UNIVERSIDADE VILA VELHA - ES

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

COMPOSIÇÃO QUÍMICA E PROPRIEDADE ANTI-INFLAMATÓRIA *IN VITRO* E *IN VIVO* DA *Renealmia petasites*

ALINE KELLER COUTO SOARES

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Dissertação apresentada à Universidade Vila Velha, como pré-requisito do Programa de Pós-graduação em Ciências Farmacêuticas, para a obtenção do grau de Mestra em Ciências Farmacêuticas.

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Banca Examinadora:

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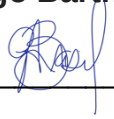

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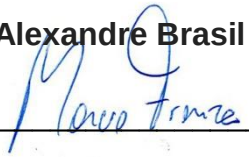

Dr. Marcio Fronza - UVV

Orientador

Dedico este estudo àqueles que mesmo sem saber me incentivaram a iniciar mais este desafio, a todos que contribuíram de forma consciente e inconsciente para mais esta realização.

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ABREVIATIONS

ABTS - 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)

AO – ostheoartrits

Cg - carrageenan

DMSO - Dimethyl Sulfoxide

DPPH - 2,2-Diphenyl-1-picrylhydrazyl

ELISA - Enzyme Linked Immuno Sorbent Assay

FRAP - Ferric Reducing Antioxidant Power

IC50 - Half maximal inhibitory concentration

IL-6 - Interleukin 6

LC/MS/MS – Liquid Chromatography coupled to Mass Spectometry

LPS - Lipopolysaccharide

MTT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

NBT - Nitroblue Tetrazolium Chloride

NF-κB - Nuclear Transcription Factor Kappa B

NO - Nitric Oxide

ROS – Reactive Oxigens Species

TNFα - Tumor Necrosis Factor Aplha

RESUMO

SOARES, Aline Keller Couto, M.Sc, Universidade Vila Velha- ES, abril de 2020. **Composição química e propriedade anti-inflamatória *in vitro* e *in vivo* da *Renealmia petasites.*** Orientador: Marcio Fronza.

Renealmia petasites (Zingiberaceae) é amplamente utilizado na medicina popular como terapia adjuvante no controle da dor e na redução da inflamação da osteoartrite. No entanto, os compostos bioativos e a relevância científica de suas propriedades farmacológicas ainda não foram revelados. O objetivo deste estudo foi investigar a composição química e a atividade anti-inflamatória dos extratos hidroetanólicos do rizoma, caule e das folhas de *R. petasites* utilizando ensaios *in vitro* e *in vivo*. A composição química do extrato foi caracterizada em um espectrômetro de massa de íons trap trap (Q-Trap). A quantificação de fenólicos, flavonóides e taninos foi realizada por análises espectrofotométricas. O sequestro do radical 2,2-difenil-1-picril-hidrazil (DPPH) e 2,2'Azino-bis (ácido 3-etilbenzotiazolina-6-sulfônico) (ABTS) e o poder antioxidante redutor férrico (FRAP) foram utilizados para estimar a atividade antioxidante. A atividade anti-inflamatória *in vitro* foi investigada em macrófagos estimulados por lipopolissacarídeos (LPS), avaliando a influência na produção de ânion superóxido (O2 • -), óxido nítrico (NO) e fator de necrose tumoral de citocinas pró-inflamatórias (TNF-α) e interleucina 6 (IL-6). Os efeitos *in vivo* foram determinados usando o modelo de bolsa de ar no qual foram inoculadas carragenina (Cg) e posteriormente tratadas com 50 mg / kg dos extratos hidroetanólicos de *R. petasites*. Após 4 e 24 h, foram avaliados o fluxo celular, exsudação de proteínas, citocinas e óxido nítrico. Oito compostos foram identificados nos extratos de R. petasites, sugerindo cinco diarilheptanóides, um flavonóide e dois álcoois graxos. Os resultados *in vitro* mostraram que os extratos foram capazes de bloquear os radicais livres e / ou inibir suas ações intracelulares, inibindo a produção de importantes mediadores do processo inflamatório, como óxido nítrico, ânion superóxido, TNFα e IL-6. *In vivo*, a atividade anti-inflamatória de *R. petasites* foi evidenciada pela redução significativa no influxo de leucócitos, principalmente neutrófilos, exsudação de proteínas, óxido nítrico, concentração de TNF-α e IL-6 na bolsa de inflamação induzida por Cg. Os resultados demonstraram cientificamente os efeitos farmacológicos associados ao uso popular de *R. petasites* e evidenciaram que a planta pode ser considerada uma terapia alternativa promissora para o tratamento e manejo da osteoartrite e outras doenças inflamatórias agudas e crônicas.

Palavras- chaves: modelo bolha de ar; osteoartrite; inflamação; macrófagos; citocinas

ABSTRACT

SOARES, Aline Keller Couto, M.Sc. University Vila Velha- ES, april 2020. **hemical composition and *in vitro* and *in vivo* anti-inflammatory properties of *Renealmia petasites***. Advisor: Marcio Fronza.

Renealmia petasites (Zingiberaceae) are widely used in the folk medicine as an adjuvant therapy of managing the pain and reducing inflammation of osteoarthritis. However, the bioactive compounds and the scientific relevance of its pharmacological properties are still unrevealed. To The aim of this study was to investigate the chemical composition and the anti-inflammatory activity of the hydroethanolic rhizomes, stems and leaves extracts of *R. petasites* using *in vitro* and *in vivo* assays. Chemical composition of the extracts characterized in a linear trap ion mass spectrometer (Q-Trap). Quantification of phenolics, flavonoids and tannins were done by spectrophotometry analyses. Scavenging radical of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and ferric reducing antioxidant power (FRAP) were used to estimate the antioxidant activity. *In vitro* anti-inflammatory activity was investigated in lipopolysaccharide (LPS) stimulated macrophages evaluating the influence on the production of superoxide anion ($O_2^{\bullet -}$), nitric oxide (NO) and the pro-inflammatory cytokines tumor necrosis factor (TNF-α) and interleukin 6 (IL- 6). *In vivo* effects was determined using the air pouch model in which were inoculated carrageenan (Cg) and thereafter treated with 50 mg/kg of the hydroethanolic extracts of *R. petasites*.

After 4 and 24 h the cellular influx, protein exudation, cytokines and nitric oxide were evaluated. Eight compounds were identified in the *R. petasites* extracts, suggesting five diarylheptanoids, one flavonoid and two fatty alcohols. The *in vitro* results showed that the extracts were capable of blocking free radicals and/or inhibiting their intracellular actions by inhibiting the production of important mediators of the inflammatory process, such as nitric oxide, superoxide anion, TNF-α, and IL-6. *In vivo*, the anti-inflammatory activity of *R. petasites* was evidenced by the significantly reduction into the influx of leukocytes, mainly neutrophils, protein exudation, nitric oxide, TNF-α and IL-6 concentration in the Cg induced inflammation air pouch. The results demonstrated that the pharmacological effects associated with the popular use of *R. petasites* has being scientific demonstrated and evidenced that the plant can be considered a promising alternative therapy for the treatment and management in osteoarthritis and other acute and chronic inflammatory diseases.

Keywords: air pouch; osteoarthritis; inflammation; macrophages; cytokines.

Artigo cientifico

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Chemical composition and *in vitro* and *in vivo* anti-inflammatory properties of *Renealmia petasites*

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ABSTRACT

Ethnopharmacological relevance: *Renealmia petasites* (Zingiberaceae) are widely used in the folk medicine as an adjuvant therapy of managing the pain and reducing inflammation of osteoarthritis. However, the bioactive compounds and the scientific relevance of its pharmacological properties are still unrevealed. **Aim of study:** To investigate the chemical composition and the anti-inflammatory activity of the hydroethanolic rhizomes, stems and leaves extracts of *R. petasites* using *in vitro* and *in vivo* assays. **Materials and Methods:** Chemical composition of the extracts was characterized in a linear iron trap mass spectrometer (Q-Trap). Total phenolic, flavonoid and tannin content were determined by spectrophotometry analyses. Scavenging radical of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and ferric reducing antioxidant power (FRAP) were used to estimate the antioxidant activity. *In vitro* anti-inflammatory activity was investigated in lipopolysaccharide (LPS) stimulated macrophages evaluating the influence on the production of superoxide anion ($O_2^{\bullet-}$), nitric oxide (NO) and the pro-inflammatory cytokines tumor necrosis factor (TNF-α) and interleukin 6 (IL- 6). *In vivo* effects were determined using the air pouch model in which were inoculated carrageenan (Cg) and thereafter treated with 50 mg/kg of the hydroethanolic extracts of *R. petasites*. After 4 and 24 h the cellular influx, protein exudation, cytokines and nitric oxide were evaluated. **Results:** Eight compounds were tentatively identified in the *R. petasites* extracts, suggesting five diarylheptanoids, one flavonoid and two fatty alcohols. The *in vitro* results showed that the extracts were capable of blocking free radicals and/or inhibiting their intracellular actions by inhibiting the production of important mediators of the inflammatory process, such as NO, $O_2^{\bullet-}$, TNF-α, and IL-6. *In vivo*, the anti-inflammatory activity of *R. petasites* was evidenced by the significantly reduction into the influx of leukocytes, mainly neutrophils, protein exudation, NO, TNF-α and IL-6 concentration in the Cg induced inflammation air pouch. **Conclusions:** The results demonstrated that the pharmacological effects associated with the popular use of *R. petasites* has being scientific demonstrated and evidenced that the plant can be considered a promising alternative therapy for the treatment and management in osteoarthritis and other acute and chronic inflammatory diseases.

Keywords: diarylheptanoids; air pouch; osteoarthritis; inflammation; macrophages.

ABREVIATIONS

ABTS - 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)

Cg - carrageenan

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NBT - Nitroblue Tetrazolium Chloride

NF-κB - Nuclear Transcription Factor Kappa B

NO - Nitric Oxide

OA - osteoarthritis

$O_2^{\bullet-}$ - superoxide anion (nitric

ROS - Reactive Oxigens Species

TNF-α - Tumor Necrosis Factor Alpha

1. INTRODUCTION

Osteoarthritis (OA) is the most common form of arthritis and affects mainly the hips, knees, hands and feet. Currently, the prevalence of OA is about 33% in the Brazilian population aged between 25 and 74 years old. The gender distribution in the population of Brazilians with OA was 40.2% for men and 59.8% for women. According to the age range, the distribution was 63.7%, 26.8% and 9.5% in the age groups >60 years, 41-60 years and <40 years, respectively (Coimbra et al., 2019). This is an alarming data, considering disability, loss of quality of life and the costs to the health system generated by this disease (Hunter and Bierma-Zeinstra, 2019).

Nowadays, OA is considered a low-grade inflammatory disease affecting the entire joint with progressive evolution to the destruction of articular cartilage. Scientific evidences indicates that OA is a multifactorial disease caused by a combination of factors producing a joint disorder as a whole, in which inflammation plays a key role in this pathogenic process (Ahmad et al., 2020; Robinson et al., 2016; Zhang et al., 2020). During the development of OA, macrophages are considered key elements for their pathogenesis. Previous studies have shown that activated macrophages were present in the majority (76%) of patients investigated with OA. The presence of pain in the joints of the fingers, wrists, ankles and toes was also significantly greater and directly correlated with the presence of activated macrophages in these locations (Kraus et al., 2017). High concentration of macrophages in patients with knee OA was also reported, and evidenced by the presence of biomarkers which could mediate structural progression (CD163 and CD14) and pain (CD14) in these patients (Scanzello et al., 2018). Moreover, therapies focus on macrophages targeting have demonstrated its ability to decrease inflammation as evidenced by the reduction of a wide variety of inflammatory mediators as interleukin 1β (IL-1β), tumor necrosis factor alpha (TNF-α), IL-8, IL-6, prostaglandin E2 (PGE_2) and oxide nitric acid (NO) and decrease on collagen synthesis followed by increase of catabolic mediators as metalloproteinases (MMPs) (Zhang et al., 2020).

Therefore, proper regulation of these mechanisms is essential to avoid uncontrolled amplification of the initial inflammatory response and a shift from tissue repair to collateral damage and disease development (Fernandes et al., 2020; Zhang et al., 2020). Currently, there is no pharmacological treatment able to reverses the

pathological process of osteoarthritis. However, many other alternatives, as the adequately dosed of aerobic exercises (Wellsandt and Golightly, 2018), acupuncture (Li et al., 2019) and the use of natural products like curcumin (Shep et al., 2019) and *Arnica montana* (Kriplani et al., 2017), have being considered important features to control the symptoms and improve the quality of life of these patients.

In the light of this scenery and based on the historical folkloric use of *Renealmia petasites* to treat OA, associated with the needs for the development of new therapeutically approach, this study was undertaken. The genus Renealmia are composed of 87 species widely distributed in the neotropical region around the world. In Brazil, it is represented by 21 species, being registered in all regions of the country, and only few species have been reported to be of ethnobotanical use and object of study regarding its chemical composition and/or pharmacological properties (Maas, 1977; Negrelle, 2015). The specie *Renealmia petasites* (Zingiberaceae) is popularly known in Brazil as “pacova” or “bananeira do mato” and it is mainly found in southern and south-eastern regions of the country (Negrelle, 2015). Decoction from rhizomes and aerial parts of *R. petasites* has been widely used in the folk medicine to alleviate the pain and reduce inflammation of osteoarthritis. The infusion of the seeds is referred as antidisenteric and against infertility. Infusion from the leaves, seeds, flowers and rhizomes are also reported as carminative medicine (Maas, 1977; Negrelle, 2015).

In this context, based on the scientific evidences addressing the involvement of macrophages in OA associated and the remarkable increase of the use of plant natural products as a medical practice in the managing of OA, our study aimed to characterize the chemical composition and to investigate the anti-inflammatory activity of the hydroethanolic rhizomes, stems and leaves extracts of *R. petasites in vitro,* using a murine macrophage cell line (RAW 264.7) and *in vivo,* using a murine air pouch model.

2 MATERIALS AND METHODS

2.1 *Chemical and biochemical reagents*

Carrageenan (Cg) agar-agar, lipopolysaccharide (LPS) from Escherichia coli O111:B4 (L630), fetal bovine serum, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), nitroblue tetrazolium chloride (NBT), gallic acid, and quercetin were obtained from Sigma Aldrich Chemical Co., St. Louis, MO, USA. Ketamine and xylazine were purchased from

Vetbrands (Paulinia, SP, Brazil). Immunoenzymatic assay (ELISA) kits for detection of cytokine TNF-α and lL-6 were obtained from eBioscience, San Diego, California, USA. All other solvents and reagents used were analytical grade and were obtained from various commercial sources.

2.2 *Cell lines*

RAW 264.7 mouse macrophages (ATCC® TIB-71™), L929 fibroblast (ATCC® CCL-1™) and murine hepatoma (Hepa 1c1c7) cells (ATTC® CRL-2026™) were obtained from the Cell Line Service, Rio de Janeiro, Brazil. The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM from Gibco-BRL Life Biotechnologies, Grand Island, NY, EUA) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin, at 37 °C, in a humidified atmosphere containing 5% CO_2.

2.3 *Animals*

The *in vivo* experiments were conducted in accordance with the Brazilian Committee for animal care and use (COBEA) guidelines. Male Swiss mice (*Mus musculus*), with 8 weeks old, weighing between 35 and 40 g, were used in the air pouch mouse experiments. Animals were supplied by the Central Animal Facility of University Vila Velha, Vila Velha, Brazil and were maintained under standard laboratory conditions with controlled temperature (22 ± 3 °C), humidity (40–60%), alternating light cycles every 12 hours, and with food and water provide ad libitum. All experiments were approved by local ethics committee (CEUA-UVV 498/2018).

2.4 Sampling and identification of plant material

The entire plant of *Renealmia Petasites*, including rhizomes, stems and leaves, was collected in the municipality Guarapari (20°36'08.7"S 40°30'32.6"W), Espirito Santo State, Brazil, in May 2018. The plant was taxonomically identified by the botanist Ms Solange Schneider and a voucher specimen (UVVES-2737) was deposited in the herbarium of Universidade Vila Velha, Brazil.

2.5 *Preparation of plant extracts*

Rhizomes, stems and leaves of *R. petasites* were selected, washed and dehydrated in a ventilated oven at 40 °C, until constant weight. Subsequently, each part of the plant was pulverized in a knife mill. The air-dried and grounded rhizomes, stems and leaves (20 g) were firstly defatted with hexane (150 mL) and thereafter subjected to the ultrasonic assisted maceration process employed three cycles of 30

min each with 150 mL of ethanol:water (80:20) as solvent. After extraction, the solvent was removed using a rotary evaporator (Fisaton 801, São Paulo, Brazil) and further subjected to the lyophilisation processes. The obtained extracts were stored in a freezer at -20ºC until chemical analyses and biological tests.

2.6 *Chemical composition of the hydroethanolic extracts of R. petasites*

The identification analyses of the main secondary metabolites in the hydroethanolic rhizomes, stems and leaves extracts of *R. petasites* were carried out through high-performance liquid chromatography coupled to a LCQ Fleet Ion Trap ThermoFisher Scientific mass spectrometer (Waltham, MA, USA) as previously described with modifications (Lorençoni et al., 2020). Briefly, the chromatographic conditions of the analysis temperature was changed to 40 °C and the mobile phase (0.1% formic acid solution (A) and methanol (B)) gradient elution was carried out as follows: 0-5 min (10% B); 5-50 min (10-95% B); 50-55 min (95% B); 55-56 min (95-10% B) and 56-60 min (10% B). In turn, any mass spectrometer parameters were also changed, as follows: capillary voltage, -1 V; capillary temperature, 400 °C; source voltage, 5,50 kV; sheat gas, 35 (arb); aux. Gas, 10 (arb). The results were processed using MzMine, generating feature peak lists in .mgf extension and dereplication was executed after data loading on the Global Natural Products Social Molecular Networking (GNPS) spectra platform (Wang et al., 2016).

2.7 Assay for total flavonoid content

The total flavonoid content was determined by spectrophotometric method after reaction with aluminium chloride as previously described (Bahiense et al., 2017). The total flavonoid content was calculated from a calibration curve using quercetin (1-12 µg/mL) as a standard and the results are expressed as g of quercetin equivalent (QE) per 100 g of dry weight plant extract.

2.8 *Assay for total phenolic and tannin content*

To quantify the total phenolic content in the *R. petasites* extracts, the Folin-Ciocalteau spectrophotometric method was used, as previously described (Bahiense et al., 2017; Krepsky et al., 2012). The determination of tannins in the extracts was based on their precipitation properties in the presence of polyvinyl polypyrrolidone (PVPP), as previously described (Bahiense et al., 2017). 100 mg PVPP were weighed and added to 1% methanolic solution of *R. petasites* extracts. This mixture was then homogenized, incubated for 10 min at 4 °C and then centrifuged for 10 min at 800 *g*. The tannin content was estimated as the difference between the total phenolic and

non-tannin phenolic content in the extract. The total phenolic and tannin content was expressed in grams of gallic acid equivalents (GAE)/100 g of crude extract.

2.9 *Free radical scavenging capacity of the R. petasites extracts*

The antioxidant activity of the *R. petasites* extracts were assayed by the colorimetric methods of DPPH free-radical scavenging (Scherer and Godoy, 2009), ABTS free-radical scavenging (Re et al., 1999) and ferric ion reducing antioxidant power (FRAP) (Benzie and Strain, 1996). Gallic acid and quercetin were used as positive controls. The experiments were executed in triplicate (n=3) and the results were expressed as IC_{50} (µg/mL).

2.10 *In vitro cytotoxicity assay*

In vitro cytotoxicity of the *R. petasites* extracts were evaluated by the standard colorimetric MTT assay with modifications (Marques et al., 2017; Mosmann, 1983). Briefly, 150 µL of RAW 264.7 (macrophage), L929 (fibroblast), and/or Hepa 1c1c7 (hepatoma) cell suspension were seeded in 96 well flat bottom plates (7×10^4 cells/mL). After overnight incubation (37 °C at 5% CO_2) cells were exposed to different concentrations (12.5 - 400.0 µg/mL) of the *R. petasites* extracts and *In vitro* cytotoxicity of the *R. petasites* extracts were evaluated by the standard colorimetric MTT assay with modifications (Marques et al., 2017; Mosmann, 1983). Briefly, 150 µL of RAW 264.7 (macrophage), L929 (fibroblast), and/or Hepa 1c1c7 (hepatoma) cell suspension were seeded in 96 well flat bottom plates (7×10^4 cells/mL). After overnight incubation (37 °C at 5% CO_2) cells were exposed to different concentrations (12.5 - 400.0 µg/mL) of the *R. petasites* extracts and camptothecin (10 µM), which was used as a positive control. After incubation (24 h, 37 °C) in CO_2 incubator, the supernatant was removed, and 100 µL of 3-(4,5-dimethylthiazol-2yl)-diphenyl tetrazolium bromide (MTT) (1 mg/mL) was added to each well and incubated again for 2 h. Then the MTT solution was carefully aspirated and DMSO (100 µL) was added with agitation (5 min) in an orbital shaker. The spectrophotometer was used at 540 nm for absorbance. The results were expressed in percent viability and the experiments were performed in triplicate (n=2).

Which was used as a positive control. After incubation (24 h, 37 °C) in CO_2 incubator, the supernatant was removed, and 100 µL of 3-(4,5-dimethylthiazol-2yl)-diphenyl tetrazolium bromide (MTT) (1 mg/mL) was added to each well and incubated again for 2 h. Then the MTT solution was carefully aspirated and DMSO (100 µL) was added with agitation (5 min) in an orbital shaker. The spectrophotometer was used at

540 nm for absorbance. The results were expressed in percent viability and the experiments were performed in triplicate (n=2).

2.11 *In vitro intracellular measurement of superoxide radical*

Superoxide radical was measured by the colorimetric nitrotetrazolium blue chloride (NBT) reduction assay (Lorençoni et al., 2020; Marques et al., 2019). Macrophages RAW 264.7 (8×10^4 cells/mL) were seed in 96-well plates and exposed to different concentrations (1, 10, 50 and 100 µg/mL) of the extracts for one hour followed by 1 µg/mL LPS for additional 20 h. Tempol (12.5 mM) was used as positive control. After incubation, 50 µL of a NBT solution at 1 mg/mL was added and incubated (5% CO_2 at 37ºC) for 1 h. Then, the cells were washed and lysed with KOH (2M):DMSO(1:1) and the absorbance of reduced NBT, formazan, was measured at 630 nm, in a microplate reader (Multi-Mode Microplate Reader, Filter Max F5, Molecular Devices Spectra, USA). The experiments were performed in triplicate (n=2) and the results were expressed as a percentage of the control without LPS.

2.12 *Carrageenan-induced air pouch experimental protocol*

The subcutaneous mouse air pouch experimental protocol and design were performance as previously described (Bahiense et al., 2017; Duarte et al., 2016). During experimental procedures, animals were anaesthetized with ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). After trichotomy and asepsis, the dorsal cervical region of the mice, the air pouch was generated via subcutaneous injection of 3 mL of sterile air, with a 25G needle through a sterile filter type Millex® GV 22 µm. After three days, a new injection of 2 mL of sterile air was applied into the pre-formed air pouches. Six days after the first injection, the animals (n=6/group) were pre-treated with 50 mg/kg of rhizomes, stems and leaves extract of *R. petasites*, indomethacin (5 mg/kg, positive control) or PBS (negative control) administered directly into the pouches (0.5 mL/pouch). One hour after the treatments, a solution containing carrageenan at 1% concentration was injected directly into the air pouch (0.5 mL/pouch) in all groups, except in the control group that receive only sterile PBS. 4 or 24 h after carrageenan injection the animals were euthanized with an overdose of the chemical anesthetics ketamine and xylazine (2-3 times the anesthetic dose), and the pouches were washed with 2 mL of sterile PBS containing 3.6% sodium citrate. The lavage fluid collected from each mouse air pouches was used for total and differential-cell count, and analyses of protein, nitrite and cytokine content.

2.13 *Total and differential leukocyte counting into the air pouches*

Total cells were counted with Turk's solution in every air pouch lavage fluid using a Neubauer chamber. Differential leukocyte counts were performed on cytospin preparations stained with using the kit for fast staining in haematology (Fast Panoptic - Instant Prov®) (Fronza et al., 2014).

2.14 *Protein quantification*

Total protein present in the cell-free fluid collected from each mouse air pouch was quantified by the colorimetric method using a commercial Pierce® kit (Labtest Diagnóstica - Lagoa Santa, MG, Brasil), according to the manufacturer's specifications.

2.15 *Quantification nitric oxide (NO)*

Nitrite (NO_2^-) was quantified in the supernatant of macrophage cell culture and in the cell-free fluid from the air pouch cavities as an indicator of nitric oxide (NO) production as previously described (Green et al., 1982; Marques et al., 2017). Briefly, macrophages (RAW 264.7) were exposed to different concentrations of the extracts (1, 10, 50 and 100 µg/mL) for one hour and then stimulated with LPS (1 µg/mL). L-NIL (50 µM) was used as a positive control. After 20 h, the cell supernatant and the cell-free fluid leakage of the mice air pouch cavity obtained was used for the quantification of nitrite using Griess reagent. Results expressed as mean ± SD of the nitrite concentration (µM) calculated by regression analysis of a standard curve of sodium nitrite (0-200 µM) by colorimetric measurements at 540 nm in an ELISA plate reader (Molecular Devices Spectra MAX 190).

2.16 Enzyme linked immunosorbent assay (ELISA)

ELISA Kits, according to the manufacturer's instructions (eBioscience, San Diego, California, USA) was used to measure the TNF-α and IL-6 production in the obtained cell-free lavage fluid of the mice air pouch cavity, and in the supernatant of LPS-stimulated RAW 264.7 macrophages cell culture exposed to different concentrations of the extracts (1, 10, 50 and 100 µg/mL) and incubated for 20 h (5% CO_2 at 37ºC). Optical densities were measured at 450 nm in a microplate reader (Multi-Mode Microplate Reader, Filter Max F5, Molecular Devices Spectra, USA). Cytokine levels were expressed in pg and assay sensitivities was >10 pg/mL.

2.17 *Acute toxicity testing using Caenorhabditis elegans*

2.17.1 Caenorhabditis elegans strains, maintenance and synchronization

In vivo assays were performed on wild-type *Caenorhabditis elegans* N2 strains genteelly provided by Dra Solange Cristina Garcia from the Faculty of

Pharmacy, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil. Strain was maintenance on nematode growth medium (NGM) plates, seeded with *Escherichia coli* OP50 and incubated at 20ºC (Brenner, 1974). The synchronization to obtain *Caenorhabditis elegans* at the L1 stage was performed from the release of eggs from pregnant nematodes (Augusti et al., 2017; Rangsinth et al., 2019). The nematodes were collected in sterile tubes with a lysis solution containing 1% NaOCl and 0.25 M NaOH, mixed and then centrifuged (3 min at 600 *g*). Then the supernatant was removed and the pellet washed with sterile water. After, the eggs were resuspended in M9 buffer solution (0.02M KH2PO4, 0.04M Na2HPO4, 0.08M NaCl, and 0.001M $MgSO_4$) (Rangsinth et al., 2019) and plated in NGM medium without bacterial addition and incubated for 16 h for egg hatching and larval release (Augusti et al., 2017; Charão et al., 2015; Rangsinth et al., 2019).

2.17.2 Survival and Development Analysis

After synchronization, 2500 worms at the L1 stage were exposed to the hydroethanolic rhizomes, stems and leaves extracts of *R. petasites*, at 50 mg concentration for 30 minutes under constant homogenization. Larvae treated with sterile deionized water were used as control (Manalo et al., 2017). After exposure, the worms were washed with 0.5% NaCl for complete sample removal and then transferred to NGM plates seeded with *Escherichia coli* OP50 and incubated at 20 °C for 24h and 48h for determination of survival and development, respectively (Avila et al., 2012). The number of surviving nematodes in each plate was determined by visual count. The development of nematodes was evaluated 48 h after treatments by measuring body area (μ^2) of adult worms. The nematodes were observed by optical microscope (Leica DMLS) and the images recorded by digital camera attached to a light microscope (Model Leica Mikroscope Type 501095). Body area was measured in 30 worms from each group using AxioVision software (version 4.8.2.0 for Windows) (Augusti et al., 2017; Rangsinth et al., 2019; Soares et al., 2019).

2.18 ***Statistical analysis***

Statistical analyses were performed using the GraphPad software v 5.0 (San Diego, CA, 176 USA). Data were expressed as mean ± standard deviation (SD). For comparison of multiple groups, we performed one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. Values of $p < 0.05$ were considered significant.

3. RESULTS

3.1 *Chemical composition of hydroethanolic extract of R. petasites*

The hydroethanolic extract of *R. petasites* rhizomes, stems and leaves were analysed in both, negative and positive electrospray ionization modes with monitoring mass range m/z from 100 to 1000. These analyses generated base peak chromatograms (BPC) (Figure 1). The processing and comparison of the spectra obtained by LC-MS-MS from hydroethanolic extracts with the GNPS database led the tentatively identification of eight compounds (Table 1), only in negative ionization mode, suggesting five diarylheptanoids, one flavonoid and two fatty alcohols. The five identified diarylheptanoids are linear, which are characterized by a 1,7-diphenylheptane skeleton (Alberti et al., 2018). The compound that had a retention time of 11.20 min (**1**) has a m/z 345 ($[M-H]^-$) is a diarylheptanoids with four phenolic hydroxyl substituents, two in each aromatic rings, while the heptane moiety has an hydroxyl and ketone groups. Just like the compound (**1**) the compounds (**3**) and (**5**) have four phenolic hydroxyl groups. Considering the heptane moiety, the compound (**3**) has a beta unsaturated ketone and the compound (**5**) two acetoxy groups. In turn, the compound (**2**) m/z 329 ($[M-H]^-$), for deprotonated molecule, has 16 Da less than the compound (**1**), that indicate the absence of one phenolic hydroxyl substituent. Finally, the compound (**4**) is a diarylheptanoid with 3 phenolics hydroxyl groups, and its heptane moiety has a beta unsaturated ketone. The identified flavonoid consists of a trimethylated flavone (**6**), while the further identified compounds are fatty alcohols. The compound (**7**) is a long-chained saturated 22-carbon aliphatic alcohol, while the compound (**8**) is an acetate form of avocadyne a lipid with a long aliphaticchain, having a terminal unsaturation of either an acetylenic (alkyne) nature and multiple hydroxylations on the opposing end (Ahmed et al., 2018).

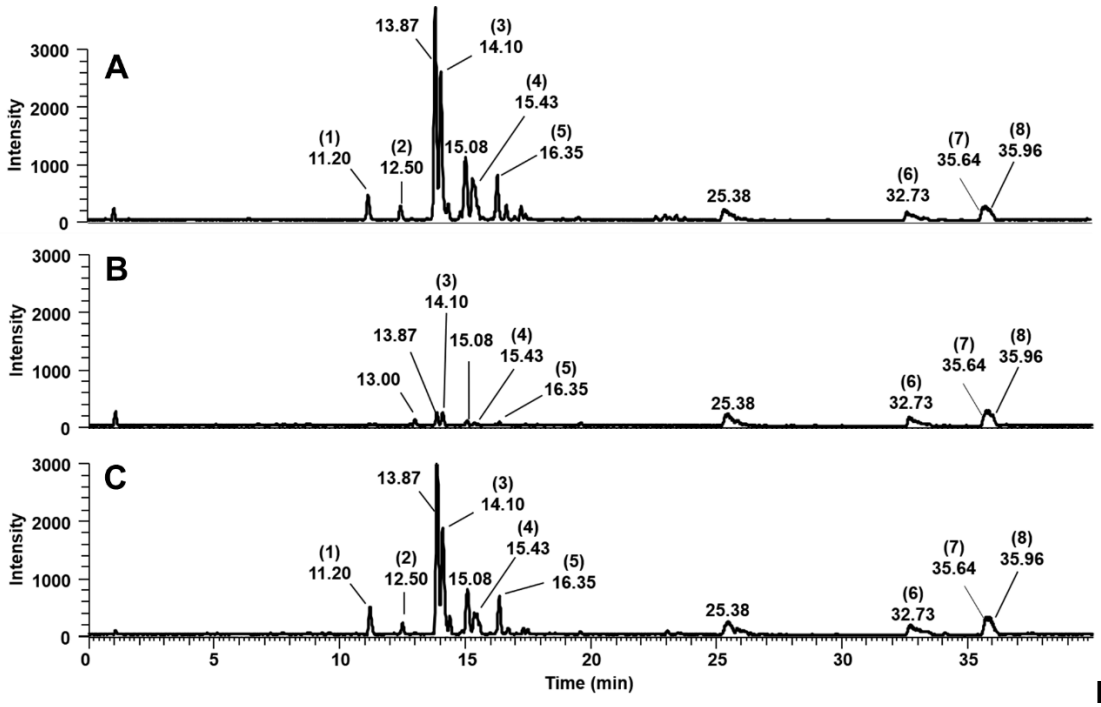

Figure 1. Representative base peak chromatograms of the hydroethanolic extract of *R. petasites* rhizomes (A), stems (B) and leaves (C) in negative ionization mode. Analytical conditions described in section 2.6.

Table 1. Compounds tentatively identified in the hydroethanolic extracts of *R. petasites*.

Number	Compound	t_R (min)	$[M - H]^-$	Fragment ions	Plant parts		
					Rhizomes	Stems	Leaves
1	1,7-bis(3,4-dihydroxyphenyl)-6-hydroxyheptan-3-one	11.20	345	327, 179, 165, 121	+	-	+
2	3-Heptanone, 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-(4-hydroxyphenyl)-	12.50	329	311, 179, 165, 121	+	-	+
3	Hirsutanone	14.1	327	205, 179, 147, 121	+	+	+
4	(E)-1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)hept-4-en-3-one	15.43	311	205, 189, 147, 121	+	+	+
5	[5-acetyloxy-1,7-bis(3,4-dihydroxyphenyl)heptan-3-yl] acetate	16.35	431	371, 311, 189, 163	+	+	+

6	5,6,2'-Trimethoxyflavone	32.73	311	247, 224, 183, 170, 119	+	+	+
7	Docosanol	35.64	325	261, 239, 225, 197, 183	+	+	+
8	Avocadyne acetate	35.96	325	308, 283, 239, 211, 183	+	+	+

t_R: retention time; $[M - H]^-$: deprotonated molecule; (+): compound presence; and (−) compound absence.

3.2 *Determination of flavonoid, phenolic and tannin content in the hydroethanolic extracts of R. petasites*

The quantitative analysis of flavonoid, phenolic and tannin content in the hydroethanolic extracts of *R. petasites* rhizomes, stems and leaves are demonstrated in Table 2. The highest total phenolic content and tannins was found in the rhizomes (33.76 ± 0.01 and 20.91 ± 0.01 g/100g of GAE, respectively) followed by the stems and leaves extracts. Flavonoid content was detected at higher concentrations in the leaves, exhibiting 1.48 ± 0.14 g/100g of QE.

Table 2. Quantification of flavonoid, phenolic and tannins in the hydroethanolic extracts of *R. petasites* rhizomes, stems and leaves.

Plant material	Flavonoid (g/100g)#	Phenolic (g/100g)*	Tannins (g/100g)*
Rhizomes	0.13 ± 0.02 [a]	33.76 ± 0.01 [a]	20.91 ± 0.01 [a]
Stems	0.01 ± 0.01 [b]	25.64 ± 0.02 [b]	17.91 ± 0.01 [a]
Leaves	1.48 ± 0.14 [c]	15.63 ± 0.01 [c]	3.22 ± 0.01 [b]

total flavonoid content is expressed as g of quercetin equivalent (QE)/100 g of dry weight plant extract. * Total phenolic and tannin content were expressed in g of gallic acid equivalents (GAE)/100 g of crude extract. Different letters in the same column correspond to significant differences ($p < 0.05$). Tests were performed in triplicate and expressed as mean ± standard error.

3.3 *In vitro evaluation of antioxidant properties*

The ferric reducing ability (FRAP), and the DPPH and ABTS assays were used to investigate the free radical scavenging capacities of *R. petasites* extracts *in vitro*. All hydroethanolic extracts exhibits a strong antioxidant activity. Among all three extracts obtained from different part of the plant, the rhizomes have lowest IC_{50} compared with the others, especial using the ABTS assay, suggesting that this part may be the most potential antioxidant extract.

Table 3. *In vitro* antioxidant activity of hydroethanolic extracts of *R. petasites* rhizomes, stems and leaves determined by DPPH and ABTS free-radical scavenging activity and by ferric reducing antioxidant power (FRAP).

		Antioxidant activity (IC_{50} µg/mL)		
Plant material		**ABTS**	**DPPH**	**FRAP**
Rhizomes		35.7 ± 10.3 [a]	72.9 ± 4.1 [a]	53.5 ± 5.1 [a]
Stems		51.2 ± 10.4 [b]	78.9 ± 12.2 [a]	51.6 ± 7.7 [a]
Leaves		53.3 ± 10.3 [b]	80.8 ± 8.3 [a]	47.9 ± 2.1 [a]
Quercetin		7.5 ± 1.2 [c]	6.9 ± 0.8 [b]	4.1 ± 0.6 [b]

Different letters in the same column correspond to significant differences ($p<0.05$) calculated by one-way ANOVA followed by Tukey´s post hoc test. Tests were performed in triplicate (n=3) and expressed as mean ± standard deviation.

3.4 *In vitro cellular viability*

The *in vitro* cytotoxicity of *R. petasites* extracts was tested against RAW 264.7 (macrophage), L929 (fibroblast) and Hepa 1c1c7 (hepatoma) cell lines using the MTT colorimetric method. No cytotoxic effects were observed for the hydroethanolic extract of rhizomes, stems and leaves in any of the tested cell lines up to 200 µg/mL (data not shown).

3.5 *In vitro inhibitory action on nitric oxide (NO) and superoxide radical ($O_2^{\bullet-}$) production in LPS-induced RAW 267.2 macrophages*

The NO production, in LPS-induced RAW 267.2 macrophages, was significant decreased after 50 and 100 µg/mL treatment with the hydroethanolic rhizomes, stems and leaves extracts of *R. petasites*. The suppression of approximately 50%, 42% and 51% in the nitrite production were observed after cellular exposure to rhizomes, stems and leaves extracts at 100 µg/mL concentration, respectively (Figure 2A). Likewise, reductions of about 44%, 33% and 27% in superoxide radical ($O_2^{\bullet-}$) concentration were observed after treatment with hydroethanolic rhizomes, stems and leaves extracts of *R. petasites* at 100 µg/mL concentrations, respectively (Figure 2 B).

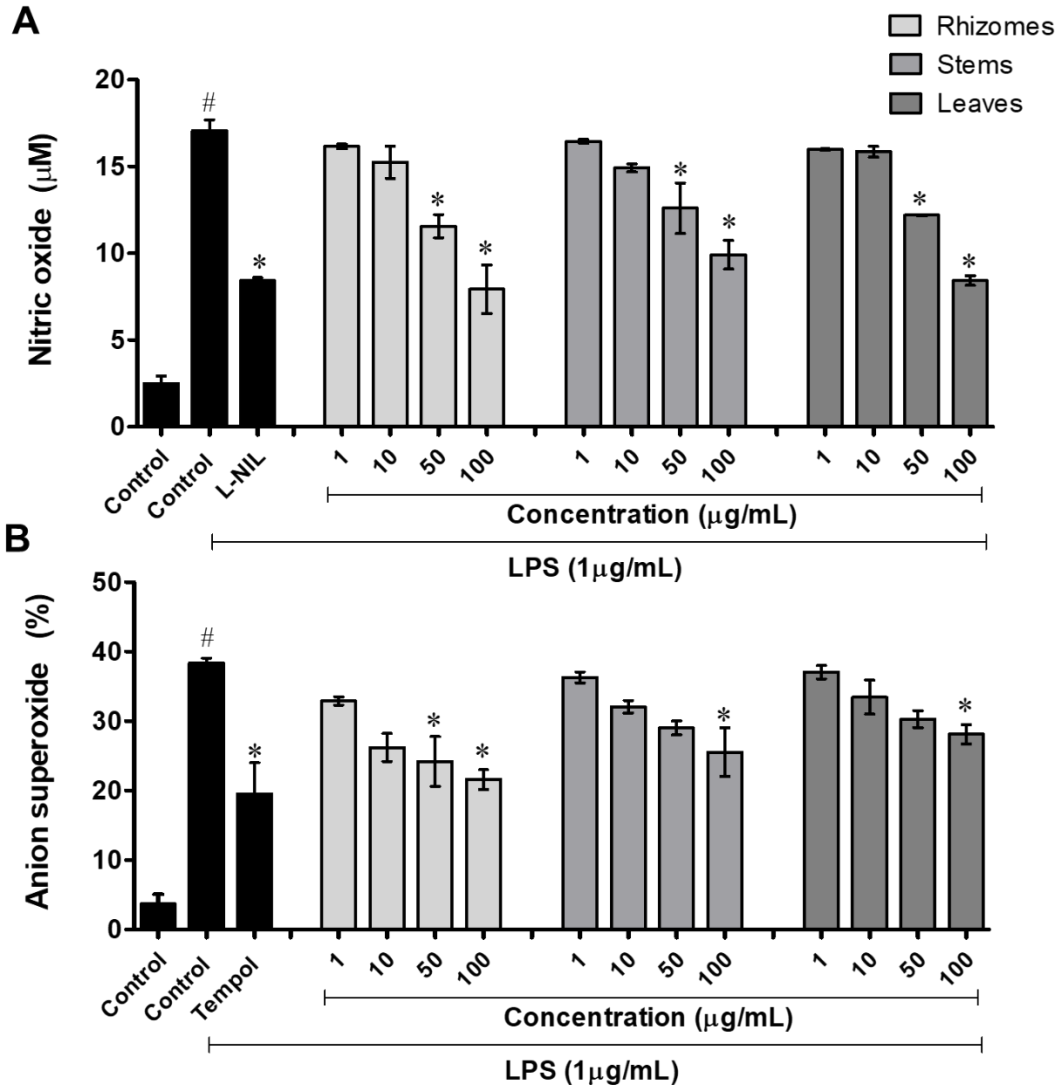

Figure 2. *In vitro* effect of the hydroethanolic rhizomes, stems and leaves extracts of *R. petasites* on the production of nitric oxide (NO) (A) and superoxide radical ($O_2^{\bullet-}$) (B). RAW 264.7 macrophages were exposed to 1, 10, 50 and 100 µg/mL of the extract and stimulated with LPS (1 µg/mL) for 20h. L-NIL (50 µg/mL) and (Tempol 12.5 mM) were used as positive control. The results were expressed as mean ± SD (n=2). #Significant ($p<0.05$) compared to the negative control without LPS. *Significant ($p<0.05$) compared to the control with LPS-induced cells by one-way ANOVA followed by Tukey´s post hoc test.

3.6 R. petasites extracts significantly inhibits IL-6 and TNF-α in vitro

The impact of the hydroethanolic rhizomes, stems and leaves extracts of *R. petasites* on the IL-6 and TNF-α production was evaluated in LPS-induced RAW 267.2 macrophages. All extracts significantly decreased the concentration of inflammatory cytokines (Figure 3). The rhizome extract showed the greatest inhibitory effect on the

production of TNF-α and IL-6 reaching a significant reduction of 87% and 80%, respectively in the concentration of these studied cytokines, after treatment with 100 µg/mL concentration (Figure 3).

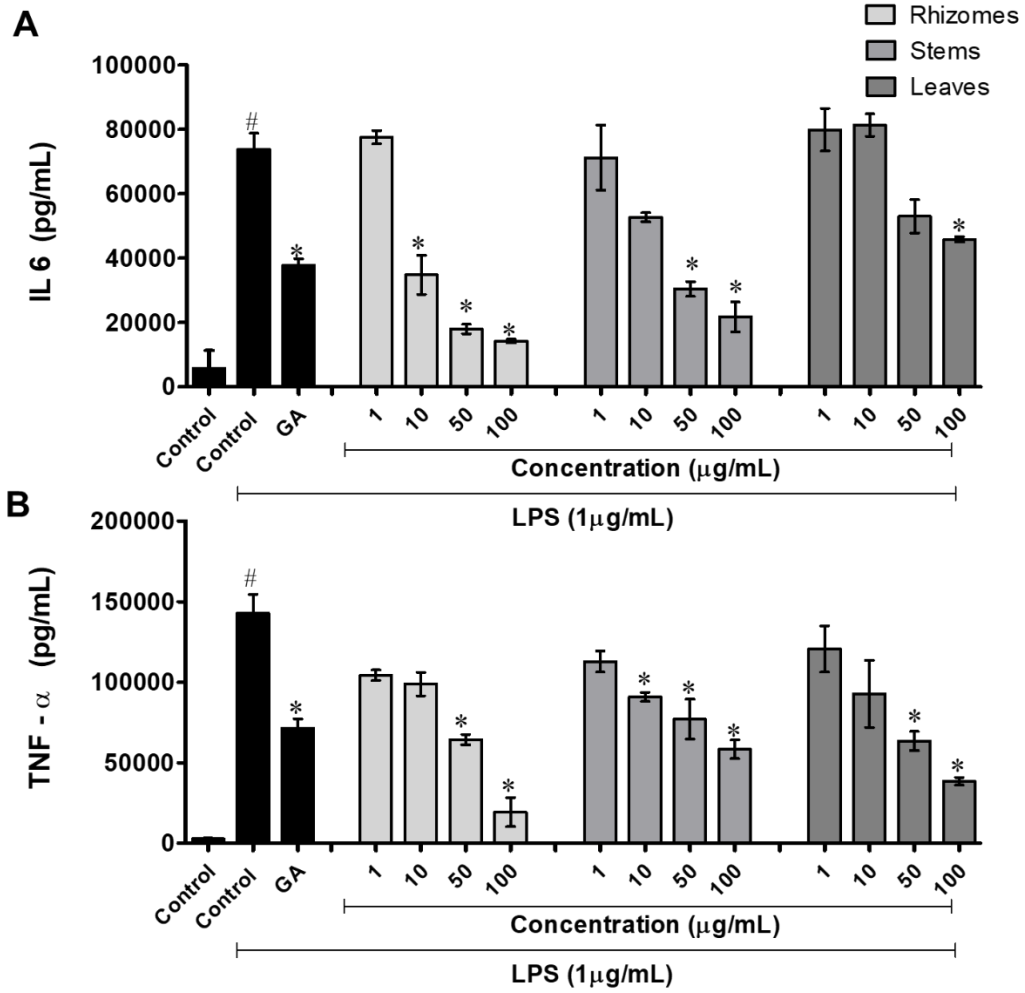

Figure 3. Effect of hydroethanolic rhizomes, stems and leaves extracts of *R. petasites* on the release of IL-6 (A) and TNF-α (B) in LPS‑stimulated RAW 264.7 cells. Cells were treated with LPS and/or the indicated concentrations of the extracts. Each value indicates the mean ± standard deviation from three independent experiments. # $p<0.05$ compared to untreated control cells; * $p<0.05$ compared to the cell treated with LPS alone.

3.7 R. petasites decreases leukocytes in carrageenan (Cg) inflamed air pouch

The animals that received only Cg directly into the pouches exhibited a massive recruitment leukocytes, neutrophils and mononuclear cells after 4 and 24 h, compared to PBS-inoculated air pouches (Figure 4). Such event highlights the establishment of the inflammatory process produced by Cg. On the other hand, treated mice with hydroethanolic rhizomes, stems and leaves extracts of *R. petasites* at 50

mg/Kg concentration one hour after Cg administration, exhibited a significant decrease in the cellular influx after 4 and 24h. Mice treated with 50 mg/Kg concentration of rhizomes, stems and leaves extracts of *R. petasites* promote reductions in total leukocyte of 79%, 60% and 62% after 4 h, and about 60% 65% and 62% after 24 h respectively, when compared to Cg control group (Figure 4A). This inhibitory effect was associated with a significant decrease in neutrophils (Figure 4 B) showing reductions of 60%, 57% and 81% after 4 h, and of 64% 65% and 78% after 24 h respectively, in the cellular influx into the air pouches treated with 50 mg/Kg concentration of rhizomes, stems and leaves extracts of *R. petasites.* As observed in Figure 4C, mononuclear cells recruitment was influenced only after 24 h with reduction of 80%, 67% and 96%, respectively. As expected, indomethacin (5 mg/kg) used as positive control, caused a significant inhibition of both total and differential leukocytes.

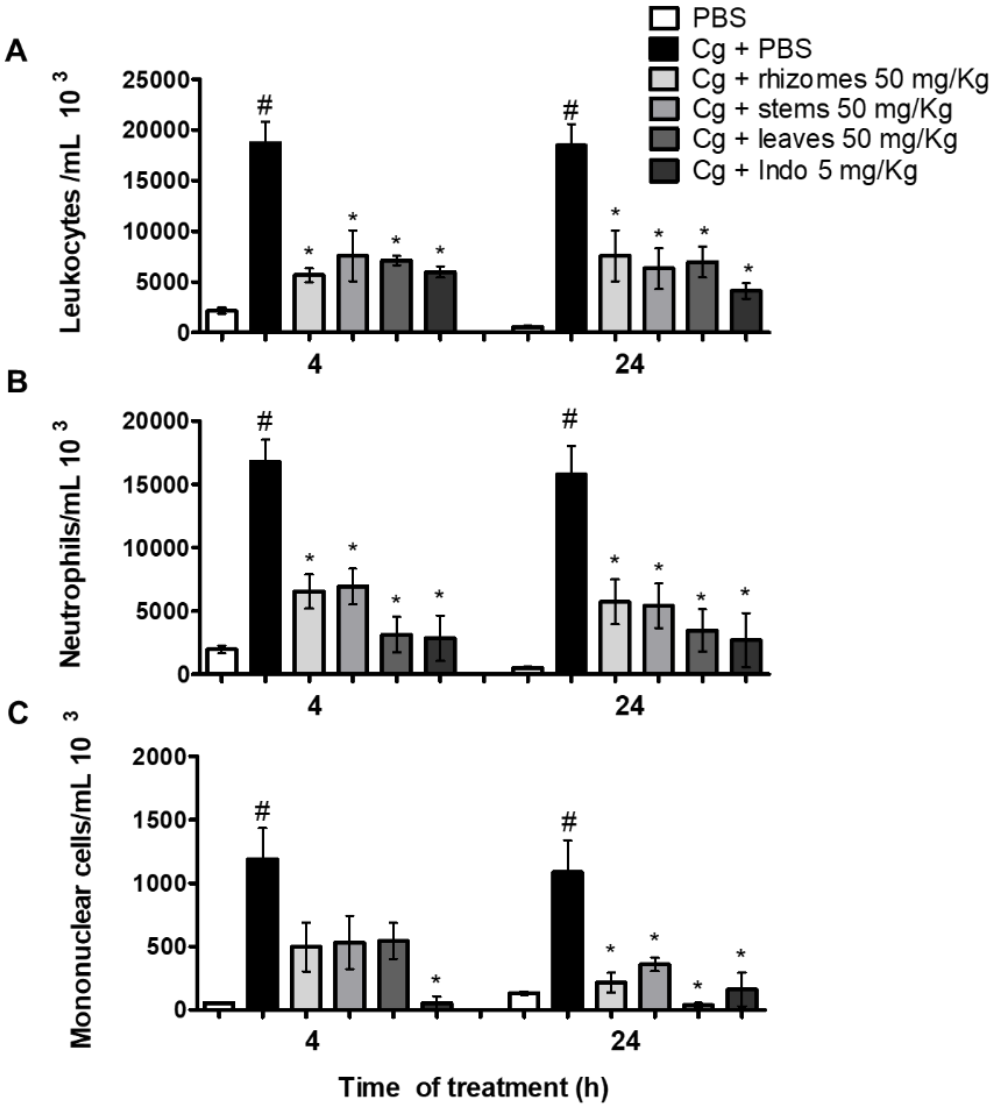

Figure 4. Effect of *R. petasites* on the influx of leukocytes in carrageenan (Cg) inflamed air pouch model. Phosphate buffered saline (PBS) or Carrageenan (Cg) were injected

directly into the pouches and one hour later treated with the rhizomes, stems and leaves extracts (50 mg/kg), indomethacin (5 mg/kg) or PBS. Total and differential cell numbers into the air pouch were determined 4 and 24 h after treatments. A) Total leukocytes; B) Neutrophils; and D) Mononuclear cells. Values express the mean ± S.E.M. (n=6 animal/group). Significantly different $p < 0.05$. * versus Cg + PBS and # versus PBS.

3.8 R. petasites reduced protein content into the air pouch

The cell-free fluid from the air pouch cavities was used to determine the total protein content after 4 and 24 h treatments (Figure 5). Rhizomes and stems extracts (50 mg/kg) treatment produce a non-significant reduction in the protein extravasation into inflamed air pouch after 4 and 24 h. However, the hydroethanolic leaves extracts promotes a significant reduction of proteins in the extravascular space compared to Cg group. Similar results were observed with indomethacin.

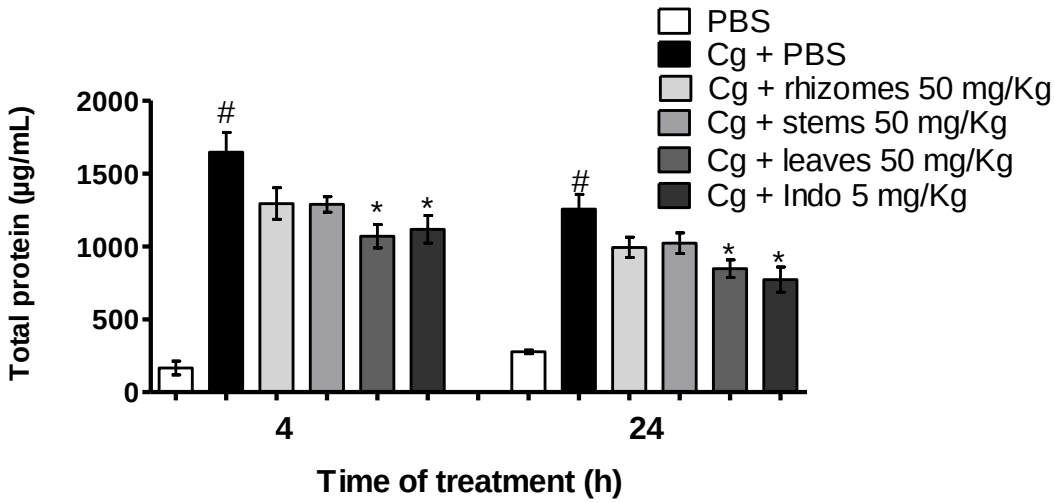

Figure 5. Effect of *R. petasites* on the protein extravasation into inflamed air pouches. The total protein content into the air pouch were determined after 4 and 24 h treatments with the rhizomes, stems and leaves extracts (50 mg/kg) indomethacin (5 mg/kg) or PBS. Values express the mean ± S.E.M. (n=6 animal/group). Significantly different $p < 0.05$. * versus Cg + PBS and # versus PBS.

3.9 R. petasites reduced pro-inflammatory mediators in the mouse model of air pouch

Nitrite (NO^{2-}) and the pro-inflammatory TNF-α and lL-6 cytokines was quantified in the cell-free fluid from the air pouch cavities after 4 and 24 h treatment with hydroethanolic rhizomes, stems and leaves extracts of *R. petasites* (50 mg/kg). As observed in Figure 6, the flogistic agent carrageenan (Cg) induced a significant

increase of these inflammatory mediators, which were significantly blocked by *R. petasists* extracts. The highest inhibition of NO, TNF-α and IL-6 was observed with the rhizomes extracts after 4 and 24 h.

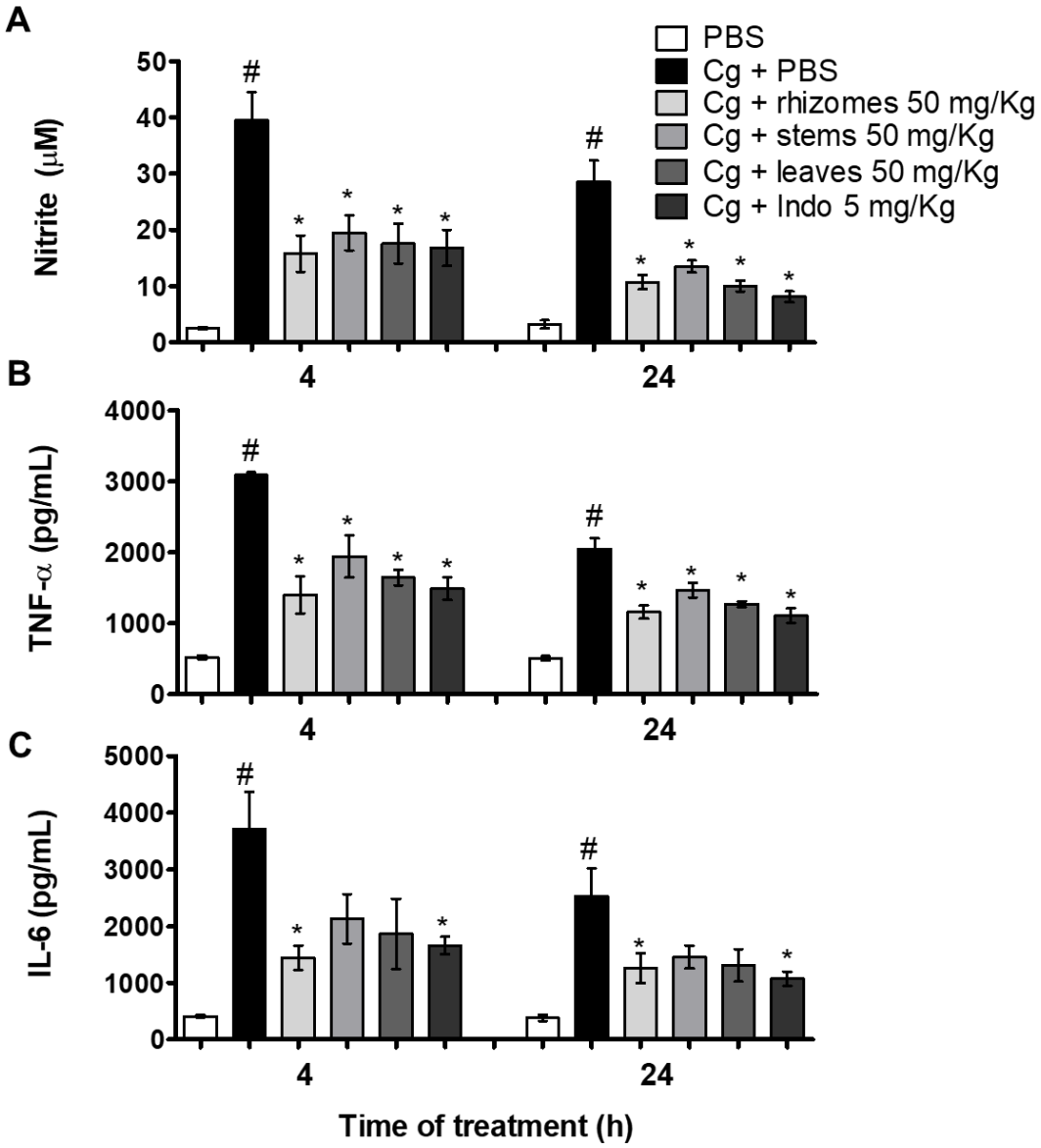

Figure 6. Effect of the rhizomes, stems and leaves extracts of *R. petasites* on the production of nitric oxide (NO) and TNF-α concentration in the air pouch model. Phosphate buffered saline (PBS) or Carrageenan (Cg) were injected directly into the pouches and one hour later treated with the rhizomes, stems and leaves extracts (50 mg/kg), indomethacin (5 mg/kg) or PBS. (A) NO, (B) TNF-α and (C) IL-6 production was measured 4 and 24 h later using Griess reagent and ELISA Kits, respectively, as described in material and methods section. Results are expressed as mean ± S.E.M. (n=6 animal/group). Significantly different $p < 0.05$. * versus Cg + PBS and # versus PBS.

3.10 R. petasites did not affect the survival and development of Caenorhabditis elegans

The acute toxicity test of hydroethanolic rhizomes, stems and leaves extracts of *R. petasites* was performed using the *in vivo C. elegans* experimental model. The effect of each extract was demonstrated by its influence on the survival rate and the development of worms (Figure 7). The extracts did not have detrimental effects on the survival rate of *C. elegans* strains. Treatments with rhizomes, stems and leaves extracts presented survival rate of 90.7 ± 5.3 %, 91.0 ± 8.9% and 98.5 ± 1.4% respectively, with no significant differences when compared to the control group (Figure 7A). Next, the influence of extracts on the development of worms also showed no difference when compared to the control group. The treatments with extracts rhizomes, stems and leaves extracts presented body area of 1451 ± 67.9 μm^2, 1521 ± 85.6 μm^2, and 1683 ± 131.9 μm^2 respectively, with no difference when compared to the control group (1652 ± 83.4 μm^2) (Figure 7B).

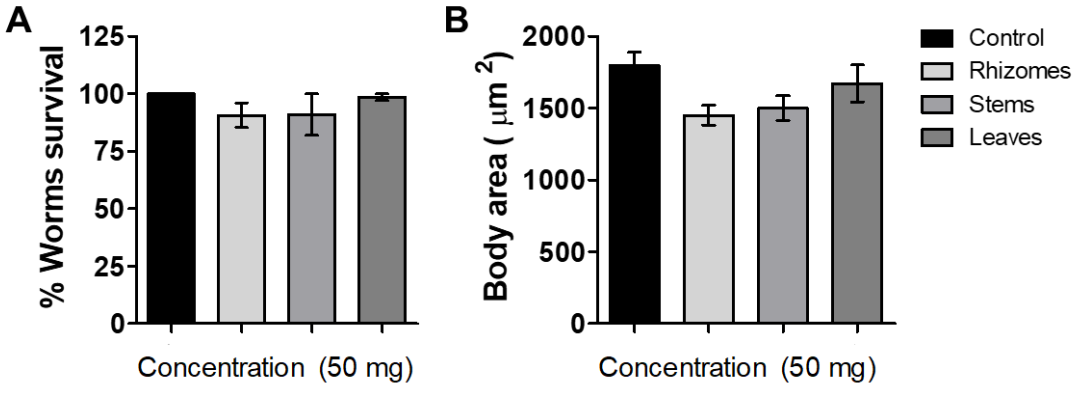

Figure 7. Effect of *R. petasites* on the survival (A) and development (B) of *Caenorhabditis elegans*. C. *elegans* worms at the L1 stage were exposed to the hydroethanolic rhizomes, stems and leaves extracts of *R. petasites* at 50 mg concentration for 24h and 48h for determination of survival and development, respectively, as described in material and methods. Values are expressed as mean ± SEM of 2 independent experiments.

4. DISCUSSION

The continuous research for new herbal medicines and plant-derived compounds as adjuvants in the treatment of chronic diseases, including osteoarthritis (OA), have drawn increasing attention in recent years, due to their anti-inflammatory activities and few side effects (Kriplani et al., 2017; Shep et al., 2019). OA is the most prevalent joint disease, and unfortunately, the existing therapeutic approaches are mainly symptomatic, without effective treatments to limit the disease progression. Thus, novel therapeutics are needed to inhibit the progress and pathology of OA. The growing knowledge about the inflammatory fundamentals and the low-grade inflammation as a key mediator of OA, keeps promise for the development of new promising anti-inflammatory strategy therapies (Fernandes et al., 2020; Robinson et al., 2016).

Reports on pharmacological properties of *R. petasites* are rare, however the rhizomes and aerial parts of *R. petasites* and other species are widely used in the folk medicine to alleviate pain and reduce inflammation (Maas, 1977; Negrelle, 2015). In this study, we could demonstrated that *R. petasites* exhibited *in vitro* antioxidant activity and significantly inhibited the production of inflammatory mediators, such as nitric oxide (NO), superoxide anion (O_2^-), TNF-α, and IL-6. Moreover, *R. petasites* also suppress the influx of leukocytes and decrease the concentration of NO, TNF-α and IL-6 in the Cg induced inflammation air pouch.

The effects presented by *R. petasites* extracts may be correlated with its phytochemical composition. The antioxidant capacity of the *R. petasites* extracts mediated by the ability to scavenge free radicals and other reactive oxygen species such as superoxide anions, nitric oxide, hydroxyl radicals, and hydrogen peroxide can be addressed to the polyphenolic compounds (Badhani et al., 2015; Kim et al., 2014). Indeed, many investigations have shown that these bioactive compounds could be therapeutically beneficial due to its antioxidant potential by preventing the oxidative stress and reducing the tissue damage which is consider the hallmark of several chronic disorders and cell death, including OA. Accumulating evidence suggested the ROS and NO, in particular, are considered the key factors to mediate chondrocyte apoptosis, cartilage destruction and cell death contributing to the pathogeneses and progression of OA (Ahmad et al., 2020; Fernandes et al., 2020; Robinson et al., 2016). Thus, the significant antioxidant activity exhibited by *R. petasites* provides the first insights for the beneficial properties of this plant. In agreement with our finds, the olive

polyphenols showed consistent evidences of its beneficial potential in preventing cartilage damage due to OA, and these effects were attributed to their antioxidant and anti-inflammatory effects (Chin and Pang, 2017). Likewise, antioxidant supplements have also shown benefits in pain relief and function in knee OA (Grover and Samson, 2015).

Without neglecting other chemical constituents, the presence of flavonoids and manly diarylheptanoids may be the major class of compounds responsible for the observed anti-inflammatory activity of *R. petasites*. The anti-inflammatory properties of diarylheptanoids have been attributed to the inhibitory activity on PGE_2, NO and TNF-α production (Ibrahim et al., 2017; Raju et al., 2019; Wohlmuth et al., 2010; Yao et al., 2018). The possible pathway for this effects are still not fully understood, but its known that such biological activity may be related to inhibition of cyclooxygenase and suppressing activities of NF-kB and AP-1 (Lai et al., 2012; Lee et al., 2005). Diarylheptanoids consists of two benzene rings linked by a linear C7-aliphatic chain with varying functional groups on the aryl and aliphatic moieties and, besides its promising anti-inflammatory activities, they showed a wide variety of biological activities, including the antioxidant, antitumoral, neuro-protective, estrogenic, anti-trypanosomal, antiviral, and leishmanicidal (Alberti et al., 2018).

Many studies describe the involvement of inflammation even in the early stages of development and progression of OA. Local inflammation response of the joint is marked by increased secretion of pro-inflammatory mediators such as tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-1β, and nitrite oxide (NO) which seems to be the main inflammatory mediators involved in the pathophysiology of OA. However, others cytokines including IL-15, IL-17, IL-18, IL-21, leukaemia inhibitory factor, and chemokines like CCL5, IL-8 and MCP-1 have also been implicated (Ahmad et al., 2020; Kapoor et al., 2011; Nees et al., 2019). Therefore, the significant impact of the hydroethanolic rhizomes, stems, and leaves extracts of *R. petasites* in LPS-stimulated macrophages and in the air pouch model of inflammation by reducing the pro-inflammatory cytokines production, regulating the leucocyte migration and edema formation, provide sustainable evidences that this herbal product could be useful to treat inflammatory diseases.

Although the *in vitro* studies have several advantages, they are criticized for being very different from the natural environment. Therefore, in order to provide a proof of concept for the anti-inflammatory activity of *R. petasites*, the air pouch model of inflammation was undertaken. This *in vivo* model is considered an acceptable animal

model for studying joint inflammation presenting many advantages such as capacity of evaluation the inflammatory cells responses and measurement of inflammatory mediators to a plant-based extracts or compounds at different time intervals (Duarte et al., 2016; Lu et al., 2020).

Similar to previous studies using the air pouch model to confirm that anti-inflammatory activities of natural compounds or plant-based remedies (Goldoni et al., 2019; Kim et al., 2017), the administration of *R. petasites* extracts into the air pouch after Cg inoculation resulted in decreased inflammatory cell infiltration and edema formation. It's known that polymorphonuclear cells (PMN), especially neutrophils, are rapidly mobilized during inflammation and are responsible to phagocyte and kill pathogens which leads to release of enzymes and ROS generation. Overwhelming infiltration of PMN and neutrophils can lead to uncontrolled inflammatory process and indiscriminate tissue damage, such as in rheumatoid arthritis (RA) (Duarte et al., 2016; Edwards et al., 1980; Lu et al., 2020). In this context, the reduction of leukocytes influx, mainly neutrophils, after *R. petasites* treatment, may represents a valid therapeutic strategy.

Many inflammatory mediators, such as IL-1β, TNF-α, IL-6, and NO and fragments of cartilage proteins including matrix metalloproteinases (MMPs), have been reported to be overexpressed in patients with OA (Kapoor et al., 2011; Robinson et al., 2016). IL-1β, TNF-α and IL-6 have been consider as pleiotropic cytokines regulating a wide range of physiological and pathological process, including cellular proliferation and differentiation, and death, as well as innate and immune responses (Hamidzadeh et al., 2017). These cytokines also contribute to excessive neutrophil accumulation leading to synovial inflammation which is another classical feature of OA progression (Fernandes et al., 2020). Data from *in vitro* and *in vivo* studies provide substantial evidence that blocking these mediators (TNF-α, IL-6 and NO) could be beneficial in counteracting the degradative mechanisms associated with OA pathology (Kapoor et al., 2011; Zhang et al., 2020).

The safety of new strategies to treat OA is also important. In this context, the analysis of acute toxicity carried out on nematode *C. elegans* showed that the extracts of *R. petasites* did not induce changes in the evaluated parameters. The nematode *C. elegans* has been widely used for toxicological evaluation of different substances (Charão et al., 2019; Soares et al., 2019). Natural products' prevention of oxidative damage in *C. elegans* has been reported as an important mechanism (Ruan

et al., 2016; Yang et al., 2019) and, therefore, justifying the absence of acute toxicity demonstrated by *R. Petasites*.

Data reported herein, based on the *in vitro* and *in vivo* experiments clearly demonstrated the effectiveness and safety of *R. petasites* in the modulation of production and release of these pro-inflammatory mediators involved in the development and progression of OA.

5. CONCLUSION

Summarizing, our results demonstrated that *Renealmia petasites* extracts, especially from the rhizomes, exhibited bioactive compounds capable to reduce the expression of reactive oxygen species, the production of pro-inflammatory mediators and to reduce the infiltration of leukocytes and edema formation, modulating the inflammatory process without exhibiting cytotoxic effects.

In conclusion, the popular use of *R. petasites* has being scientific demonstrated and evidenced that the plant can be considered a promising alternative therapy for the treatment and management in osteoarthritis and other acute and chronic inflammatory diseases. However, further studies are necessary to identify specific compounds and the associated biological activities, to understand the mechanism of action of *Renealmia petasites* extracts.

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CONFLICT OF INTERESTS

The authors have no conflicts of interest to report.

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