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Effect of *Arrabidaea Chica* Verlot Hydroalcoholic Extract on Monosodium Iodoacetate-Induced Osteoarthritis of Rat Knees

Efeito do Extrato Hidroalcoólico de *Arrabidaea chica* Verlot na osteoartrite induzida por Monoiodoacetato de Sódio em joelhos de ratos

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ABSTRACT
Introdution: The *Arrabidaea chica* Verlot (*A. chica*, ACV), with well-demonstrated anti-inflammatory properties, appears as an option with therapeutic potential for the osteoarthritis; thus, validating its use is highly relevant. Method: 72 rats were allocated to 3 groups: control, osteoarthritis and phytotherapy {these last two were subjected to osteoarthritis induction, and treated orally with 0.9% normal saline (0.1 mL/100 g) and ACV hydroalcoholic extract (500 mg/kg), respectively, from days 7 to 28}. The 3 groups were subjected to weekly (days 7, 14, 21, 28) assessments including clinical tests (weight-bearing and von Frey), radiological and histopathological analyses. Fractionation of the ACV´s hydroalcoholic extract was performed and it´s fractions were analysed. Results: The evaluation of the values of the osteoarthritis and phytotherapy groups showed significant difference, with $p < 0,05$: weight-bearing- on days 14 (29,64 x 35,52), 21 (32,62 x 42,53) and 28 (33,56 x 47,14), von Frey- on days 14 (31,12 x 37,80), 21 (30,24 x 41,48) and 28 (35,78 x 46,09), x-ray- on days 21 (2,17 x 1,20) and 28 (2,33 x 1,40), and histopathological analysis- on day 28 (0,03 x 2,20). The fractionation of the extract obtained the FH (hexane), FC (chloroform), FAE (ethyl acetate) and FB (butanolic) fractions. The FAE had highest total polypnenolic contents and the FH had the highest concentration of total flavonoids. Conclusion: The ACV extract promoted a reduction in static incapacitance, allodynia, radiological score and degree of synovitis, and FAE and FH fractions are probably the fractions responsible for the anti-inflammatory and analgesic activities of the ACV extract.

Keywords: Osteoarthritis, Knee, *Arrabidaea chica* Verlot, Rats, Pain.

RESUMO
Introdução: *Arrabidaea chica* Verlot (*A.chica*, ACV), com propriedade anti-inflamatórias bem demonstradas, aparece como uma opção com potencial terapêutico para osteoartrite, assim a validação de seu uso é altamente relevante. Metodologia: 72 ratos foram divididos em 3 grupos: controle, osteoartrite e fitoterapia {estes dois últimos foram submetidos à indução de osteoartrite e tratados oralmente com solução salina 0,9% (0,1mL/100g) e extrato hidroalcoólico de ACV (500 mg/Kg), respectivamente, dos dias 7 a 28 do estudo}. Os 3 grupos foram submetidos semanalmente (dias 7, 14, 21 e 28) a abordagem clínica

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(testes de weight-bearing e von Frey), radiológica e análise histopatológica. O extrato hidroalcoólico de ACV foi submetido a fracionamento e suas frações foram analisadas. Resultado: A avaliação dos valores dos grupos osteoartrite e fitoterapia mostraram diferenças estatísticas significantes, com p<0,05: teste de weight-bearing nos dias 14 (29,64 x 35, 52), 21 (32,62 x 42,53) e 28 (33,56 x 47,14), teste de von Frey nos dias 14 (31,12 x 37,80), 21 (30,24 x 41,48) e 28 (35,78 x 46,09), raio-X nos dias 21 (2,17 x 1,20) e 28 (2,33 x 1,40), e análise histopatológica no dia 28 (0,03 x 2,20). O fracionamento do extrato obteve as frações FH (hexânica), FC (clorofórmica), FAE (acetato de etila) e FB (butanólica). A fração FAE teve o maior conteúdo total de polifenóis e a FH teve a maior concentração total de flavonóides. Conclusão: O extrato hidroalcoólico de ACV promoveu redução na incapacitância estática, alodinia, escore radiológico e grau de sinovite, e as frações FAE e FH são provavelmente as responsáveis pelas atividades analgésicas e anti-inflamatórias do extrato.

Palavras-chaves: Osteoartrite, Joelho, *Arrabidaea chica*, Ratos, Dor.

1 INTRODUCTION

Osteoarthritis (OA) comprises a set of conditions involving signs and symptoms of joint pain associated with defects in the integrity of the joint and underlying bone (1), affects approximately 9.6% of men and 18% of women older than 60 (2) and accounts for billions of dollars spent every year in medications, therapies and surgery, as well as work absenteeism (3). The prevalence and high costs associated with osteoarthritis have awakened interest in obtaining a thorough knowledge of its pathophysiology. It is the result of a dynamic process characterized by coexisting destruction and repair triggered by biochemical and mechanical insult, activationof the imune system and production of cytokines and metalloproteinases that degrade the extracellular matrix (4).

Although articular cartilage is the main location of abnormalities in OA, fibrosis of the periarticular tissues and synovitis also occur (5). And, as cartilage doesn´t have innervation, the pain, wich can be devasting, limiting and which can compromise productivity and quality of life in patients with osteoarthritis, probably originates in the synovial membrane, involving central and peripheral modulation. Synovitis is probably caused by the release of cartilage matrix into synovial fluid, resulting in the production of inflammatory mediators that further the cartilage degradation, giving rise to a visious circle (6, **7**).

Treatment includes education of the disease, reduction of the joint overload through weight loss, physical therapy, muscle reinforcement, use of walkers, insoles, surgery (endoscopic removal of debris and cartilage fragments) and analgesia (8). Many drugs and therapies might be used for the treatment of OA, however in addition to their cost making

them inaccessible to most of the population, the drugs and therapies meet the 2 criteria for drug failure: they do not work (i.e., do no fully meet the needs of patients with OA and do not modify the course of disease) and pose risk (adverse effect) (9).

The high cost and limiting adverse effects of drugs led to the search for phytotherapeutic alternatives. *Arrabidaea chica* Verlot (*A. chica*, ACV) is one of the available phytotherapy options. This plant is found in the Amazon rainforest and has long been used by the indigenous population to paint the body and utensils. Known as "crajirú", ACV has anti-inflammatory, wound healing and immunomodulating properties that are widely used at the popular level and have been scientifically proven via the isolation of anthocyanin, flavonoids and steroids (10).

The oral use of ACV has been scarcely investigated, the pathophysiology of osteoarthritis depends on inflammation, and its clinical presentation is overwhelmingly characterized by joint pain.

1.1 OBJECTIVE

Establish whether ACV administered orally has analgesic and anti-inflammatory action in a model of rat knee OA induced by intra-articular injection of monosodium iodoacetate (MIA). And identify substances in ACV extract and its fractions that may be responsible for their action.

2 METHODS

2.1 ANIMALS

Following approval by the ethics committee of Federal University of Maranhão (Universidade Federal do Maranhão; ruling no. 23115006040/2013-04), adult male *Rattus norvegicus* rats of the Wistar lineage weighing 180 to 200 g were used (n=72). For eight days, the animals were housed in polypropylene cages of 46 x 31 x 16 cm^3, with wire cap, forage for wood, receiving ration and water *ad libitum*, and undergoing light-dark cycles of 12 hours. During this period they were accustomed to the devices of the clinical tests and to the handling by the researchers. The animals were obtained from the vivarium at Federal University of Maranhão and allocated to three groups: control, osteoarthritis and phytotherapy, with 24 animals each, being subdivided into subgroups day 7, day 14, day 21 and day 28 (n = 6, each subgroup, according to the day programmed for euthanasia).

2.2 *ARRABIDAEA CHICA* VERLOT

Adult leaves of *Arrabidaea chica* Verlot were collected in natural habitat during dry and rainy periods, RDC 26/14, at the Medical Garden of the Federal University of Maranhão (UFMA). A sample was prepared for the preparation of exsicata, which is registered and cataloged under the number 1067, in the Herbarium Attic Seabra of the Department of Pharmacy of UFMA.

2.3 PREPARATION OF THE HYDROALCOHOLIC EXTRACT OF ACV

The collected plant material was dried at 40ºC in an air circulating oven and then pulverized in an electric mill to obtain the powder, which was soaked in 92% ethyl alcohol in a ratio of 1: 4 and put under maceration under daily manual shaking. The alcoholic extraction of the macerate was carried out by three successive changes every 72 hours, with the renewal of the solvent. At the end of this process, the extract was filtered with gauze. The filtrate was concentrated in a rotary evaporator (MARCON®) under reduced pressure and at a temperature of 44 ° C. From this process the hydroalcoholic extract was obtained, which was packed in an amber bottle and kept under refrigeration (5 ° C).

Three aliquots of 0.5 mL of the hydroalcoholic extract were used to determine the dry weight in dry pre-dried beakers. The aliquots had their solvent evaporated under a stream of hot air and the beakers, after cooling, were weighed in analytical balance (SARTORIUS®) to determine the dry weight of the residues. This operation was repeated successively until obtaining constant weights. When necessary, small aliquots, previously determined according to the dry weight of the extract and the weight of the animals, were pipetted into beakers, and the solvent was evaporated under a stream of hot air and then the volume was completed to the desired concentration with distilled water or physiological solution. Ethyl alcohol and the dose programmed for our study (500 mg / kg) have already been used in other studies without presenting toxicity to animals (11).

2.3.1 Fractionation and chemical characterization of the ACV hydroalcoholic extract

Part of the ACV extract (9,08 g) was dissolved in 100 mL of the methanol/water mixture (70:30, v/v) by shaking and subjected to the liquid-liquid partition using hexane, chloroform, ethyl acetate and n-butane. The extrative solutions were filtered (anhydrous Na_2SO_4) and concentrated in a rotary evaporator under vacuum to give hexane (FH), chloroform (FC), ethyl acetate (FAE) and butanolic (FB) fractions.

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The content of phenolics and flavonoids was determined using the Folin-Ciocalteu reagent and 20% sodium carbonate and the colorimetric method with the methanolic solution of aluminum chloride, according to the methodology described by Dutra *et al* (12).

The HPLC analysis was performed on a Thermo Finnigan Surveyor Autosampler liquid chromatograph equipped with a 25 μL injector and UV detector. The mobile phases consist of Milli-Q water containing 0.1% formic acid (A) and aceto nitrile (B). The linear gradient was applied: 0-35 min, 5-30% B; 35-50 min, 30-70% B; 50-60 min, 70-100% B. The column was rebalanced for 10 min before the next run. The injection volume in the system was 25 μL, and UV-VIS detection was performed at 254 nm. The compounds were identified based on the co-injection of gallic acid, B-sitosterol, caffeic acid, coumaric acid, rutin, myricetin and apigenin standards. In the absence of available standards, compounds were identified based on literature data.

2.4 INDUCTION OF OSTEOARTHRITIS

The control group has not undergone to the induction of osteoarthritis, nor to any treatment (n=24). On days 7, 14, 21 and 28 of study, this group was undergone to clinical tests. After euthanasia, were collected right hind paw to execution the x-ray and collection of synovial membranes for histopathological study.

On day zero of the experiment, the animals of the osteoarthritis and phytotherapy groups (n=48) were anesthetized by intraperitoneal injection (sterile syringe and sterile insulin disposable needle - BD®) of thiopental sodium (Cristália, Brazil) at a dose of 40 mg / kg (12) for induction of osteoarthritis with injection of MIA, (2 mg) into the right knee.

2.5 TREATMENT

The treatment was administered orally from days 7 to 28 and consisted of 0.9% normal saline (0.1 mL/100 g of animal weight) for the osteoarthritis group (n=24) and 500 mg/kg (animal weight) of ACV extract for the phytotherapy group (n=24).

2.6 ASSESSMENT

The animals were assessed using clinical, radiological and histopathological tests on study days 7, 14, 21 and 28.

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2.6.1 Weight-bearing Test

The weight-bearing test (WB) or static incapacitance, analyzes the body weight distribution across the pelvic limb paws and is used to assess joint discomfort or the primary hyperalgesia caused by osteoarthritis. The test involves a sensor connected to 2 independent platforms (scales), which measure how much of the animal weight is applied to each paw separately; the values are shown on a digital display and used in an equation that calculates the results as percentages: (weight on the right paw / total weight of the animal X 100) (14).

2.6.2 von Frey Test

The von Frey, or mechanical allodynia, test assesses the threshold for paw withdrawal when presented with an innocuous stimulus. Rats are placed in an acrylic box on a wire platform that provides access to the plantar face of the hind paws. The device includes a calibrated polypropylene tip connected to a strength meter (digital analgesiometer). The tip is pushed into the animals' plantar surface until the animal performs a withdrawal response or shakes the limb. The analgesiometer measures the strength required to elicit that response. Repeated measurements are performed until 3 similar responses occur, and the average is calculated as the withdrawal threshold (14).

2.6.3 x-Ray

Following the clinical tests, the animals were euthanized through an intraperitoneal injection of 150 mg of sodium thiopental; the right pelvic limbs were surgically removed and fixed on a Styrofoam plaque and assigned a number corresponding to each animal from left to right.

Simple x-rays were taken on 2 planes, anteroposterior and latero-lateral, in conventional apparatus Raiano SH 300®. The computer screen was photographed, and the images were saved in digital format. Three observers blinded to the groups of allocation scored each knee according to the Kellgren-Lawrence (K-L) method for grading arthrosis (0-normal, 1-doubtful, 2-minimal, 3-moderate and 4-severe), and the average was calculated for comparison (15) (Table 1).

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Table 1. Radiological classification of joints

CLASSIFICATION	RADIOLOGICAL FINDINGS
Grade 0	Without arthrosis: normal radiology
Grade I	Doubtful arthoris: doubtful joint narrowing, possible marginal osteophyte
Grade II	Minimal arthrosis: possible narrowing, definite osteophyte
Grade III	Moderate arthrosis: definite narrowing, multiple osteophytes, some subchondral sclerosis, possible boné deformity
Grade IV	Severe arthrosis: marked articular narrowing, severe subchondral sclerosis, large osteophytes, definite deformity

Source: Kellgren and Lawrence, 1957.

2.6.4 Histopathology

For histopathological examination, the patellar tendon was identified and folded superiorly to expose and remove the synovial membrane (SM). The tissue was fixed with 10% formalin for 48 hours and then sent to the pathology laboratory for hematoxylin-eosin staining and embedding in paraffin blocks. The SM abnormalities were classified based on the increase in the number of synovial cell layers, subsynovial tissue proliferation and inflammatory infiltrate (16) (Table 2).

Table 2. Degree of synovial membrane inflammation

Parameter	Grade	Description
Synovial membrane inflammation	0	Without changes 1-2 layers of sinovial cells
	1	Increased number of cells (≥ 3-4 layers) or Mild subsynovial proliferation
	2	Increased number of cells (≥ 3-4 layers) and/or subsynovial proliferation
	3	Increased number of cells (> 4 layers) and/or subsynovial proliferation and infiltrate of few inflammatory cells
	4	Increased number of cells (> 4 layers) and/or subsynovial proliferation and infiltrate of many inflammatory cells

Source: Gerwin *et al.*, 2010.

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2.7 STATISTICAL ANALYSIS

The results are presented in mean +/- standard deviation. The data were entered into the program Graph Pad Prism 5. The Shapiro-Wilk test indicated a non-normal distribution of the data; therefore, the means among the various experimental groups were compared using the non-parametric Kruskal-Wallis test followed by the Dunn test. The significance level was set as $p<0.05$.

3 RESULTS

3.1 CHEMICAL CHARACTERIZATION OF THE ACV HYDROALCOHOLIC EXTRACT

After fractionation were obtained the fractions hexane (FH), chloroform (FC), ethyl acetate (AED) and butanolic (FB).

The contents of polyphenol in descending order was: FAE (51,221 +/- 3,927), FC (35,462 +/- 2,149), FH (19,597 +/-2,904), FB (7,170 +/- 0,492). And the contents of flavonoids in descending order was: FH (1,977 +/- 0,026), FC (1,708 +/- 0,186), FAE (0,941 +/- 0,177), FB (flavonoids not detected).

The FAE presented the highest total polyphenolic contents, while the FH, the highest concentration of total flavonoids. Based on these results, liquid chromatography of these two fractions was performed. In FH, the following substances were identified: carajuflavone, diglycosylated derivative of carajurine, malvidin-3-O (6-O-acetylglucoside-4-vinylphenol), peonidin-3-O- (6 "-acetyl glucoside), jacarandic acid, jacoumaric acid, jacoumaric acid isomer, apigeninidine and dimethoxy apigeninidine (Table 3).

Table 3 - Analysis of the hexane fraction (FH) by high performance liquid chromatography coupled to a mass spectrometer.

Number of substances	**tR (min)**	**PM**	**[M – H]$^-$ (m/z)**	**EM/EM (m/z)**	**Identified substance**
1	7,3	317,27	316,67		Carajuflavone
2	43,6	602,87		298,79	Diglycosylated derivative of carajunine
3	43,7	651,57			Malvidin-3-O(6-O-acetyl-glucoside-4-vinylphenol)
4	47,8	504,89	502,98		Peonidin-3-O-(6” -acetil-glucoside)
5	61,8	488,86	487,06	469,11	Jacarandic acid
6	77,8	618,39	617,09		Jacoumaric acid
7	78,3	618,39	617,10		Isomers of jacoumaric acid

8	86,3	254,80	-	-	Apingeninidine
9	87,5	284,01	281,88		Dimetoxy Apigeninidine

Note: rR= retention time; min= minutes; PM= molecular weight; $[M-H]^-$= molecular ion; EM/EM= mas spectrum/mass spectrum. Source: author.

In FAE were identified: gallic acid, β-sitosterol, caffeic acid, coumaric acid, rutin, myricetin and apigenin were identified (Table 4).

Table 4 - Identified substances in FAE by high performance liquid chromatography with visible ultraviolet detector at 254 nm

Peak number	**Substances**
1	Gallic acid
2	β-sitosterol
3	Caffeic acid
4	Coumaric acid
5	Rutine
6	Myricetin
7	Apigenin

Source: author.

3.2 WEIGHT-BEARING TEST

All animals showed good general condition and normal behavior before the experiments. After the injection of sodium iodoacetate, the animals presented a lower weight distribution on the right paw (from 50,30 ± 0,67% to 25,48 ± 1,14% on osteoarthritis group , and from 50,83 ± 0,56% to 24,99 ± 0,84% on phytotherapy group), with statistical significance in comparison with the control group (p=0,0043 in both), proving the efficacy of the MIA osteoarthritis induction method.

Treatment with oral ACV extract improved the distribution of weight on the right paw (from 35,52 ± 3,12% on day 14, to 42,53 ± 2,11% on day 21, and to 47,14 ± 0,84% on day 28) in the comparisons with group osteoarthritis, on days 14 , 21 and 28 (p=0,0022 in the three moments) (Figure 1A).

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3.3 VON FREY TEST

The induction of osteoarthritis by sodium monoiodoacetate produced mechanical allodynia, with reduction of nociceptive paw withdrawal (from 50,44 ± 2,00% to 32,15 ± 3,93% on osteoarthritis group, and from 48,78 ± 1,12% to 30,02 ± 1,30 % on phytotherapy group), with a statistically significant reduction on day 7 when compared induced groups and control group (p=0,0022 for both). Treatment with the ACV extract promoted a progressive elevation of the nociceptic threshold (or reduction of allodynia) with significant statistical difference on days 14 (37,80 ± 1,99%), 21 (41,48 ± 2,65%) and 28 (46,09 ± 4,19%) (p=0,0050, p=0,0050, p=0,0087, respectively) when compared with the osteoarthritis group. Phytotherapy and control groups presented similar results at day 28 (p=0,3095) (Figure 1B).

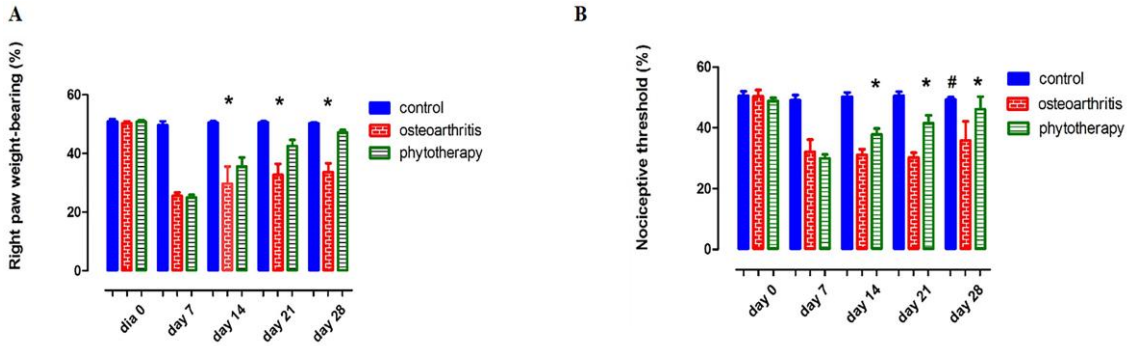

Figure 1. Effect of ACV hydroalcoholic extract on distribuction of weight on the right paw and nociceptive threshould of rats with knee osteoarthritis induced by MIA. **A** – Distribution of weight on the right paw of rats knee osteoarthritis by MIA treated daily with hydroalcoholic extrac of ACV (500 mg/Kg by gavage) from days 7 to 28. Comparison between osteoarthritis and phytotherapic groups on days 14, 21 and 28 (*): p< 0,05 (p=0,0260; p=0,0020; p=0,0025 respectively). **B-** Nociceptive threshould of rats knee osteoarthritis by MIA treated daily with hydroalcoholic extrac of ACV (500 mg/Kg by gavage) from days 7 to 28. Comparison between osteoarthritis and phytotherapy groups on days 14, 21 and 28 (*): p<0,05 (p=0,0050; p=0,0055; p=0,0087 respectively); and between control and phytotherapy groups on day 28 (#): p=0,30.

3.4 X-RAY

After induction, the averages of the radiological classification of the osteoarthritis (1,75 ± 0,27) and phytotherapic (1,66 ± 0,40) group were higher than those of the control group (0,66 ± 0,51), with statistical significance in the comparisons with the control group (p = 0,0043 e p=0,0099, respectively). The treatment with the ACV extract promoted an improvement in the radiological classification of the right knees of rats on day 21 (1,33 ± 0,51) and 28 (1,50 ± 0,54) of the study, with statistically significant differences in the comparisons with the osteoarthritis (2,16 ± 0,40 and 2,33 ± 0,51, respectively) group (p = 0,0256 and p = 0,0396, on days 21 and 28, respectively). No significant difference was found between the phytotherapy (1,33 ± 0,51 and 1,50 ± 0,54) and control (1,16 ± 0,25 and 1,56 ± 0,34) groups on days 21 (p=0,7745) and 28 (p=0,6758) (Figure 2A)

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3.5 HISTOPATHOLOGY

Seven days after induction, there was a statistically significant difference in the comparison of the groups induced (2,75 ± 0,97 on osteoarthritis, and 3,16 ± 1,16 on phytotherapy groups) with the control (0,05 ± 0,08) group (p = 0,0043 and p=0,0099 respectively). The osteoarthritis group exhibited progressive worsening until the end of the study (2,33 ± 1,03; 2,40 ± 1,20 and 3,83 ± 0,40 on days 14, 21 and 28, respectively). Treatment with oral extract of *A. chica* (phytotherapeutic group) improved the degree of synovitis (1,40 ±1,02; 1,66 ± 0,51 and 2,20 ± 0,40 on days 14, 21 and 28, respectively). A comparison indicated a statistically significant difference between the osteoarthritis and phytotherapy groups only on day 28 (p=0.0047) (Figure 2B).

Figure 2. Effect of ACV hydroalcoholic extract on radiological abnormality scores and histological grading of synovitis. **A-** Radiological abnormality score to assess the effect of the ACV hydroalcoholic extract on the MIA-induced osteoarthritis in rat knees._Comparison between the control and phytotherapy groups on days 21 and 28 (*: p>0,05) (p=0,7745 and p=0,6758 respectively) and between the osteoarthritis and phytotherapy groups on days 21 and 28 (#: p<0,05) (p= 0,0256 and p=0,0396 respectively). The columns and vertical bars represent the mean and standard deviation (±) of the means. **B-** Histological grading of synovitis to assess the effect of the ACV hydroalcoholic extract on the MIA-induced osteoarthritis in rat knees._Comparison between the osteoarthritis and phytotherapy groups on day 28 (*: p=0,0047) and between the control and phytotherapy groups on day 14 (#: p=0,0250). The columns and vertical bars represent the mean and standard deviation (±) of the means.

3.6 ADVERSE EFFECTS

No adverse effects (vomiting, weight loss, diarrhea, bleeding) or death were observed in animals until the day of euthanasia.

4 DISCUSSION

All the fractions of the EEAC analyzed in this study showed phenolics compounds, and that had a larger fraction of polyphenols was FAE, and the bigger concentration of total flavonoids was the FH. Previous studies have also detected these metabolites in leaf extracts of ACV and their fractions, in lower concentrations, which may be a variation of the

specimen, since that research was performed with samples collected in another brazilian state (17)

According to the results of the chemical analysis, the pharmacological activity of *A. chica* fractions can be attributed mainly to flavonoids, anthocyanidins, and triterpenes, compounds that are related to the anti-inflammatory, antinociceptive and analgesic effects. (18). The ACV extract inhibited the nuclear transcription factor Kappa B, avoiding the production of cytokines and inflammatory enzymes, and that these actions were probably promoted by the flavonoids and anthocyanins that inhibited COX1, COX2, NO, reducing damage to the articular cartilage (19).

MIA-induced osteoarthritis is a model that induces dose- and time-dependent inflammation. The acute stage begins 3 days after the intra-articular injection, which is followed by established osteoarthritis (14 days), and the chronic stage, which is characterized by a neuropathic component and abnormalities of the subchondral bone. Thus, the lesions present in humans are reproduced, making this model useful for assessing the therapeutic efficacy of various drugs (20).

The statistically significant difference found in the comparison between the control and the other 2 groups on day 7 on all assessments (clinical tests, radiology and histopathology) demonstrates the efficacy of the technique of inducing osteoarthritis via a single injection of MIA.

Treatment was associated with the progressive improvement of the results on the WB test, i.e., better use of the affected limb, until study day 28 in both the osteoarthritis (which might reflect the natural progression of disease) and phytotherapy (which might reflect the therapeutic efficacy of the ACV extract) groups; however, the improvement was clearly better for the latter, which did not exhibit a statistically significant difference compared with that in the control group on days 21 and 28 ($p>0.05$). We were not able to locate any scientific publication reporting the use of the ACV hydroalcoholic extract per the oral route for the treatment of osteoarthritis. One study conducted with another phytotherapy drug, cat's claw (*Uncaria guianensis*), that was applied topically to treat OA of the knee did not detect a statistically significant difference on the WB test between the osteoarthritis and treatment groups. The authors attributed these findings to the fact that the animals in the phytotherapy group had a higher body mass index, contributing to the poorer outcomes exhibited by this group (21). Adipose tissue cytokines, such as adiponectin and leptin, might influence the development of OA via direct joint injury or local inflammatory action, with

obesity being a positive risk factor for the development of OA and contributing to an insufficient response to treatment (22, 23). Improvement of primary hyperalgesia, measured by the WB test, was more evident in the group treated with ACV on day 14 (stage of established osteoarthritis). One study that tested the use of diclofenac (30 mg/kg) and paracetamol (300 mg/kg) found a better response during the acute stage of OA (24). Therefore, the analgesic action of the ACV extract does not seem to be related to early anti-inflammatory activity.

The osteoarthritis group exhibited a reduction in the nociceptive threshold up to day 21 and a slight improvement on day 28, indicating a prolonged clinical progression; compared to the results of the other tests, the worse results were detected on day 7. In 1 study of osteoarthritis induced by ligament transection (24), allodynia exhibited progressive worsening until day 14, whereas in the model of MIA-induced OA, the paw withdrawal threshold decreased to its minimum on days 4 to 7 and then progressively increased starting on day 11 (21). Allodynia, i.e., a painful sensitivity in response to an innocuous stimulus, is due to dynamic abnormalities of the excitability of the spinal cord posterior horn neurons (central sensitization) after persistent nociceptive afferent stimulation of the central nervous system. This process requires time to establish and demands multimodal treatment; as a rule, the condition does not respond to monotherapy with anti-inflammatory agents (25, 26). One study (24) assessed the treatment of allodynia in MIA-induced OA using drugs administered subcutaneously. The results showed that diclofenac and paracetamol were inefficacious at all the tested time-points (early, established and late stages), morphine caused almost 100% improvement on days 14 and 28 (established and late stages), and gabapentin caused 76.4% improvement on day 14 (established stage). These results confirm that the common anti-inflammatory and analgesic drugs are not adequate for treating allodynia, whereas opioids and anticonvulsants have better results in the established and late stages of OA. In the present study, the phytotherapy group exhibited better performance starting on day 14, with progressive elevation of the nociceptive threshold to achieve results statistically similar to those of the control group at the end of the study ($p=0.2539$ on day 28). We could not locate any study in which allodynia improved in the treatment group to the point of not exhibiting a statistically significant difference relative to the control group at the end of the study.

Radiography is very useful to follow up the progression of OA and is considered the gold standard. In study that employed the K-L classification of radiological severity, which is widely accepted for diagnosis of OA (27), synovitis was associated with advanced

radiological stages, i.e., K-L grade 4. These findings agree with those of the present study, in which poorer radiological scores and higher degrees of synovitis occurred on days 21 and 28. A recent study did not find radiological abnormalities 7 days after induction (28). In contrast, the present study detected abnormal findings on the radiographs of the investigated right knees, thus agreeing with the results of other studies that also detected early bone involvement, with areas of bone resorption indicating the onset of bone remodeling (29, 30).

The radiological abnormalities agreed with the results of the histopathological assessment (which demonstrated synovitis 7 days after the induction of osteoarthritis) and with the clinical tests (which showed a reduction in the use of the affected limb and of the nociceptive threshold 7 days after induction). These findings demonstrate that the method of inducing oa using MIA triggers intense and early inflammation, which was evident on radiographs, as osteophytes and subchondral sclerosis might appear months before joint space narrowing can be measured on radiographs. One possible reason is that the bone has rich vascularization and thus would respond faster to joint abnormalities, while the response of the avascular cartilage would be comparatively slower (31, 32).

The scale for grading the histopathological abnormalities of the SM employed in the present study has high sensitivity to detect the effects of various treatments on the severity of disease. An abnormal SM is described and characterized according to the inflammation type and the extension and severity of lesions. Synovitis occurs frequently in patients with advanced OA but also appears in earlier stages (16, 33). Biopsies from 70 patients subjected to total prosthesis or knee arthroplasty exhibited synovitis; this finding was not restricted to the patients with extensive joint damage on simple radiographs. It has not yet been established whether joint damage is the cause of synovitis, as the products of cartilage degradation are released into the synovial fluid, or whether the inflammatory infiltrate in the SM causes the chondral lesion via production of metalloproteinases. SM might play a role in the perpetuation of the joint lesion through the synthesis and release of multiple inflammatory mediators (metalloproteinases, interleukins, tumor necrosis factor, free oxygen radicals, etc.), which interfere with the activity of chondrocytes and thus favor the occurrence of bone resorption. Synovitis is known to be a part of the process of OA and is considered a potential therapeutic target because pain is the main symptom, and cartilage has no innervation (33).

Upon histopathological assessment, the control group did not show any changes, and the osteoarthritis group had high degrees of synovitis throughout the study period. The ACV

extract was efficacious for reducing the degree of synovitis for all 28 days and exhibited a better effect on day 14 (7 days after the onset of treatment). Upon studying orally administered doxycycline for the treatment of MIA-induced OA of rat knees, some investigators found that the intensity of the histopathological changes was similar in the animals from groups I (osteoarthritis induction, without treatment) and II (osteoarthritis induction, treated) on study days 7 and 14 and that doxycycline only reduced synovitis after 21 days of treatment (29), while in the present study, ACV showed beneficial effects after 7 days of treatment (day 14).

4.1 CONCLUSION

Upon analyzing the effect of the ACV extract administered orally on the MIA-induced OA of rat knees, we found that the extract promoted a reduction in the static incapacitance, allodynia, the radiological score and the degree of synovitis during the study.

Four fractions of the ACV specimen were obtained: FH, FC, FAE, FB. The phytochemical approach tests detected the presence of phenolic compounds, tannins, triterpenes and steroids in the extract and fractions. The FAE fraction had the highest total polyphenolic contents, while the FH had the highest concentration of total flavonoids; being probably the fractions responsible for the anti-inflammatory and analgesic activities of the ACV extract.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Elizabeth Servin was the main author, Dr Nicolau Czeczko was the research supervisor and Dr João Batista Garcia the co-supervisor. Dra Maria do Socorro Cartágenes and Dr Fernando Cezar Vilhena Moreira Lima coordinate the collection, preparation, storage and use of the herbal. Dr Osvaldo Malafaia e Dr Orlando Torres are postgraduate coordinators and participate in the critical analysis of the manuscript. Dr Gyl Eanes Silva is the pathologist responsible for histopathology.

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DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

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