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Original Article

In vivo anti-hyperuricemic activity of sesquiterpene lactones from *Lychnophora* species

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ABSTRACT

Hyperuricemia is the main cause of gout, an inflammation induced by uric acid deposition in joints. Drugs available present side effects, so there is a need for new treatment alternatives. *Lychnophora* species are used in folk medicine to treat inflammation, rheumatism and muscle pain. Goyazensolide (10 mg/kg), eremantholide C (25 mg/kg) and lychnopholide (25 mg/kg), sesquiterpene lactones isolated from *Lychnophora* species were previously studied and showed anti-hyperuricemic effects in mice. However, the mechanisms of this effect were not elucidated. The methodology of this study consisted in treatment of hyperuricemic-induced rats, and comparison between control groups, clinically used anti-hyperuricemic drugs and sesquiterpene lactones. Urine and blood were collected for uric acid quantification. Xanthine oxidase inhibition was measured in liver homogenates. Results showed that all evaluated sesquiterpene lactones presented anti-hyperuricemic activity at the doses of 5 and 10 mg/kg and can act through one or both mechanisms, depending on the dose administrated. Goyazensolide and lychnopholide at dose of 5 mg/kg showed important uricosuric effect. Goyazensolide and lychnopholide at dose of 10 mg/kg, and eremantholide C (5 and 10 mg/kg) presented notable inhibition of hepatic xanthine oxidase activity and uricosuric effect. Thus, these sesquiterpene lactones are promising hypouricemic agent to treat hyperuricemia and gout.

Introduction

Hyperuricemia is associated to gout, an inflammatory arthritis caused by deposition of uric acid in joint. Patients with gout (90%) have hyperuricemia and 10% with hyperuricemia develop gout (Smith et al., 2010). Gout is a result of a metabolic disorder in purine catabolism leading to increased uric acid blood concentration (Choi et al., 2004). The strategies to treat gout and hyperuricemia involve using a xanthine oxidase inhibitor to reduce urate production or using a uricosuric agent to increase uric acid excretion. Long-term gout therapy mainly involves reducing blood levels of urate (Rees et al., 2013). There are two drugs capable of inhibiting this enzyme, allopurinol and febuxostat. Febuxostat because it possesses essentially hepatic metabolism is a choice for patients with chronic renal diseases (Harrold et al., 2012). However, it can cause changes in liver function tests, undesirable cardiovascular effects and has a high cost (Rees et al., 2014). Thus, allopurinol is the therapy of choice because it is effective in about 90% of patients, is administered in a single daily dose and has a low cost (Zhang et al., 2006). Adverse effects may occur in 20% of patients in use of allopurinol and are characterized by gastrointestinal intolerance, nausea, rash, hypersensitivity to allopurinol syndrome involving eosinophilia, fever, hepatitis and progressive renal failure (Kim et al., 2013).

Benzbromarone and probenecid, both uricosuric agents, are a second choice treatment and are used in cases of intolerance to xanthine oxidase inhibitors (Reinders et al., 2009). These drugs predispose users to renal calculi by significantly elevating the uric acid concentration in the collecting ducts (Crittenden and Pillinger, 2012). Therefore, there is a need for new drugs to treat hyperuricemia, inflammation and gout.

Nature provides to humans uncountable resources for basic needs and treatment of several diseases. Evidences from approximately 2600 BC demonstrate the use of medicinal plants and derived compounds by people from Mesopotamia region (Cragg and Newman, 2013). Plants' defense mechanisms include production of secondary metabolites. To therapeutics, these metabolites have many potentials, like flavonoids, triterpenes and sesquiterpene lactones found in *Lychnophora* species (Asteraceae) which present many biological activities, like anti-inflammatory and anti-hyperuricemic actions (Gobbo-Neto and Lopes, 2007; De Souza

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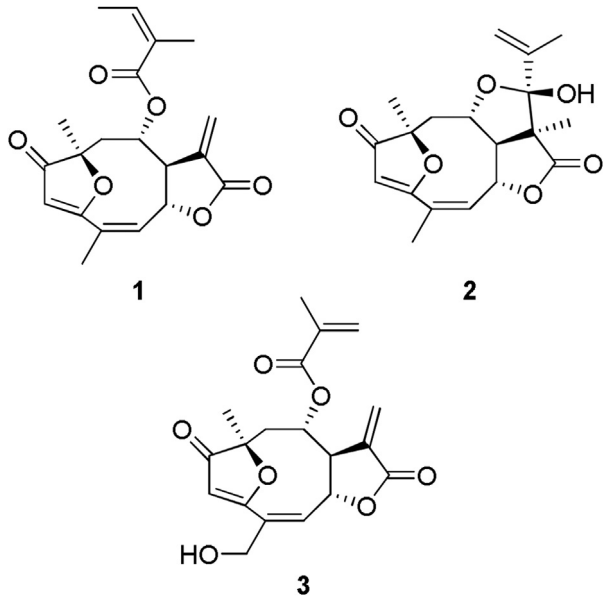
et al., 2012). Species from *Lychnophora* genus are known in Brazil as "arnica" and are used by Brazilian population to treat inflammation, pain, rheumatism and insect bites (Saúde et al., 1998; Chaves et al., 2015) and because of that, they have been studied in order to learn about its chemical composition and biological activities.

Sesquiterpene lactones are found in 90% of species from subtribe *Lychnophorinae*, Asteraceae, including *Lychnophora* species (Keles et al., 2010). Previous studies with sesquiterpene lactones lychnopholide (**1**) and eremantholide C (**2**) isolated from *Lychnophora trichocarpha* showed their anti-inflammatory effect in experiments *in vitro* using J774A.1 macrophage cell line stimulated by LPS. These sesquiterpene lactones also demonstrated *in vivo* anti-inflammatory activity by carrageenan and monosodium urate crystal-induced mouse paw edema models (De Souza et al., 2012; Ferrari et al., 2013), antitumor (Saúde-Guimarães et al., 2014) and antibacterial (Saúde et al., 2002) activities. Another sesquiterpene lactone, goyazensolide (**3**), isolated from several *Lychnophora* species, is an important chemical marker for the genus (Chicaro et al., 2004). *Lychnophora passerina* ethanolic extract presented anti-inflammatory and anti-hyperuricemic activities, being the goyazensolide one of the compounds responsible for these actions (Ugoline et al., 2017). In previous studies, lychnopholide and eremantholide C reduced serum uric acid in swiss mice at dose of 25 mg/kg (De Souza et al., 2012) and goyazensolide at dose of 10 mg/kg (Ugoline et al., 2017). The anti-hyperuricemic mechanism of action of these lactones was not yet elucidated.



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In this study, it was investigated the anti-hyperuricemic effect of lychnopholide, eremantholide C and goyazensolide, at doses of 2.5; 5 and 10 mg/kg, in hyperuricemic Wistar rats and the mechanisms involved in this effect: uricosuric effect and hepatic xanthine oxidase inhibition activity.

Materials and methods

Chemicals and reagents

Xanthine, potassium oxonate, uric acid, probenecid, benzbromarone and allopurinol were purchased from Sigma–Aldrich (USA). Lychnopholide (**1**) (colorless solid, mp 128–129 °C, ethanol) and eremantholide C (**2**) (colorless solid, mp 214–215 °C, ethyl acetate) were previously isolated from *Lychnophora trichocarpha* Spreng. ethanolic extract as described by Saúde-Guimarães et al. (2014). Goyazensolide (**3**) (colorless solid, mp 168.7 to 169.5 °C, hexane/ethyl acetate) was previously isolated from *Lychnophora passerina* (Mart ex DC.) Gardn chloroformic extract as described by Ugoline et al. (2017). Ketamine and xylasine were purchased from Sespro Industria e Comercio Ltda (Brazil). Uric acid assay was purchased from Bioclin (Brazil). All other chemicals used were of analytical grade.

Animals

The anti-hyperuricemic experimental procedure was carried out on male Wistar rats (180–280 g) provided by Animal House of Federal University of Ouro Preto (Ouro Preto, Minas Gerais, Brazil). Animals were divided in groups of six, maintained on plastic cages at a 12 h light/12 h dark cycle with access to water and food *ad libitum*. The experimental protocol was approved by the Ethical Committee on the Use of Animals of the Federal University of Ouro Preto, Brazil (number: 2016/54) and are compliant with NIH guide for the care and use of laboratory animals, published by the US National Institute of Health (NIH Publication No. 80-23, revised in 1978).

Anti-hyperuricemic activity in rats

Anti-hyperuricemic activity was evaluated following the animal model previously described by Murugaiyah and Chan (2009). To induce hyperuricemia, rats received potassium oxonate (200 mg/kg), uricase inhibitor, by intraperitoneal injection and uric acid (1 g/kg) by gavage. The animals were deprived of food and water 12 h before starting the experiment. Each sesquiterpene lactone (goyazensolide, lychnopholide and eremantholide C) was prepared in a mixture of 10% DMSO in 10% Tween 80 aqueous solution at 2.5, 5 and 10 mg/kg doses. Clinically used drugs probenecid and benzbromarone (uricosuric agents) and allopurinol (xanthine oxidase inhibitor) were prepared in a mixture of 10% ethanol in 20% Tween 80 aqueous solution and administered at doses of 50, 10 and 10 mg/kg, respectively. Thirty minutes after hyperuricemia induction, treatments were administered intraperitoneally. Animals from normal and hyperuricemic control groups received only vehicle. The rats were placed individually in metabolic cages and were provided with 100 ml of water. After 5 h, urine was collected in graduated tubes and the water intake was measured. Urine was stored at −20 °C for further uric acid quantification and water discarded. Animals were anesthetized with an association of ketamine and xylasine (80 and 20 mg/kg, respectively) intraperitoneally. After anesthesia, the blood was collected from abdominal aorta and kept at room temperature until coagulation. The blood samples were then centrifuged at 3000 × *g* for 15 min at 4 °C, supernatant serum collected and stored at −20 °C for further uric acid quantification. Right after collecting the blood, liver was removed and washed in 0.9% saline solution. They were stored at −80 °C for further xanthine oxidase quantification.

Uric acid assay

Uric acid was quantified in blood and urine samples by a colorimetric technique using a diagnostic kit (Bioclin, Brazil) following manufacture's instructions.

Xanthine oxidase assay

Allopurinol, a xanthine oxidase inhibitor, was administered to positive control group. Livers were homogenized in 5 ml sodium phosphate buffer 50 mM, pH 7.4. Then, they were centrifuged at 3000 × *g* for 10 min at 4 °C. The lipidic layer was discarded and the supernatant was centrifuged at 10,000 × *g* for 60 min at 4 °C. The

Table 1
Effects of sesquiterpene lactones, probenecid and benzbromarone on water intake, urine output, uric acid excretion and serum uric acid in hyperuricemic rats.

Group	Dose (mg/kg)	Water intake (ml)	Urine output (ml)	Uric acid excretion (mg/(kg 5 h))	Serum uric acid (mg/dl)
Normouricemic control	–	5.7 ± 2.473	5.62 ± 1.550	2.68 ± 0.416	2.87 ± 0.376[h]
Hyperuricemic control	–	6.83 ± 0.830	7.58 ± 0.398	6.03 ± 1.849	9.04 ± 2.281[d]
Benzbromarone	10	6.10 ± 1.194	6.16 ± 1.042	25.33 ± 12.917[d,f]	4.47 ± 1.350[f]
Probenecid	50	6.00 ± 1.527	7.50 ± 2.588	27.29 ± 12.310[d,g]	4.81 ± 1.699[f]
Allopurinol	10	4.82 ± 3.021	8.60 ± 0.894	17.22 ± 6.269[a]	2.27 ± 0.513[h]
Goyazensolide	2.5	6.83 ± 1.213	5.57 ± 2.491	17.88 ± 6.306[a]	6.22 ± 2.067
Goyazensolide	5	3.37 ± 2.310	7.50 ± 2.363	33.86 ± 8.454[d,h]	5.29 ± 1.455[e]
Goyazensolide	10	6.33 ± 2.478	4.33 ± 2.640	20.93 ± 5.367[b,e]	5.61 ± 0.962[e]
Lycnopholide	2.5	4.92 ± 1.426	5.33 ± 1.599	16.66 ± 2.856[a]	5.97 ± 3.744
Lycnopholide	5	3.67 ± 2.285	9.67 ± 3.350	33.93 ± 7.578[d,h]	4.46 ± 1.827[f]
Lycnopholide	10	5.50 ± 3.819	4.83 ± 2.034	20.89 ± 6.589[c,e]	3.92 ± 1.276[g]
Eremantholide C	2.5	6.50 ± 2.500	6.67 ± 3.197	16.93 ± 8.171[a]	5.73 ± 0.808
Eremantholide C	5	3.33 ± 1.886	6.17 ± 0.898	21.72 ± 2.153[c,e]	4.32 ± 2.603[f]
Eremantholide C	10	3.95 ± 0.883	6.00 ± 1.155	22.79 ± 9.352[c,f]	3.27 ± 0.432[h]

Values were expressed as mean ± S.E.M. of six animals. One-way ANOVA was used followed by Dunnett's test for statistical significance.
[a] $p < 0.05$, *vs.* normouricemic group.
[b] $p < 0.01$, *vs.* normouricemic group.
[c] $p < 0.001$, *vs.* normouricemic group.
[d] $p < 0.0001$, *vs.* normouricemic group.
[e] $p < 0.05$, *vs.* hyperuricemic group.
[f] $p < 0.01$, *vs.* hyperuricemic group.
[g] $p < 0.001$, *vs.* hypeuricemic group.
[h] $p < 0.0001$, *vs.* hypeuricemic group.

liver homogenates resultants were assayed to evaluate liver xanthine oxidase residual activity as previously described by Hall et al. (1990) and Ferrari et al. (2016) with modifications. This spectrophotometric assay aims to monitor uric acid formation from xanthine. To begin the assay, 100 μl of liver homogenate was mixed with 5.4 ml of 1 mM potassium oxonate solution in 50 mM sodium phosphate buffer. The mixture was pre-incubated for 15 min at 37 °C. Then, the reaction started by adding 1.2 ml of 250 mM xanthine solution. The reaction was stopped by adding 0.5 ml of 0.6 M HCl at times 0 and 30 min. Samples were centrifuged at 3000 × *g* for 5 min and 3 ml of the supernatant were read by spectrophotometer Varian 50 Bio UV/VIS at 295 nm. Protein total content was quantified by spectrophotometer as described by Bradford (1976) using bovine serum albumin (BSA) as standard. The assays were performed in triplicates. Results for enzyme activity were expressed as nmoles of uric acid produced per minute by 1 mg of protein.

Statistical analysis

Results were expressed as mean values ± S.E.M. Experimental data were analyzed using GraphPad Prism version 6.01 Scientific Software (USA). Statistical significance was determined by One-way analysis of variance (ANOVA) followed by Dunnett's test. *p* values ≤0.05 were considered statistically significant.

Results

Serum uric acid concentration

Hyperuricemic group presented serum uric acid concentration of 9.04 mg/dl, indicating a significant hyperuricemia increase in relation to the normal control. Treatment with allopurinol, positive control at dose of 10 mg/kg, reduced serum uric acid levels to 2.27 mg/dl (Table 1). The group without induced hyperuricemia (normouricemic control) presented 2.87 mg/dl uric acid serum concentration and animals treated with benzbromarone and probenecid (uricosuric drugs) had their serum uric acid levels reduced to 4.47 and 4.81 mg/dl, respectively. Quantification by spectrophotometer showed that serum uric acid concentration decreased in all treated animals with sesquiterpene lactones at doses of 5 and 10 mg/kg, in comparison with hyperuricemic control group (Table 1 and Fig. 1). Treatment with lychnopholide (**1**) at doses of 5 and 10 mg/kg decreased serum uric acid concentration to 4.46 and 3.92 mg/dl, respectively, value close to normouricemic control group. Eremantholide C (**2**) promoted a decrease in serum uric acid levels to concentration of 4.32 mg/dl at dose of 5 mg/kg and 3.27 mg/dl at dose of 10 mg/kg, close to normouricemic group. Goyazensolide (**3**) at dose of 5 mg/kg reduced blood uric acid concentration to 5.29 mg/dl, which was close to benzbromarone and probenecid results. A similar situation occurred with goyazensolide at dose of 10 mg/kg, which was able to decrease serum uric acid to 5.61 mg/dl.

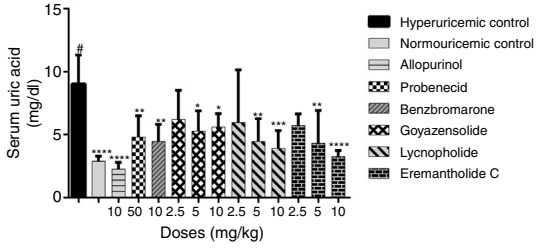

Fig. 1. Effects of treatment with sesquiterpene lactones and clinical used drugs on serum uric acid in hyperuricemic rats. Data represent mean ± S.E.M. of six animals. One-way ANOVA was used followed by Dunnett's test for statistical significance. #$p < 0.0001$, *vs.* normouricemic group; *$p < 0.05$, *vs.* normouricemic group; **$p < 0.01$, *vs.* hyperuricemic group; ***$p < 0.001$, *vs.* hyperuricemic group; ****$p < 0.0001$, *vs.* hyperuricemic group.

Uric acid excretion

Uric acid excretion was expressed in mg/kg for 5 h (Table 1 and Fig. 2). Animals treated with uricosuric drugs, benzbromarone and probenecid, showed uric acid excretion levels of 25.33 mg/kg and 27.29 mg/kg, respectively, in 5 h. Goyazensolide and lychnopholide at dose of 5 mg/kg promoted a uric acid excretion of 33.86 and 33.93 mg/kg in 5 h, respectively, which was a higher elimination comparing to the uricosuric drugs. Goyazensolide, lychnopholide and eremantholide C at dose of 10 mg/kg showed a uric acid excretion of 20.93, 20.89 and 22.79 mg/kg in 5 h, respectively, similar to the clinically used drug benzbromarone. Eremantholide C at dose of

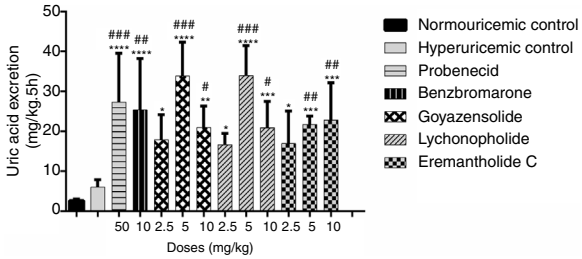

Fig. 2. Uric acid excretion results after treatment of hyperuricemic rats with sesquiterpene lactones and clinical used drugs. Data represent mean ± S.E.M. of six animals. One-way ANOVA was used followed by Dunnett's test for statistical significance. $^{*}p < 0.05$, *vs.* normouricemic group; $^{**}p < 0.01$, *vs.* normouricemic group; $^{***}p < 0.001$, *vs.* normouricemic group; $^{****}p < 0.0001$, *vs.* normouricemic group; $^{\#}p < 0.05$, *vs.* hyperuricemic group; $^{\#\#}p < 0.01$, *vs.* hyperuricemic group; $^{\#\#\#}p < 0.001$, *vs.* hyperuricemic group; $^{\#\#\#\#}p < 0.0001$, *vs.* hyperuricemic group.

5 mg/kg also demonstrated a similar response by uric acid excretion of 21.72 mg/kg in 5 h.

Xanthine oxidase inhibition

Liver homogenates were quantified to allow analysis of liver xanthine oxidase residual activity, enzyme responsible for uric acid synthesis. Hyperuricemic control group compared to normal group showed that there are no interferences by hyperuricemia inducing process on xanthine oxidase activity (Table 2). Allopurinol is known to be a xanthine oxidase inhibitor and was used in this experiment for means of comparison with the sesquiterpene lactones response. Allopurinol inhibited 72.81% of the residual hepatic xanthine oxidase activity. Goyazensolide at dose of 10 mg/kg showed a 36.26% hepatic xanthine oxidase inhibition whereas on the other two doses evaluated was not able to inhibit this enzyme. Lychnopholide was able to inhibit the activity of hepatic xanthine oxidase in 28.31% at dose of 10 mg/kg. Eremantholide C at doses of 5 and 10 mg/kg promoted an inhibition of the enzyme of 29.56% and 31.95%, respectively.

Discussion

Increased serum uric acid concentration is the essential condition leading to the development of gout arthritis, which can cause inflammation and be painful for patients suffering with this disease. Nowadays, there are limited options to treat gout, and these drugs essentially consist of reducing hyperuricemia. Uricosuric agents and xanthine oxidase inhibitors are often used, but they can cause side effects (Reinders et al., 2009). Probenecid is an uricosuric agent that requires multiple daily doses and can lead to problems such as urolithiasis, besides increased bioavailability of allopurinol and other drugs (Dubchack and Falasca, 2010). Benzbromarone is the uricosuric agent of choice for hyperuricemia treatment. However, due to the risk of hepatotoxicity associated with its use, the use of benzbromarone is not permitted in some countries. The hepatotoxic effect of this drug is related to its metabolism, which occurs *via* the cytochrome P450 system in the liver (Terkeltaub, 2003; Reinders et al., 2009; Dubchack and Falasca, 2010). Allopurinol, uricostatic drug most commonly used in the chronic treatment of gout, present as main adverse effects renal and hepatic dysfunction, nephropathies and rashes. In addition, allopurinol is ineffective in episodes of acute gouty arthritis, even aggravating them (Dubchack and Falasca, 2010). Therefore, it is important to look for new therapeutic options.

Lychnophora species are used in Brazilian folk medicine to prepare ethanolic extracts for treatment of diseases such as inflammation, pain, bruises and rheumatism (Saúde et al., 1998). In previous studies, lychnopholide and eremantholide C reduced serum uric acid in swiss mice at dose of 25 mg/kg and were not able to inhibit the activity of hepatic xanthine oxidase (De Souza et al., 2012). In another study, goyazensolide obtained from *L. passerina* demonstrated anti-inflammatory and anti-hyperuricemic effects. Goyazensolide, at dose of 10 mg/kg, was able to decrease serum uric acid levels in mice, by inhibition of hepatic xanthine oxidase activity, hence inhibiting uric acid production (Ugoline et al., 2017).

Another treatment approach to hyperuricemia is by increasing uric acid excretion, which is the uricosuric agent mechanism chose mainly for treatment of chronic gout. Besides increased uricemia, gouty arthritis is also related to inflammation, which is why it is common to use combination of uricosuric agents and/or xanthine oxidase inhibitors with Nonsteroidal Anti-inflammatory Drugs. None of the drug options available nowadays is able to interfere in the reduction of serum uric acid levels and the resolution of inflammation caused by the accumulation of uric acid in the joints (Zhang et al., 2006). The sesquiterpene lactones were proved to be efficient lowering serum uric acid in hyperuricemic rats at doses of 5 and 10 mg/kg.

Goyazensolide, lychnopholide and eremantholide C (5 and 10 mg/kg), demonstrated a decrease in serum uric acid levels, statistically significant comparing to non-treated hyperuricemic control group. It is valid to point out that lychnopholide and eremantholide C promoted a reduction of blood uric acid to levels like those observed on normouricemic control group. This finding is important, because excessive reduction on uric acid level is

Table 2
Effects of sesquiterpene lactones and allopurinol on xanthine oxidase activity in rats with induced hyperuricemia.

Group	Dose (mg/kg)	Xanthine oxidase activity (nmol/min/mg protein)	Inhibition (%)
Normouricemic control	–	10.89 ± 1.033	–
Hyperuricemic control	–	18.97 ± 2.696	–
Allopurinol	10	5.16 ± 1.002[c]	72.81
Goyazensolide	2.5	21.95 ± 3.438	–
Goyazensolide	5	19.67 ± 1.909	–
Goyazensolide	10	12.09 ± 2.174[b]	36.26
Lycnopholide	2.5	22.03 ± 2.146	–
Lycnopholide	5	20.36 ± 2.559	–
Lycnopholide	10	13.60 ± 2.748[a]	28.31
Eremantholide C	2.5	19.56 ± 2.922	–
Eremantholide C	5	13.36 ± 2.230[a]	29.56
Eremantholide C	10	12.91 ± 2.016[a]	31.95

Values were expressed as mean ± S.E.M. of six animals. One-way ANOVA was used followed by Dunnett's test for statistical significance.
[a] $p < 0.05$, *vs.* hyperuricemic control group.
[b] $p < 0.01$, *vs.* hyperuricemic control group.
[c] $p < 0.0001$, *vs.* hyperuricemic control group.

not desirable, since uric acid antioxidant activity and its ability to protect DNA from damage is well documented (Haidari et al., 2008).

The experiments performed in the present study also allowed to evaluate the anti-hyperuricemic mechanisms of the sesquiterpene lactones and to establish the lowest dose in which they exert the hypouricemic effect. Goyazensolide and lychnopholide at dose of 5 mg/kg promoted an intense uric acid excretion and did not inhibit xanthine oxidase. Therefore, uricosuric activity was responsible for lowering blood uric acid concentration. Goyazensolide (10 mg/kg), lychnopholide (10 mg/kg) and eremantholide C (5 and 10 mg/kg) showed both uricosuric effect and inhibition of hepatic xanthine oxidase activity, with a prevalence of the second one. However, goyazensolide and lychnopholide uricosuric effect at dose of 10 mg/kg was even more efficient than the drugs currently used for this purpose.

Conclusion

All evaluated lactones exerted hypouricemic effects. Anti-hyperuricemic activity of the three tested sesquiterpene lactones is related to both evaluated mechanisms: xanthine oxidase inhibition and uricosuric effects. Thus, goyazensolide, eremantholide C and lychnopholide can be considered promising hypouricemic agents to treat hyperuricemia and gout. This study made possible to elucidate the anti-hyperuricemic mechanisms of action of three sesquiterpene lactones obtained from *Lychnophora* species: lychnopholide, eremantholide C and goyazensolide and to determine the lowest dose at which the hypouricemic effect was obtained.

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.

Conflicts of interest

The authors declare no conflicts of interest.

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