sigma-Aldrich, ≥ 95%) and chlorogenic acid (Sigma- Aldrich,> 95%) were determined with calibration curves constructed from the serial dilution of a stock solution of standards prepared at a concentration of 1 mg / mL in methanol.

Antioxidant Activity by 2,2-diphenyl-1-picryl-hydrazyl (DPPH)

Radical scavenging antioxidant activity was assessed using the DPPH assay. This test is based on the measurement of the absorbance decay at 515 nm of the DPPH solution containing antiradical substances. The test was performed on a 96-well micro-dilution plate according to the method described by Huang *et al.*[22] and Cabral *et al.*[23] Serial dilutions were made for each sample to obtain a final sample concentration in the wells between 200 µg / mL - 6 µg / mL, plus 100 µL of 0.1 M acetate buffer (pH 5.5) and 50 µL of a methanolic solution of DPPH (2.77 x 10-4). As a positive control, a curve of quercetin was used, and the diluents were used as a negative control. The progress of the reaction was measured by absorbance using a spectrophotometer (λ = 517 nm) taking measurements every 10 min for a total of 60 min. The percentage of reduction in DPPH was calculated (%DPPH) = [(abs sample- abs blank)/abs control]x100. Subsequently, the hyperbola equation was calculated from the value of the percentage of reduction of the DPPH, and with these data the EC_{50} value is calculated for each sample. All experiments were carried out in triplicate.

Antioxidant Activity by Oxygen Radical Absorbance Capacity (ORAC)

ORAC assays were performed according to the method described by Prior *et al.*[24] with slight modifications. All samples, standards and reagents were prepared in a 75 mmol potassium phosphate buffer solution, pH 7.4. Reactions were performed by adding 20 µL of Trolox standard or previously diluted extracts, 120 µL of fluorescein (0.378 µg/mL) and 60 µL of AAPH [2,2-Azobis-(2-methylamidinopropane)-dihydrochloride] (108 mg/mL) in each well of fluorescence-specific polystyrene microplates containing 96 compartments (Corning Co®, NC, USA). After AAPH addition, the fluorescence intensity was monitored at 37° for 80 cycles of 60 sec via NOVOstar Microplate Reader (BMG Labtech®, Offenburg, Germany) with fluorescence filters (excitation and emission at 485 and 520 nm, respectively), accompanied by the data analysis software MARS Data Analysis version 1.3 (BMG Labtech®, Offenburg, Germany). This assay was performed in triplicate and results were expressed as µmol Trolox equivalent (TE)/g.

Dystrophic Primary Skeletal Muscle Cell Culture

Mdx mice (C57BL/10-Dmdmdx/PasUnib) were housed in the animal house of the UNICAMP, with food and water being available *ad libitum*. The animal experiments described here were conducted in accordance with the guidelines of the Brazilian College for Animal Experimentation and the guidelines set forth by our institution. The protocol (#5754- 1/2021,) was approved by the Committee on the Ethics of Animal Experiments of UNICAMP, São Paulo, Brazil. Animals were sacrificed at 28 days of age by decapitation. The quadriceps femoris, tibialis anterior, extensor digitorum longus, gastrocnemius, soleus, and plantaris muscles were removed and used to prepare the primary culture of skeletal muscle cells.[20] Muscles were sliced using a pair of scissors and enzymatically digested with collagenase and trypsin solutions at 37°C. The satellite cells ($5x10^4$ cells/cm^2) were plated in 1% Matrigel-coated dishes. The myoblasts were cultured in a proliferation and growth medium containing DMEM with glucose (5.5 mM), L-glutamine (2 mM), fetal bovine serum (10% v/v), horse serum (10% v/v) and penicillin/streptomycin (1% v/v) for 2 days. Myogenesis (myotube differentiation) was induced by the addition of a fusion medium (FM) that consisted of DMEM

with glucose (5.5 mM), L-glutamine (2 mM), and horse serum (10% v/v). The culture was maintained at 37°C and 5% CO_2 and the differentiated muscle cells with contractile properties were observed at 7–8 days of culture in the FM. Skeletal muscle cell cultures at 7-8 days were used in all experiments and all measurements were obtained from triplicate cultures.

MTT Cell Proliferation Assay

The quantification of mitochondrial metabolism and activity of the respiratory chain of cells were assessed by tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT; Sigma) assay. Briefly, primarily skeletal muscle cells were washed in PBS, treated with MTT solution (5 mg/ml, tetrazolium salt) and incubated for 4hr at 37°C. After 4hr, the cell supernatants were discarded, MTT crystals were dissolved with acid isopropanol and the absorbance was measured at 570nm. Plates were analyzed in a multi-mode microplate reader model Synergy H1M (Bio Tek Instruments, Washington) at 570 nm with a 655 nm reference wavelength to quantify the amount of formazan product, which reflects the number of cells in culture. Wells that did not contain cells were used as a zero point of absorbance. All assays were performed in triplicate.

Neutral Red

The metabolic activity of cells was assessed by neutral red assay. Briefly, the entire cell medium was removed from primary skeletal muscle cells and replaced by an equal amount of solution (25: 1 medium + Neutral Red); the cells were incubated for 3hr at 37°C. Plates were washed in PBS and they were incubated respectively with solutions of 1% calcium chloride in formaldehyde 0.5% and 1% solution of 50% acetic acid in ethanol. Plates were analyzed in a multi-mode microplate reader model Synergy H1M (Bio Tek Instruments, Washington) at 540nm. Wells that did not contain cells were used as a zero point of absorbance. All assays were performed in triplicate.

Western Blotting

Proteins were extracted in a buffer containing Tris–HCl (100 mM), pH 7.5; EDTA (10 mM), pH 8.0; sodium pyrophosphate (10 mM); sodium fluoride (0.1 mM); sodium orthovanadate (10 mM); PMSF (2 mM); and aprotinin (10 µg/ml). The cell extracts were sonicated for 30 sec at 4°C. The homogenates were centrifuged at 11,000 g for 20 min at 4°C and the supernatants were treated with Triton X-100 (1%) and transferred to a −80°C freezer until Western blotting analysis. An aliquot from the supernatant was used to determine the total protein content by the Bradford method. Thirty µg of total protein homogenate was loaded on 6%–15% SDS- polyacrylamide gels. Proteins were transferred from the gels to a nitrocellulose membrane using a submersion electrotransfer apparatus (Bio-Rad Laboratories, Hercules, California). The membranes were incubated with the primary antibodies overnight at 4°C, washed in TBST, incubated with the peroxidase-conjugated secondary antibodies for 2hr at room temperature and developed using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology, Rockford, Illinois). To control protein loading, Western blot transfer and nonspecific changes in protein levels, the blots were stripped and reprobed for β-actin. Band intensities were quantified using the GeneTools software (SynGene – A Divison of Synoptics, Cambridge, England). The following primary antibodies were used for Western blotting: anti-SOD-2 (rabbit, Sigma-Aldrich, St. Louis, MO, USA); anti-GSR (rabbit, Sigma-Aldrich, St. Louis, MO); anti-catalase (mouse monoclonal, Sigma-Aldrich, St. Louis, MO, USA); anti-GPx1 (rabbit, Sigma-Aldrich, St. Louis, MO), TNF-α (rabbit anti-mouse polyclonal antibody; Millipore, CA, USA); NF-κB (goat polyclonal,Santa Cruz Biotechnology, Santa

Table 1: Antioxidant activity (EC_{50} in µg/mL) of *M. laevigata* (Mlet) extract and fractions, *M. glomerata* (Mget) extract and fractions, quercetin – control, by DPPH.

Samples	EC_{50}		Samples	EC_{50}
Mlet crude	73.55		Mget crude	20.46
Mlet Fr2	n		Mget Fr2	n
Mlet Fr3	n		Mget tFr5	n
Mlet Fr4	n		Mget Fr8	n
Mlet Fr5	n		Mget Fr11	n
Mlet Fr8	n		Mget Fr13	n
Mlet Fr10	n		Mget Fr18	n
Mlet Fr13	n		Mget Fr19	n
Mlet Fr16	n		Mget Fr22	4.67
Mlet Fr18	503.64		quercetin- control	1.35
Mlet Fr21	n			
Mlet Fr23	12.65			
Mlet Fr24	30.33			

Table 2: Identified and quantified compounds in selected fractions of *M. glomerata* and *M. laevigata* (µg/mg).

Number	Compound	Mlet-Fr13	Mget-Fr 22	Mlet-Fr24
I	Chlorogenic acid		12.97	3.68
II	Caffeic acid		1.53	
III	Melilotoside			0.84
IV	Dicaffeoylquinic acid		25.01	42.45
V	Coumarin			4.51
VI	Grandifloric acid	2.88		
VII	Kaurenoic acid	55.84		

Table 3: Antioxidant capacity of *M. laevigata* (Mlet) extract and fraction 24, *M. glomerata* (Mget) extract, fractions 13 and 22 by ORAC assay (µmol TE/g).

Sample	Average	% Variation
Mlet crude	1894.93	0.32
Mget crude	4038.50	2.96
MGet Fr 13	505.07	9.37
MGet Fr 22	4417.64	1.22
MLet Fr 24	5051.71	7.33

Cruz, California); IL-1β (rabbit polyclonal antibody Santa Cruz Biotechnology, Santa Cruz, California) and β- actin (mouse, Sigma-Aldrich, St. Louis, MO, USA). The secondary antibody was peroxidase- labelled affinity-purified goat, rabbit and mouse IgG antibody (Bio-Rad, Hercules, CA, USA).

Glutathione (GSH) Content

Total GSH content was determined by Ellman's reaction using 5′5′-dithio-bis-2- nitrobenzoic acid (DTNB) as described by Anderson 1985. The intensity of the yellow color was read at 412 nm. The results were expressed as nmol per mg of protein. All assays were performed in triplicate.

RESULTS

Fraction Selection based on Composition and Antioxidant Activity

The 24 fractions of the crude extracts hydroethanolic extracts of *M. laevigata* (Mlet) and the 22 fractions of *M. glomerata* (Mget) obtained by CCC were analyzed via thin layer chromatography to determine which fractions had the same components and could be pooled (results not shown). These extracts and their pooled fractions were then submitted to the DPPH test to evaluate their antioxidant activity, with results shown in Table 1.

In the DPPH assay, the lower the EC_{50} concentration, the higher the antioxidant activity. The Mget crude extract presented a strong activity (EC_{50} 20.46 µg/mL), and its fraction, Mget-Fr22, was responsible for this activity, presenting an even stronger antioxidant activity (EC_{50} 4.67 µg/mL). Three phenolic compounds were identified and quantified in this fraction (caffeic acid and two caffeoylquinic acids) as shown in Table 2, which justify its strong antioxidant activity. A similar result was observed in the ORAC assay results (Table 3), where the Mget crude extract and Mget-Fr22 fraction demonstrate high antioxidant potential, with values of 4038.50 and 4417.64 µmol TE/g, respectively.

The Mlet crude extract presented a lower antioxidant activity in the DPPH assay (EC_{50} 73.55 µg/mL) and its fractions Mlet Fr23 and Fr24 were responsible for this activity as they were more active than the crude fraction (Table 1). As the composition of fraction Mlet Fr23 was similar to Mget Fr22 it was not selected. However, Mlet Fr24 contained coumarin and melilotoside, as well as two caffeoylquinic acids, so it was selected for further studies (Table 2). In the ORAC assay the results presented a similar pattern, with fraction Mlet Fr24 presenting a higher antioxidant capacity (5051.71 µmol TE/g) than the crude Mlet extract (1894.93 µmol TE/g) (Table 3).

Mlet Fr13 did not present antioxidant activity in the DPPH assay and the lowest result in the ORAC assay (505.07 µmol TE/g). As it was composed of less polar terpenes (kaurenoic and grandifloric acids) which could also be responsible for the activity of this medicinal plant, it was selected for further studies. All compounds quantified in Fractions Mlet-Fr 13, Mget-Fr 22 and Mlet-FR 24 are shown in Table 2. The chromatograms of the crude extracts and selected fractions are shown in Figure 1.

Determination of Cell Viability and Fraction Concentration

Initial studies of the cytotoxicity of extracts and fractions were performed in acetate buffer, which did not dissolve the components adequately and stressed the cells over the four-day study. Therefore, in a second test the samples were fully dissolved in propylene glycol, which was less toxic to the cells. Cell viability was evaluated by both MTT and NR (neutral red) reagents to determine the concentrations which could be used (Table 4). These were defined as 62.5 µg/mL for the extracts and 12.5 µg/

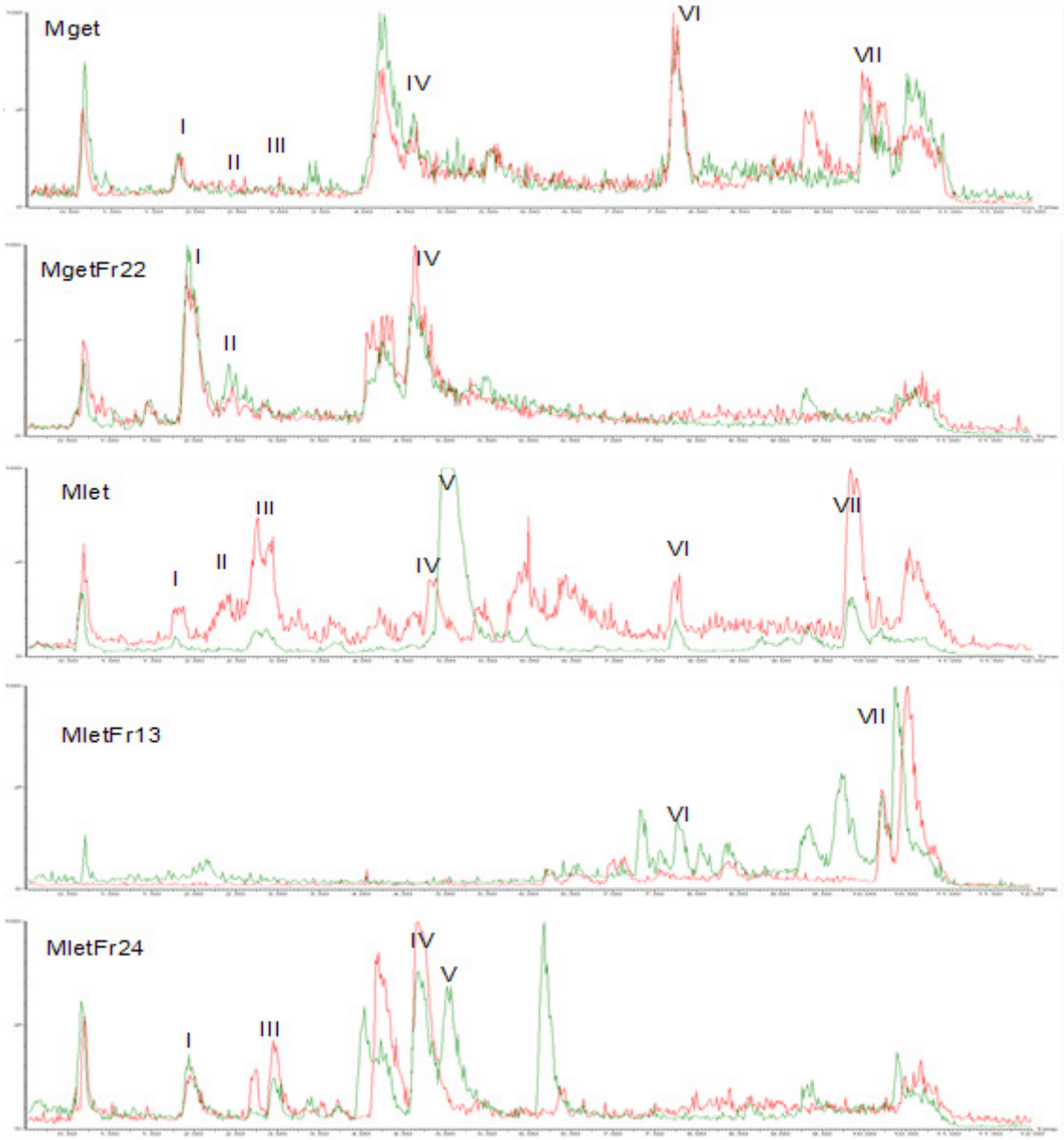

Figure 1: UHPLC-MS chromatograms of *M. glomerata* (Mget) hydroethanolic extract and its fraction (Mget Fr22); *M. laevigata* (Met) hydroethanolic extract and its fractions (Mlet Fr13 and Mlet Fr24). Red – ESI negative ion mode and green – ESI positive ion mode. Identified compounds: (I) chlorogenic acid, (II) caffeic acid, (III) melilotoside, (IV) dicaffeoylquinic acid (V) coumarin, (VI) grandifloric acid and (VII) kaurenoic acid.

mL for the fractions, as in these concentrations the cell growth was equivalent to or superior to the control (defined as 100%).

Determination of the Anti-inflammatory Activity of the Fractions

The defined concentrations of the three fractions were tested in dystrophic primary muscle cells, to assess their potential anti-inflammatory effects. The inflammatory process in the dystrophic primary muscle cells was evaluated by determining the TNF-α, NF-κB and IL-1β content. Immunoblotting revealed no significant differences in TNF-α, NF-κB and IL-1β levels between the experimental groups (Figure 2 A-D). This means that these fractions showed no potential anti-inflammatory effect on the dystrophic primary muscle cells.

Determination of the Antioxidant Activity of the Fractions in Cells

To analyze the fractions` effects on oxidative stress in dystrophic primary muscle cells, the content of 4-hydroxynonenal (4-HNE)-protein adduct; superoxide dismutase (SOD); catalase, glutathione peroxidase (GPx); glutathione reductase (GR) and glutathione (GSH) were determined. Bands of 4-HNE-protein adducts are shown in Figure 2 A, proteins from 17 to 250 kDa were observed. The dystrophic primary muscle cells treated with Mget-Fr22, Mlet- Fr13, and Mlet-Fr24 showed a significant

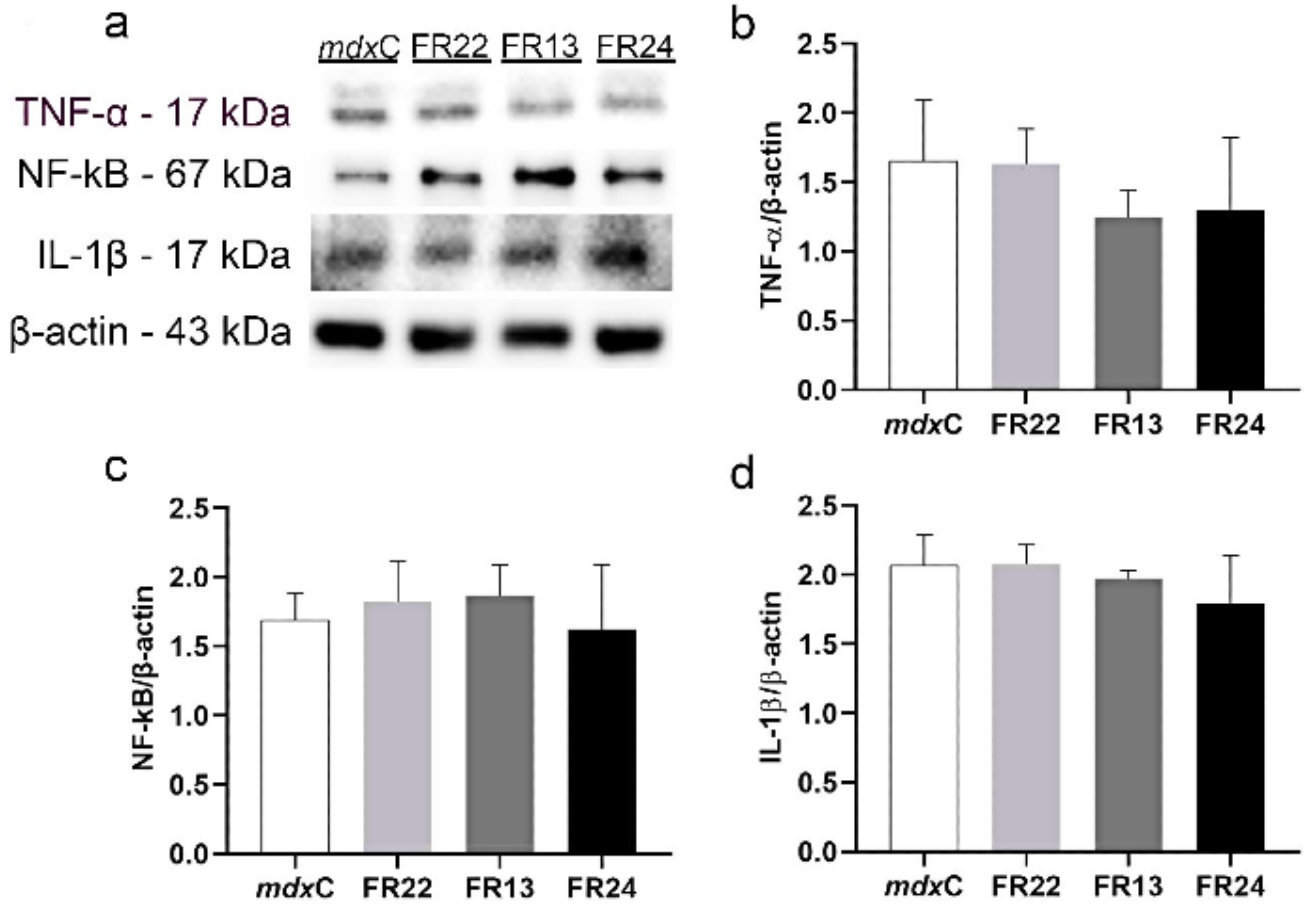

Figure 2: Western blotting analysis of TNF-α, NF-κB and IL-1β (A) in muscle cells from mdxC (dystrophic untreated cells) and treated at different fractions (FR22, FR13 and FR24). The graphs show western blotting analysis of TNF-α (B), NF-κB (C) and IL-1β (D) in muscle cells from mdxC (dystrophic untreated cells) and treated at different fractions (FR22, FR13 and FR24). β-actin was used as a loading control. The relative value of the band intensity was quantified and normalized by the corresponding control. Data expressed as mean ± SD.

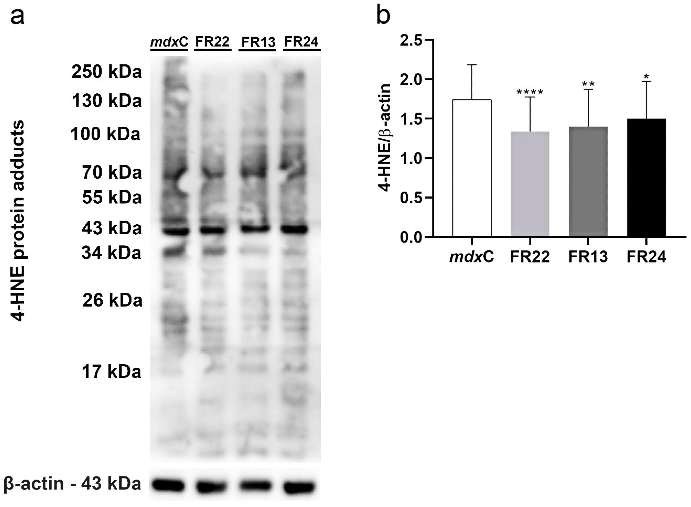

Figure 3: Western blotting analysis of 4-HNE protein adducts (A) in muscle cells from mdxC (dystrophic untreated cells) and treated at different fractions (FR22, FR13 and FR24). The graphs show western blotting analysis of 4-HNE protein adducts (B) in muscle cells from mdxC (dystrophic untreated cells) and treated at different fractions (FR22, FR13 and FR24). β-actin was used as a loading control. The relative value of the band intensity was quantified and normalized by the corresponding control. *p< 0.05 versus mdxC; **p< 0.001 versus mdxC; ***p< 0.0001 versus mdxC. Data expressed as mean ± SD.

decrease in the 4-HNE protein adduct levels (by 23.2, 19.6, and 13.9%, respectively) compared to the mdxC group (Figure 3A–B). This means that all three fractions showed potential antioxidative effects in dystrophic primary muscle cells, albeit in different proportions.

Regarding the fractions` effects on the enzymatic antioxidant defense system, there were no significant differences between the levels of GSH and Catalase between the control and treatments (Figure 4 A, C, F). However, the levels of SOD-2, GR and GP-X were significantly lower in the treatments with all three fractions (Figure 4 A, B, D, E). The level of SOD-2 was reduced by 34.4% by Mget-FR22; 58.9% by Mlet-FR13 and 46.6% by Mlet- FR24. Mlet-Fr13 was the most effective although the result was not significantly different from Mget-FR22 and Mlet-FR 24. The level of GSR was reduced by 27.9% by Mget-FR22; 42.5% by Mlet-FR13 and 34.9% by Mlet-FR24. Again, Mlet-Fr13 was the most effective although the results were not significantly different between samples. The level of GPX-1 was reduced by 54.1% by FR22; 50.1% by FR13 and 39.2% by FR24. In this case, Fr22 was the most effective; although the results were not significantly different between samples.

Table 4: Percentage of cell viability evaluated by MTT and NR assays.

Sample	Extract Conc. µg/mL	Abs. (540 nm)	MTT %	Abs. (570 nm)	NR %
Control	0	1.16	100.00	0.17	100.00
Mlet crude	62.5	1.14	98.81	0.21	122.88
Mget crude	62.5	1.49	128.43	0.18	104.73
Mlet-Fr13	12.5	1.50	129.82	0.21	119.98
Mget-Fr22	12.5	1.46	126.46	0.19	110.33
Mlet-Fr24	12.5	1.62	140.16	1.95	113.13

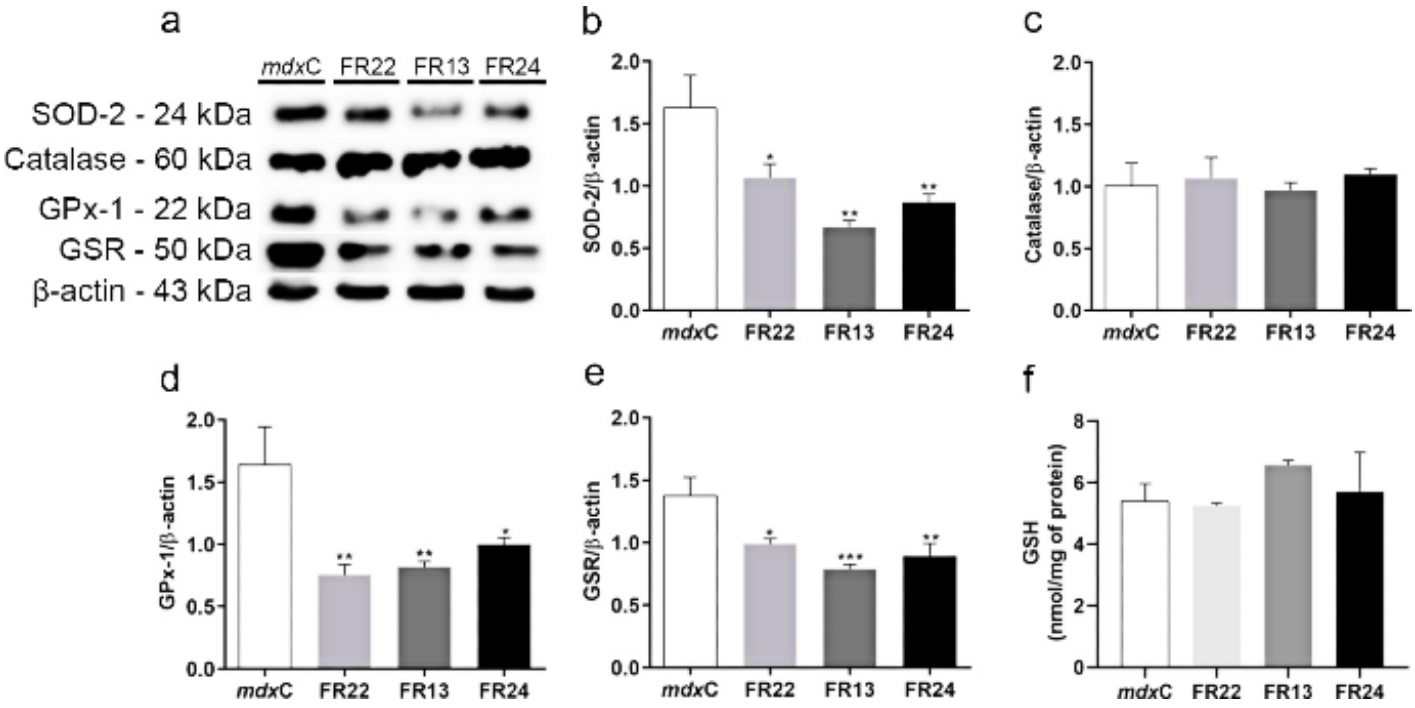

Figure 4: Western blotting analysis of SOD-2, Catalase, GPx-1 and GSR (A) in muscle cells from mdxC (dystrophic untreated cells) and treated at different fractions (FR22, FR13 and FR24). The graphs show western blotting analysis of SOD-2 (B), Catalase (C), GPx-1 (D) and GSR (E) in muscle cells from mdxC (dystrophic untreated cells) and treated at different fractions (FR22, FR13 and FR24). β-actin was used as a loading control. The relative value of the band intensity was quantified and normalized by the corresponding control. The graphs show the glutathione (F, GSH) content. *p< 0.05 versus mdxC; **p< 0.001 versus mdxC;
***p< 0.0001 versus mdxC. Data expressed as mean ± SD.

DISCUSSION

The fractions obtained by CCC from the hydroethanolic extracts of *M. glomerata* and *M. laevigata* were chosen due to the difference in their composition, which represented the main known components found in these species.

Among the three analyzed fractions, Mlet Fr13 presented mainly terpenes (kaurenoic and grandifloric acids) in its composition, which are described in the literature as having an anti- inflammatory activity.[11] Although this fraction showed a tendency to reduce TNF-α levels in dystrophic muscle cells, this reduction was not deemed significant under our experimental conditions. This fraction did not present activity in the DPPH antioxidant assay, but it was active in the ORAC assay. Furthermore, it effectively reduced three enzymatic anti-oxidant defense system components, such as SOD-2, GR, and GPX1, showing a reduction in cellular oxidative stress; indicating that the anti-inflammatory effect of kaurenoic acid results mainly from a reduction in cellular oxidative stress, rather than the inactivation of pro-inflammatory enzymes, in the DMD model used. These terpenes, kaurenoic and grandifloric acids, are found in extracts of both *M. laevigata* and *M. glomerata* (Figure 1).

In comparison, fractions Mget-FR22 and Mlet-FR24 were more polar and contained mainly phenolic and caffeoylquinic acids (I to IV). These substances have known antioxidant activity[12,13] and presented strong activity in both the DPPH and ORAC assays. Furthermore, Mlet- Fr24 contained coumarin, which is often deemed responsible for the anti-inflammatory activity of

these species' extracts. Coumarin, by itself, was responsible for inhibiting carrageenan-induced paw edema, compound 48/80 paw edema and leukocyte migration.[10] However, the present results indicate that this effect is not due to direct inflammatory pathway inhibition, but rather is mediated by oxidative stress reduction; because the enzymatic antioxidant defense system components, such as SOD-2, GR, and GPX1, were reduced. Except for coumarin, found in high concentrations only in *M. laevigata,* varying proportions of the other phenolic compounds are found in both species.

CONCLUSION

Fractions of *M. laevigata* and *M. glomerata* containing phenolic compounds and coumarin effectively reduced antioxidant defense system components, such as SOD-2, GR, and GPX1, possibly due to direct antioxidant activity as indicated by the DPPH and ORAC *in vitro* tests. Moreover, even the fraction containing kaurenoic and grandifloric acids, which did not present an antioxidant effect in the DPPH assay and low activity in the ORAC test, also effectively reduced the same enzymes. None of these fractions significantly reduced TNF-α, NF-κB and IL-1β levels in the DMD model, indicating that the reported anti-inflammatory activity of these species is not related to direct inflammatory pathway inhibition, but rather mediated by oxidative stress reduction. Except for coumarin, found in high concentrations only in *M. laevigata*, varying proportions of the other phenolic compounds are found in both species` extracts and the terpenes, kaurenoic and grandifloric acids, are also present in both species. Therefore, it is clear that both species present therapeutic potential and coumarin is not exclusively responsible for this effect. These results highlight the importance of the antioxidant activity of natural products for the control of inflammation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

AAPH: [2,2-Azobis- (2-methylamidinopropane)-dihydrochloride]; **CCC:** Countercurrent Chromatography; **DMD:** Duchenne muscular dystrophy; **DMEM:** Dulbecco's Modified Eagle's Medium; **DPPH:** 2,2-diphenyl-1-picryl-hydrazyl; **DTNB:** 5′5′-dithio-bis-2- nitrobenzoic acid; **EDTA:** 2,2',2'',2'''-(Ethane -1,2-diyldinitrilo)tetraacetic acid; **ESI:** electrospray ionization source; **GPx:** Glutathione peroxidase; **GR:** Glutathione reductase; **GSH:** Glutathione; **HEMWat:** Hexane-ethyl acetate-methanol-water system; **4-HNE:** 4- hydroxynonenal; **HPLC:** High-Efficiency Liquid Chromatography; **IL-1β:** Interleukin-1β; **Mget-Fr22:** *M. glomerata* Fraction 22; **Mlet-Fr13:** *M. laevigata* Fraction 13; **Mlet-Fr24:** *M. laevigata* Fraction 24; **MTT:** [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; **NF-κB:** Nuclear factor kappa-B; **ORAC:** oxygen radical absorbance capacity; **ROS:** Reactive oxygen species; **SDS:** Sodium Dodecyl Sulfate; **SOD:** Superoxide dismutase; **TBST:** Tris Buffered Saline with Tween 20; **TLC:** thin-layer chromatography; **TNF-α:** tumor necrosis factor α; **UHPLC-MS:** Ultra High-Efficiency Liquid Chromatography- Mass Spectrometry.

SUMMARY

M. glomerata and *M. laevigata (guaco)* extracts are used indistinctly for asthma, cough and anti-inflammatory activity. As these species present different chemical composition, the specific components and the cellular mechanisms are not fully identified.Crude extracts and 3 selected fractions presented antioxidant capacity and reduced levels of antioxidant defense system enzymes.However, none of these fractions significantly reduced inflammatory enzymes.The results indicate that their anti-inflammatory activity may be mediated by oxidative stress reduction.Ethical ApprovalThe animal experiments described here were conducted in accordance with the guidelines of the Brazilian College for Animal Experimentation and the guidelines set forth by our institution. The protocol (#5754-1/2021,) was approved by the Committee on the Ethics of Animal Experiments of UNICAMP, São Paulo, Brazil.

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