

Original Article

Campomanesia velutina leaves extracts exert hypouricemic effects through inhibition of xanthine oxidase and ameliorate inflammatory response triggered by MSU crystals



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ABSTRACT

Gout is a destructive arthritis with a high prevalence worldwide. However, the available therapy is not able to increase life quality in many patients. *Campomanesia velutina* (Cambess) O. Berg, Myrtaceae, is used in Brazilian folk medicine to treat pain, inflammation and rheumatism. The aim of this study was to evaluate the potential of ethanolic and aqueous extracts from *C. velutina* leaves to treat hyperuricemia and inflammation in gout arthritis model. Ethanolic extract of leaves and aqueous extract of leaves were *in vitro* assayed on xanthine oxidase inhibitory effect and *in vivo* on an experimental model of oxonate-induced hyperuricemia in mice, liver xanthine oxidase inhibition and monosodium urate crystal-induced paw edema model. The extracts at both tested doses (100 and 300 mg/kg) reduced serum urate levels. They were also able to inhibit xanthine oxidase *in vitro* and *in vivo*, demonstrating that this might be the mechanism of action underlying the urate-lowering effects. In addition, the extracts showed significant anti-inflammatory activity on monosodium urate crystal-induced paw edema, especially aqueous extract (100 and 300 mg/kg) that reduced edema at all evaluated times. Rutin and myricitrin were identified in ethanolic and in aqueous extracts. In this study, myricitrin was able to reduce serum uric acid levels and inhibit liver xanthine oxidase at the dose of 15 mg/kg. The anti-hyperuricemic activity of rutin has been previously reported. Thus, rutin and myricitrin seem to contribute to the observed effects of ethanolic and aqueous extracts. The results demonstrated the ability of aqueous and ethanolic extracts to lower serum urate levels and to reduce edema induced by monosodium urate crystals. Therefore, they may contribute to the management of gout in the future.

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Introduction

Gout is an inflammatory disorder that arises when supersaturation of body tissues with urate occurs, leading to the formation and deposition of monosodium urate (MSU) crystals in articular and periarticular tissues (Roddy and Choi, 2014). The prevalence of both hyperuricemia and gout has risen in the last decades and therefore the burden of gout has increased (Perez-Ruiz et al., 2015). Clinical manifestations of this disease include acute gouty arthritis flares characterized by severe pain, swelling, warmth and erythema. If hyperuricemia persists, MSU crystal deposits further induce chronic inflammatory responses that may lead to the formation of tophaceous MSU crystal deposits in joints and other body

tissues, chronic joint damage, renal stone formation with potential renal insufficiency and cardiovascular problems (Perez-Ruiz et al., 2015; Roddy and Choi, 2014).

The therapies for treating gout's pain and inflammation include nonsteroidal anti-inflammatories (NSAID), colchicine and oral corticosteroids (Edwards and So, 2014). However, MSU crystal deposits must be considered the most important target for gout management. By lowering MSU levels below 6 mg/dl, dissolution of pathogenic MSU crystals is achieved and disappearance of clinical features of gout can be obtained (Perez-Ruiz et al., 2015). The urate-lowering therapies are based on the use of allopurinol and probenecid since the 1960s. Recent studies have showed how inadequate is the traditional approach to this destructive arthritis. After all, patients do not experience a significant reduction of pain and intolerance to allopurinol and probenecid means that the patient would go untreated. These problems have led to the recognition that gout's treatment requires better and more specific agents (Edwards and So, 2014).

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Plants have been used for centuries to treat numerous pathological conditions and diseases and even nowadays, they still provide a rich source for new drug discoveries due to a tremendous chemical diversity of compounds. *Campomanesia* species are used in Brazilian folk medicine to treat a wide range of clinical conditions, including their use to treat rheumatism (Alice et al., 1995; Cravo, 1994). The term rheumatism includes a wide range of disorders marked by inflammation, degeneration and pain, affecting connective tissues structures, specially joints and related structures (Dorland's Medical Dictionary, 2007). The specie *Campomanesia velutina* (Cambess.) O. Berg, Myrtaceae, can be found in the Brazilian cerrado bioma and there are reports about its use by the population of its occurrence area (Dias and Laureano, 2009; Giraldo and Hanazaki, 2010; Oliveira et al., 2010). Previous studies with this specie assessed its anti-inflammatory and antinociceptive activities *in vivo* and lead to the isolation of the active constituent myricitrin from the ethanolic extract of leaves (Michel et al., 2013).

Since *Campomanesia* species are used to treat rheumatism and previous studies demonstrated the anti-edematogenic and antinociceptive activity of the specie, this study was conducted in order to evaluate the role of *C. velutina* in gout, a known and prevalent rheumatic disease. Thus, the aim of this study is to evaluate the biological effects of aqueous and ethanolic extracts from *C. velutina* leaves over the hyperuricemia and inflammation triggered by MSU crystals. The ability of extracts to inhibit xanthine oxidase (XO) was also evaluated both *in vitro* and *in vivo*. In addition, it was evaluated myricitrin ability to inhibit XO and thus reduce uric acid levels *in vivo*.

Materials and methods

Chemicals

Xanthine oxidase from cow's milk, xanthine, potassium oxonate, uric acid, allopurinol and indomethacin were purchased from Sigma–Aldrich (USA). Uric acid assay kit was purchased from Bioclin (Brazil). Ketamine and xylazine were obtained from Sesprou Indústria e Comércio Ltda (Brazil). Water was purified using Milli-Q apparatus from Millipore (USA). Ethanol, dimethylsulphoxide (DMSO) and Tween 80 were of analytical grade. HPLC solvents were purchased from Tedia (Brazil) and standards were purchased from Sigma–Aldrich (USA).

Plant material

Leaves from *Campomanesia velutina* (Cambess.) O. Berg, Myrtaceae, were collected in Lagoa Santa city, Minas Gerais state, Brazil, in December of 2012, with permission of Chico Mendes Institute of Biodiversity Conservation (Instituto Chico Mendes de Conservação da Biodiversidade – ICMBio/Sistema de Autorização e Informação em Biodiversidade – SISBIO), license no. 17021–5. The plant botanical identification was realized by Dr. Marcos E. Guerra Sobral from the Department of Natural Sciences, Federal University of São João Del-Rei (Universidade Federal de São João Del-Rei (UFSJ)), Minas Gerais, Brazil. A voucher specimen (HUFSJ 4637) was deposited at the herbarium of UFSJ.

Preparation of plant extracts

The leaves were air-dried and powdered. Part of the leaves powder (540 g) was exhaustively extracted with ethanol at room temperature by percolation. Solvent was removed under reduced pressure, at 40 °C, yielding 41 g of the dried ethanolic extract of leaves (EEL). In order to obtain the aqueous extracts, 450 g of leaves powder was percolated with 4.5 l of water. The water was removed

by lyophilization, yielding 19 g of the aqueous extract of leaves (AEL).

Characterization of the extracts by HPLC-UV/DAD

HPLC-UV/DAD analysis were performed on a Waters Liquid Chromatography (model Alliance 2695) equipped with a vacuum degasser, a quaternary pump, an auto sampler, a diode array detector (DAD Waters 2996) and reversed phase C18 column (Shimadzu ODS – 250 mm × 4.6 mm, 5 µm).

To assign compounds to the peaks, it was used the retention time and $UV_{\lambda_{max}}$ of standards eluted on the same conditions as the extracts. The following standards were used: oleanolic acid, chlorogenic acid, caffeic acid, galocatequin, quercetin, pinocembrin, rutin, kaempferol, crisin and myricitrin. To obtain the HPLC profiles, the UV-DAD detector was set to record between 200 and 400 nm and UV chromatograms were recorded at 254 nm.

The extracts and the standards were solubilized in methanol to yield a concentration of 5 mg/ml and 1 mg/ml, respectively. Then, they were filtered through a 0.45 µm Millex syringe filters. The volume injected was 25 µl. EEL was eluted in a system with 5% of methanol and 95% of water, taking 60 min to reach 100% of methanol and another 5 min to return to the initial condition. The flow rate was kept constant at 1 ml/min. AEL was eluted in a system with 100% of water, taking 30 min to reach 20% of methanol, another 10 min to reach 40% of methanol and 15 min to reach 100% of methanol. The system was returned to the initial condition in 5 min. The flow rate was kept constant at 0.8 ml/min. In both cases, the separation temperature was 25 °C.

Inhibition of XO activity *in vitro*

To evaluate the effect of the extracts over XO activity, it was used the method described by Ferraz-Filha et al. (2006) with modifications. The EEL was dissolved in DMSO:Tween 80:Water (1:1:8) and the AEL was dissolved in water. The assay mixture consisted of 500 µl of extract solution, 1125 µl of 1/15 M phosphate buffer (pH 7.5) and 187.5 µl of enzyme solution (0.28 units/ml in buffer). The reaction was initiated by adding 1375 µl of xanthine substrate solution (0.15 mM in water). The assay mixture was incubated at 25 °C and the absorbance (295 nm) was measured spectrophotometrically every minute for 12 min using a Cary 50 Bio Spectrophotometer (Varian – Australia). A blank (0% of XO inhibition) was prepared without the extracts solutions. Allopurinol was used as a positive control. XO inhibitory activity was expressed as the percentage of XO inhibition in the assay mixture system and calculated as: % inhibition = $(1 - \text{test inclination}/\text{blank inclination}) \times 100$, where test inclination is the linear change in absorbance of test material per minute and blank inclination is the linear change in absorbance of blank per minute. To calculate IC₅₀, final concentrations of extracts were 10, 20, 30, 40 and 50 µg/ml and final concentrations of allopurinol were 0.1, 0.25, 0.5, 0.75 and 1 µg/ml. All assays were performed in triplicate.

Animals

Animal Science Center of Universidade Federal de Ouro Preto supplied the male albino Swiss mice (25–30 g) for the experiments. Animals were divided into experimental groups ($n=6$ or $n=9$), housed in plastic cages and maintained on a 12-h light/12-h dark cycle. They were given standard chow and water *ad libitum*. The experiments with animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institute of Health (NIH). The Ethical Committee on Animal Experimentation of UFOP (no. 2012/69) approved all experimental procedures.

Preparation of drugs and test solutions

Allopurinol, indomethacin, myricitrin, *Campomanesia velutina* extracts and potassium oxonate were prepared according to the average weight of each experimental group. Potassium oxonate (250 mg/kg) and MSU crystals (80 mg/ml) were suspended in 0.9% sterile saline. Allopurinol (10 mg/kg), indomethacin (3 mg/kg) and EEL (100 and 300 mg/kg) were solubilized in DMSO:Tween 80:water (1:1:8). AEL (100 and 300 mg/kg) was solubilized in water and myricitrin (15 mg/kg) was solubilized in DMSO:water (5:95). All solutions and suspensions were prepared on the day of their use.

Anti-hyperuricemic effects and inhibition of liver XO residual activity in oxonate-induced hyperuricemic mice

The anti-hyperuricemic activity of myricitrin, AEL and EEL was evaluated using an experimental animal model of hyperuricemia induced by potassium oxonate, an uricase inhibitor, as previously described by Hall et al. (1990) and modified by others (de Souza et al., 2012; Lemos et al., 2015). Animals were divided into experimental groups ($n=6$) and fasted 1 h before drug administration. Potassium oxonate was administered intraperitoneally to animals in the first and third day of the experiment 1 h before oral administration of test solutions. Mice of normal control group were not treated with potassium oxonate. All treatments were orally administered by gavage once a day for three consecutive days. Mice of normal control and hyperuricemic control groups received only vehicle (DMSO:Tween 80:water or DMSO:Water). Animals from treated control group received allopurinol. Animals of remaining groups were treated with myricitrin, EEL and AEL. On the third day, 1 h after the last oral test administration, mice were anesthetized with a mixture of ketamine and xylazine (100 and 20 mg/kg, respectively) and the blood was collected through cardiac puncture. The blood was allowed to clot for approximately 45 min at room temperature and then centrifuged at $3500 \times g$ for 10 min. Sera were separated and stored at -20°C until assay for uric acid quantification. The liver was removed, washed in 0.9% saline and stored at -80°C .

Uric acid assay

Serum uric acid concentration was spectrophotometrically (Varian Cary 50 Bio Spectrophotometer, Australia) determined by enzymatic colorimetric method (UOD-PAP) using a standard diagnostic kit (Bioclin, Brazil) according to manufacturer's instructions. This test is based on uric acid oxidation by uricase producing allantoin and hydrogen peroxide which is used by peroxidase to produce a red chromogen through the reaction of 4-aminoantipyrine with the hydroxyl-dichloro-benzene sulphononic acid (HDBS). The color intensity is proportional to the concentration of uric acid in the sample with maximum absorption at 505 nm.

Liver XO activity assay

Enzyme extraction from liver was carried out according to previously described (Zhu et al., 2004; Haidari et al., 2008). Liver XO residual activity was assayed spectrophotometrically by monitoring uric acid formation from xanthine according to method described by Hall et al. (1990) and modified by others (de Souza et al., 2012; Ferrari et al., 2016). Briefly, 100 μL of livers final supernatant were pre-incubated for 15 min at 37°C with 5000 μL of 50 mM phosphate buffer (pH 7.4) containing 1 mM of potassium oxonate. The presence of potassium oxonate prevents the oxidation of uric acid to allantoin. Then, the reaction was initiated by the addition of 1200 μL of 250 mM xanthine solution. The addition of 500 μL of 0.6 M HCl solution to the reaction medium after

0 and 30 min stopped the reaction. The reaction mixtures were then centrifuged at $3000 \times g$ for 5 min. The supernatants were separated and the absorbance measured at 295 nm (Varian Cary 50 Bio Spectrophotometer, Australia). The amount of uric acid formed was quantified by the difference of absorbance from 30 and 0 min using a uric acid calibration curve. XO residual activity was expressed as nanomoles of uric acid formed per minute per milligram of protein. Protein concentration was determined spectrophotometrically using method described by Bradford (1976).

Monosodium urate crystal-induced inflammation in mice

The anti-inflammatory activity of *Campomanesia velutina* leaves extracts was evaluated on an experimental model of gout according to previously described by (Rassol and Varalakshimi, 2006) and modified by others (de Souza et al., 2012; Lemos et al., 2015; Ferrari et al., 2016). Animals were divided into seven experimental groups ($n=9$) and fasted 1 h before drug administration. Inflammation was induced on the first day of the experiment by intradermal injection of 50 μL of MSU crystal suspension into the mice right hind paw. MSU crystals were prepared according to previously described method (Rassol and Varalakshimi, 2006). All animals received MSU injection, except those from normal control group (group 1), which were administered only saline. All treatments were orally administered by gavage 1 h before MSU injection on the first day and repeated daily, at the same time, for three more days. Animals from group 1 and 2 were treated with the vehicle and served as normal control group and MSU-induced control group, respectively. Mice of group 3 were treated with the standard anti-inflammatory drug indomethacin. Animals of remaining groups (4–7) were treated with EEL and AEL (100 and 300 mg/kg). Paw thickness was measured with a caliper rule (150 mm–6 in., Vonder, China) before and 4, 24, 48 and 72 h after MSU injection. Inflammatory swelling was expressed as percentage of thickness variation.

Statistical analysis

Experimental data was analyzed using GraphPad Prism 5.0 Software (Inc., San Diego, CA, USA). IC_{50} values were calculated by linear regression of plots on an XY graph of inhibition versus concentration values, assuming a 95% confidence interval. Results from *in vivo* experiments were presented as mean values $\pm$ S.E.M. One-way analysis of variance (ANOVA) was used followed by Newman–Keuls' multiple comparison test. p values < 0.05 were considered statistically significant.

Results and discussion

Brazilian traditional medicine uses *Campomanesia* species to treat pain, inflammation and rheumatism. Rheumatism is a generic term used to describe various clinical conditions that affect muscles, bones or articulations causing reduction or loss of mobility, as arthritis, gout, arthrosis, among others.

Gout is one of the most common metabolic diseases and affects thousands of people worldwide. Hyperuricemia characterizes gout and leads to the deposit of MSU crystals in joints, resulting in a marked local inflammatory process with progressive loss of function (Roddy and Choi, 2014). Recent data have shown that hyperuricemia and gout are increasing worldwide. Such an epidemic has deleterious consequences on not only joint function, health resources utilization, and quality of life, but may also increase cardiovascular mortality. Therefore, reducing MSU levels is the main strategy to treat gout and implementation of anti-inflammatory prophylaxis prior to or at the initiation of urate-lowering therapy to prevent the occurrence of flares is highly recommended (Perez-Ruiz et al., 2015). Thus, anti-inflammatory and anti-hyperuricemic properties are convenient for compounds

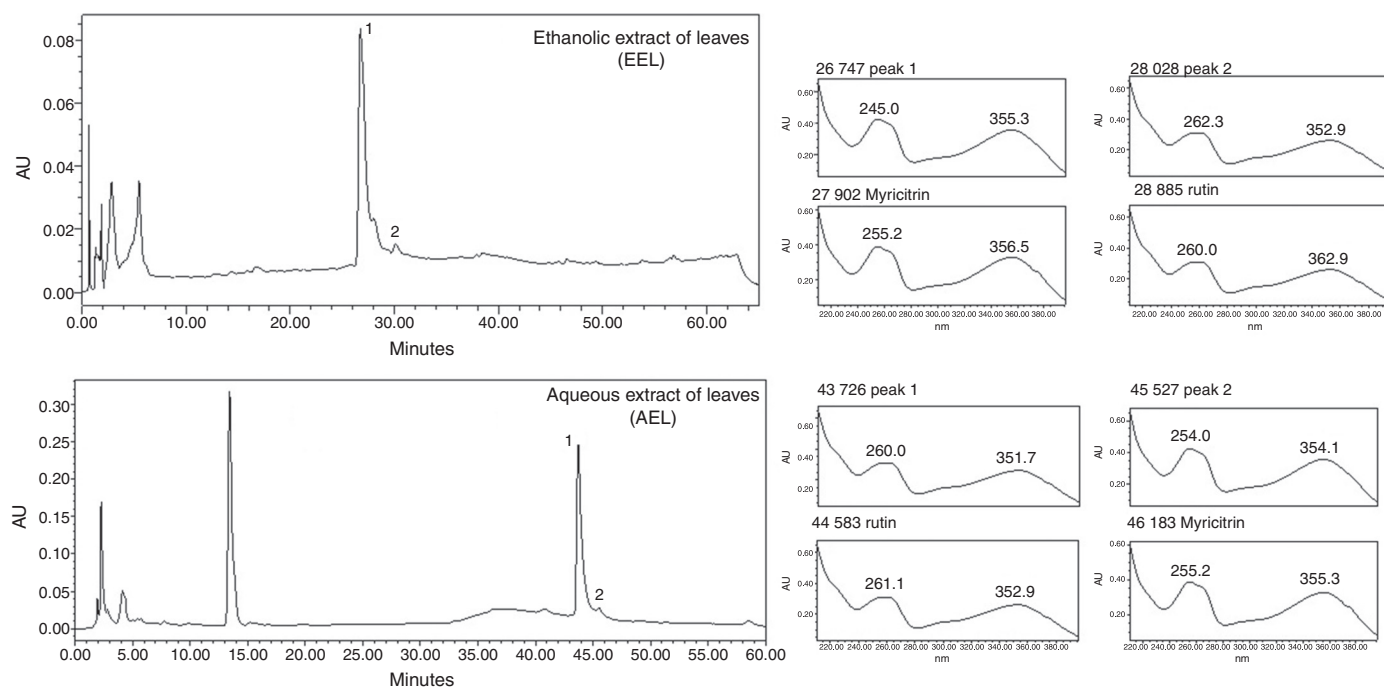


Fig. 1. HPLC chromatogram at 254 nm of EEL and AEL of *Campomanesia velutina* with extracted UV spectrum of identified and standards substances myricitrin and rutin.

intended to treat gout (Ahmad et al., 2008), and in this context, natural products are a potential source of new agents (Kong et al., 2002).

Previous studies with *Campomanesia velutina* demonstrated that the specie possess antinociceptive and anti-inflammatory properties (Michel et al., 2013). The aim of this study was to evaluate the ability of the specie to lower serum urate levels and investigate the mechanisms underlying this effect. In addition, was investigated its ability to act over the inflammatory process triggered by MSU crystals. This way, the results obtained may help develop an alternative therapy to treat gout, since the traditional approach to this disease has low efficacy and various side effects and restrictions.

Flavonoids are naturally occurring plant compounds with antioxidant, anti-inflammatory and XO inhibitory properties (Nagao et al., 1999; Tung et al., 2015). Furthermore, their consumption has been associated with the protective effects of certain diets and herbs against some of the complications of hyperuricemia and gout, such as cardiovascular disease and diabetes (Sampson et al., 2002). A study also demonstrated the ability of the flavonoid quercetin to prevent kidney injury associated with hyperuricemia (Wang et al., 2012). As flavonoids possess important biological effects in inflammation and hyperuricemia, HPLC analysis was carried out in order to verify the presence of some common flavonoids and correlated phenolic substances. AEL and EEL profile showed at retention time of approximately 43 and 30 min, respectively, the presence of substances that absorb energy at two different wavelengths, like flavonoids. The extraction of the chromatograms at 254 nm and the comparison of retention time and $UV_{\lambda_{max}}$ of the peaks with standards revealed the presence of two distinct flavonoids at those retention times (Fig. 1). The presence of the flavonoid myricitrin was confirmed in EEL and found in AEL. In addition, another flavonoid, rutin, was found in EEL and AEL.

Myricitrin and rutin have been reported to possess a wide range of biological activities and many of these activities are related to the biological effects investigated in this study. In previous studies, myricitrin-3-O- α -D-glucopyranoside (myricitrin) was able to modulate the release and/or production of NO, TNF- α and IL-10 on macrophages

(Ferreira et al., 2013; Michel et al., 2013). *In vivo*, myricitrin has been reported as a nitric oxide (NO) and protein kinase C inhibitor that exerts antinociceptive effect (Meotti et al., 2006) and its oral administration reduced TNF- α and COX-2 expression in mice livers (Domitrović et al., 2015). Myricitrin also showed inhibitory effects against TNF- α production in RAW264.7 macrophages (Shimosaki et al., 2011) and irreversibly inactivated myeloperoxidase activity, closely related to the progression of chronic inflammatory diseases (Meotti et al., 2011).

Rutin is a typical flavonoid with several biological effects demonstrated *in vitro* and *in vivo* including antioxidative, anti-inflammatory, anticancer, antidiabetic, antimicrobial, antifungal, anti-allergic, among others. Most of these activities are attributed to the potent antioxidant property of rutin, particularly as a free radical scavenger (Chua, 2013). Rutin can inhibit XOD *in vitro* (Chen et al., 2011) and a three-day oral pretreatment with rutin produced a dose-dependent decrease on serum urate levels in hyperuricemic mice and these effects were partly due to inhibition of XDH/XO activities in mouse liver (Zhu et al., 2004). Rutin also have the ability to inhibit NO production induced by LPS (Shen et al., 2002) and the anti-inflammatory activity of rutin was found to be beneficial for the treatment of rheumatoid arthritis and osteoarthritis (Umar et al., 2012).

XO is the enzyme responsible for the conversion of hypoxanthine into xanthine and of xanthine into uric acid. Assays with this enzyme are used to test compounds that may inhibit the enzyme and thus be useful to the treatment of gout and others diseases related to XO (Haidari et al., 2008). Two different extracts obtained from *Campomanesia velutina* leaves (EEL and AEL) were assayed for XO inhibitory activity *in vitro*. The results are shown in Table 1. At final concentration of 100 μ g/ml, the two extracts produced an inhibition greater than 60% over XO. The IC₅₀ values were determined for both of them since the extracts presented an inhibition bigger than 25% at 100 μ g/ml. EEL showed an IC₅₀ value of 35.63 μ g/ml. For AEL, the IC₅₀ was 47.33 μ g/ml. Allopurinol IC₅₀ was 0.3287 μ g/ml. According to Schmeda-Hirschmann et al. (1996), compounds with IC₅₀ values lower than 50 μ g/ml should be further investigated. Thus, both extracts were *in vivo* assayed to verify if

Table 1

Xanthine oxidase inhibitory activity of ethanolic and aqueous extracts from *Campomanesia velutina* leaves.

Extract	Inhibition at 100 µg/ml (% ± S.E.M.)	IC ₅₀ µg/ml (Confidence interval – 95%)
EEL	66.43 ± 3.17	35.63 (30.14 to 42.84)
AEL	66.82 ± 1.19	47.33 (45.06 to 49.89)
Allopurinol	–	0.33 (–0.5981 to 0.6105)

EEL, ethanolic extract of leaves; AEL, aqueous extract of leaves.

the activity observed *in vitro* would produce important biological effects.

In most mammals, uricase is an enzyme that catalyses the conversion of uric acid into allantoin, thus, serum uric acid levels are typically low. However, human and other primates lost the ability to express uricase and the result is higher serum uric acid levels. Potassium oxonate is the most used uricase inhibitor in animal models of hyperuricemia, since it is a low-cost compound and produces a rapid effect. Therefore, the hyperuricemic model produced by oxonate is the most suitable to the preliminary study of new drugs (Kong et al., 2002). Thus, to assay the hypouricemic activity of *Campomanesia velutina*, hyperuricemic oxonate-induced animals were treated for three days with EEL and AEL at 100 and 300 mg/kg. Fig. 2 shows the results.

Treatment with uricase inhibitor potassium oxonate significantly increased serum urate levels when compared to normal group, showing that the model was effective to induce hyperuricemia. A three-day treatment with the two extracts, at both doses (100 and 300 mg/kg) significantly reduced serum urate levels compared to hyperuricemic control group. As observed on *in vitro* assays, extracts were also able to inhibit XO residual activity *in vivo*. As shown in Table 2, treatment with EEL and AEL for three days was able to inhibit liver XO residual activity in hyperuricemic mice at both doses when compared to control group. Since the reduction of serum uric acid levels was followed by XO inhibition, these results indicate that the anti-hyperuricemic activity of the extracts is strongly related to XO inhibition and this might be the mechanism of action.

Statistical analysis revealed that there were no differences between the doses concerning uric acid levels and XO inhibition. This is probably because the maximum response was reached with

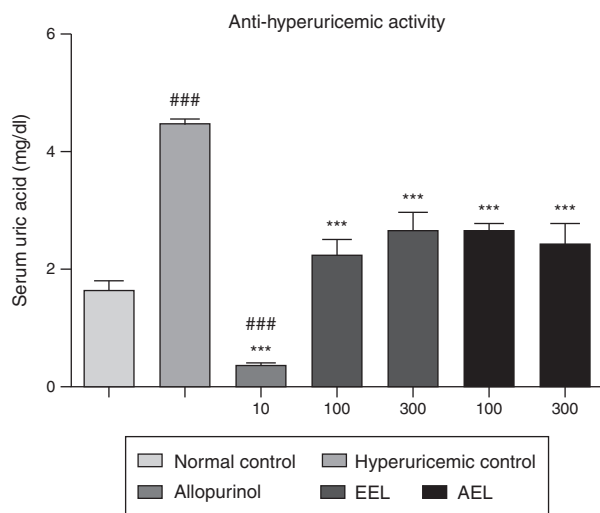


Fig. 2. Anti-hyperuricemic effects of ethanolic and aqueous extracts from *Campomanesia velutina* leaves in mice treated with potassium oxonate. Data represents means ± S.E.M. of six animals. One-way ANOVA followed by Newman–Keuls' multiple comparison test was used for statistical significance. *** $p < 0.001$ compared with hyperuricemic control group. ### $p < 0.001$ compared with normal control group.

Table 2

Effects of ethanolic and aqueous extracts from *Campomanesia velutina* leaves on liver xanthine oxidase residual activity after a three-day oral administration.

Treatment	Dose (mg/kg)	XOD (nm/min/mg protein)	% Inhibition
Vehicle	–	14.86 ± 0.802	–
Allopurinol	10	4.21 ± 0.429 ^a	71.67
EEL	100	7.76 ± 1.452 ^a	47.78
	300	7.49 ± 0.496 ^a	49.59
AEL	100	5.89 ± 0.569 ^a	60.36
	300	5.28 ± 0.345 ^a	64.47

EEL, ethanolic extract of leaves; AEL, aqueous extract of leaves; nm, nanomoles of uric acid.

Data represents mean ± S.E.M. of six animals. One-way ANOVA followed by Newman–Keuls' multiple comparison test was used for statistical significance.

^a $p < 0.001$ compared to hyperuricemic control group.

the dose of 100 mg/kg. This way, an increase in the dose did not produce a better response. In addition, results of uric acid and XO inhibition exhibited no statistical differences between AEL and EEL. This probably means that the active compounds are present on both extracts. In fact, myricitrin and rutin were found in AEL and EEL. Previous studies with rutin demonstrated the ability of this flavonoid to decrease serum urate levels in hyperuricemic mice

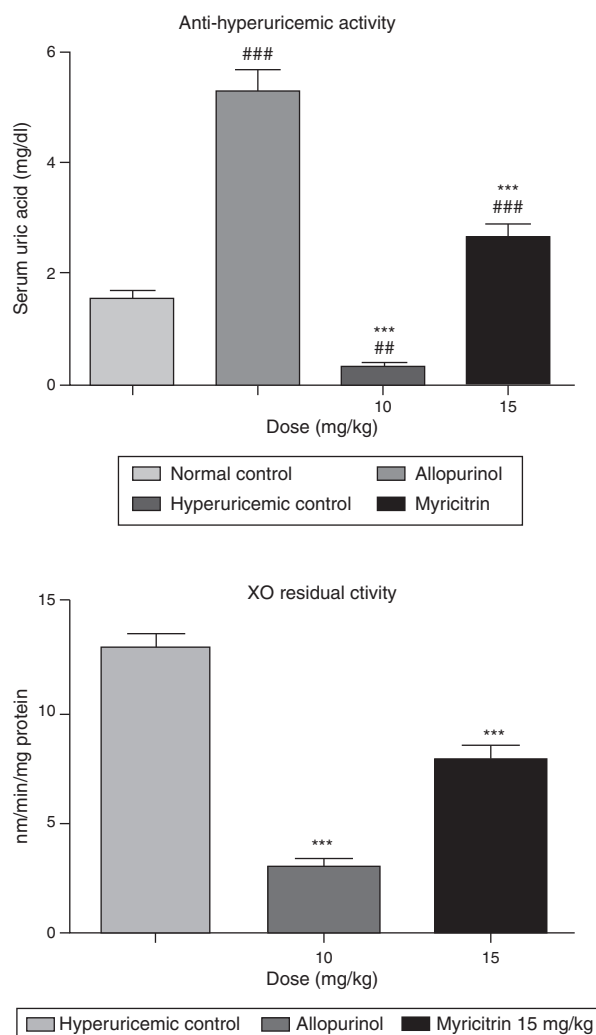


Fig. 3. Myricitrin effects over serum uric acid levels and XO residual activity in mice treated with potassium oxonate. Data represents means ± S.E.M. of six animals. One-way ANOVA followed by Newman–Keuls' multiple comparison test was used for statistical significance. *** $p < 0.001$ compared with hyperuricemic control group. ## $p < 0.01$ and ### $p < 0.001$ compared with normal control group.

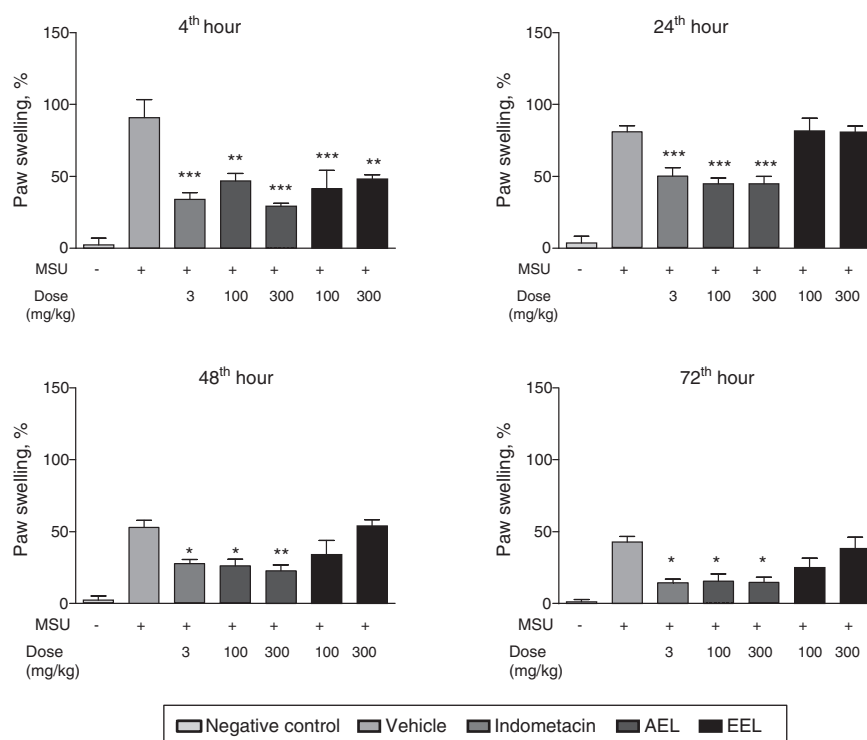


Fig. 4. Effects of aqueous and ethanolic extracts from the leaves of *Campomanesia velutina* on MSU crystal-induced paw edema in mice. Data represents means $\pm$ S.E.M. of nine animals. One-way ANOVA followed by Newman–Keuls' multiple comparison test was used for statistical significance. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with vehicle control group.

with a three-day oral pretreatment (Zhu et al., 2004) and to inhibit XO *in vivo* (Zhu et al., 2004) and *in vitro* (Chen et al., 2011). Because the lack of studies about the anti-hyperuricemic activity of myricitrin, this flavonoid was further investigated and the results showed that myricitrin was able to significantly reduce serum uric acid levels and to inhibit XO residual activity after a three-day treatment at the dose of 15 mg/kg (Fig. 3). Thus, it is reasonable to assume that myricitrin and rutin are related to the effects of AEL and EEL over serum uric acid levels and XO residual activity. However, other compounds can also contribute to the observed effect, since the full composition of the extracts is not known.

Furthermore, the results of uric acid levels and XO inhibition from animals treated with the extracts were not significantly different of those observed on normal group. Allopurinol (10 mg/kg) reduced serum urate levels of hyperuricemic mice to values lower than that found in normal group (Fig. 2) and inhibited 71.67% of XO activity (Table 2). However, the fact that the extracts did not produce such reduction can be considered an advantage. Despite elevated serum uric acid levels can trigger gout and other metabolic disorders, the antioxidant activity of uric acid, particularly its ability to inhibit DNA damage, is well documented (Stinefelt et al., 2005). Therefore, an excessive decrease in uric acid levels may even be harmful (Haidari et al., 2008). Thus, as the extracts reduced serum urate to the same levels observed in normal animals, a possible therapy with them could lead to fewer side effects.

Previous studies with EEL demonstrated its ability to inhibit edema formation after a carrageenan injection (Michel et al., 2013). However, there were no data concerning the aqueous extract or the role of the specie over a gout-like inflammation. Therefore, AEL and EEL were tested about their ability to prevent edema formation triggered by MSU crystals. MSU crystal injection starts a local inflammatory reaction with symptoms similar to those observed clinically in gout, suggesting that this model is able to predict clinical efficacy of new agents (Getting et al., 2002). MSU crystals stimulate innate immune system through the production and

release of several inflammatory mediators, such as kinins, interleukins and TNF- α . Some of these mediators are chemotactics and amplify the inflammatory response leading to neutrophil infiltration followed by the release of oxygen free radicals, lysosomal enzymes, prostaglandin-E₂, leukotrienes and interleukin-1 β . If not treated, the inflammation can lead to structural damage (Martinon et al., 2006).

MSU crystals injection caused a significant increase in paw thickness when compared to negative control (saline administration). Indomethacin (3 mg/kg) promoted a significant reduction on paw swelling observed during the entire experiment. The two extracts at both doses reduced edema formation at 4th hour after MSU injection, but only AEL (100 and 300 mg/kg) was able to maintain the activity throughout the experiment. EEL limited only initial inflammatory response, evaluated 4 h after MSU-crystal injection (Fig. 4). These results indicate that EEL acts only on the initial phase of the inflammatory process initiated by MSU crystals while AEL can act both in the initial and in the late phases of the inflammatory process. Myricitrin and rutin can be involved in the anti-inflammatory activity of the extracts too. As previously detailed, several studies demonstrated the ability of these flavonoids to inhibit the production and/or the release of inflammatory mediators.

The prevalence of gout and hyperuricemia are increasing and the deleterious outcomes of these diseases demonstrate the necessity to treat properly those patients. Gout usual treatment includes the use of anti-inflammatory drugs for symptom relief, especially pain, and the use of XO inhibitors or uricosuric agents to reduce serum uric acid. The problem with this approach is the numerous side effects, the poor control of pain and the high cost. Therefore, the use of a unique agent to treat inflammation, pain and hyperuricemia would be the ideal scenario of gout treatment (Yao et al., 2012). The results of this study are very promising, since the extracts were able to inhibit XO and thus reduce serum uric acid levels. Furthermore, the extracts were able to inhibit edema formation after MSU crystal injection, indicating a potential anti-inflammatory activity. It is also

valid to point out that these effects were observed at the same doses and after an oral treatment, indicating that the active substances are well absorbed in the intestinal tract. This way, aqueous and ethanolic extracts from *Campomanesia velutina* leaves can act over crucial points of gout management: decrease uric acid serum levels by inhibiting xanthine oxidase activity and reduce paw edema induced by MSU. Therefore, these extracts are a promising alternative to treat gout and could be used for the development of an herbal medicine or as a source of new molecules to treat this deleterious disease and thus contribute to increase the armamentarium to achieve a properly management of gout and a good life quality for those patients. However, more studies are necessary to establish all the substances in the extracts that are responsible for the activity, identify and propose the molecular mechanisms under these effects.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

MCPMA (PhD student) contributed in running the laboratory work, analysis of the data and drafted the paper. ZSFF and FCF contributed to the development, implementation and realization of the assays. DASG raised the necessary funds for work development, designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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