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Pharmacological basis for use of Lychnophora trichocarpha in gouty arthritis: Anti-hyperuricemic and anti-inflammatory effects of its extract, fraction and constituents

Maíra Ribeiro de Souza [a], Carmen Aparecida de Paula [a], Michelle Luciane Pereira de Resende [a], Andrea Grabe-Guimarães [b], José Dias de Souza Filho [c], Dênia Antunes Saúde-Guimarães [a,*]

[a] Laboratório de Plantas Medicinais, Escola de Farmácia, Universidade Federal de Ouro Preto, Ouro Preto, Minas Gerais 35400-000, Brazil.
[b] Laboratório de Farmacologia Experimental, Escola de Farmácia, Universidade Federal de Ouro Preto, Ouro Preto, Minas Gerais 35400-000, Brazil.
[c] Departamento de Química, Instituto de Ciências Exatas, Universidade Federal de Minas Gerais, Brazil.

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ABSTRACT

Ethnopharmacological relevance: The ethanolic extract of *Lychnophora trichocarpha* Spreng. is used in Brazilian folk medicine to treat bruise, pain and inflammatory diseases.
Aim of the study: The present study aimed at investigating whether ethanolic extract of *L. trichocarpha*, its ethyl acetate fraction and its main bioactive compounds could be useful to treat gouty arthritis by countering hyperuricemia and inflammation.
Materials and methods: L. trichocarpha ethanolic extract (LTE), ethyl acetate fraction from ethanolic extract (LTA) and isolated compounds were evaluated for urate-lowering activity and liver xanthine oxidase (XOD) inhibition in oxonate-induced hyperuricemic mice. Anti-inflammatory activity in monosodium urate crystal-induced paw oedema, an experimental model of gouty arthritis, was also investigated.
Results: Crude ethanolic extract and its ethyl acetate fraction showed significant urate-lowering effects. LTE was also able to significantly inhibit liver xantine oxidase (XOD) activity in vivo at the dose of 250 mg/kg. Luteolin, apigenin, lupeol, lychnopholide and eremantholide C showed the anti-hyperuricemic activities among tested compounds. Apigenin also showed XOD inhibitory activity in vivo. Luteolin, lychnopholide, lupeol and eremantholide C, in turn, did not shown significant inhibitory activity towards this enzyme, indicating that this mechanism is not likely to be involved in urate-lowering effects of those compounds. LTE, LTA, lupeol, β-sitosterol, lychnopholide, eremantholide, luteolin and apigenin were also found to inhibit monosodium urate crystals-induced paw oedema in mice.
Conclusions: Ethanolic extract of *Lychnophora trichocarpha* and some of its bioactive compounds may be promising agents for the treatment of gouty arthritis since they possesses both anti-hiperuricemic and anti-inflammatory properties.

1. Introduction

Gout is a worldwide-distributed inflammatory arthritis, which is caused by the precipitation of monosodium urate crystals (MSU) in the joints (Lioté and Ea, 2006). Hyperuricemia is kown to be the major risk factor for the development of gout and has also been related to the development of cardiovascular diseases, hypertension, nephrolithiasis and diabetes (Dalbeth and So, 2010; Vázquez-Mellado et al., 2004).

In humans, uric acid forms in the final step of purine catabolic pathway, as a product of the oxidation xanthine by the enzyme xanthine oxidoreductase (XDH/XOD). This enzyme exists in two interconvertible forms: xanthine dehydrogenase (XDH) and xanthine oxidase (XOD) (Chung et al., 1997).

The control of hyperuricemia and the treatment of inflammation are the major therapeutic approaches against gouty arthritis (Liu et al., 2008). At present, allopurinol is the only drug with clinical application in hyperuricemia that acts by inhibiting XOD activity. Although allopurinol is effective in reducing serum urate levels, it is not an appropriate choice to treat acute gout attacks (Dubchak and Falasca, 2010). Moreover, this drug has been associated with adverse effects, such as allergic reactions, skin rashes, fever, hepatitis and nephropathy (Dubchak and Falasca, 2010; Haidari et al., 2009). Nonsteroidal anti-inflammatory drugs

* Corresponding author. Tel.: +55 31 35 59 16 26; fax: +55 31 35 59 16 28.
E-mail addresses: saude@ef.ufop.br, saudeguima@gmail.com (D.A. Saúde-Guimarães).

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such as indomethacin are often used as first-line therapies for acute inflammation in gout. However, they also present some adverse effects such as gastrointestinal toxicity, renal toxicity, or gastrointestinal bleeding (Sabina et al., 2011). Therefore, the search for new anti-inflammatory and urate-lowering drugs, including xanthine oxidase inhibitors, which could be useful in gouty arthritis therapy, has motivated a number of recent studies focused on natural products (Ahmad et al., 2008; Haidari et al., 2008, 2009; Huang et al., 2011; Liu et al., 2008; Mo et al., 2007; Sabina et al., 2011; Zhu et al., 2004).

Species of the genus *Lychnophora* (Asteraceae), popularly known as "arnicas", occur only in Brazilian "cerrado", in Minas Gerais, Goiás and Bahia States. Aerial parts of those species are often used in folk medicine to treat pain, bruise, rheumatism, and inflammatory diseases (Cerqueira et al., 1987; Saúde et al., 1998). Antinociceptive and anti-inflammatory activities of *Lychnophora trichocarpha* ethanolic extract have been evaluated in previous studies (Guzzo et al., 2008). This extract was also shown to inhibit XOD activity in vitro (Ferraz-Filha et al., 2006), although the constituents capable of inhibiting this enzyme have not been identified. Therefore, the aim of this study was to evaluate the in vivo anti-inflammatory, anti-hyperuricemic and liver XOD inhibitory activities of *L. trichocarpha* and its isolated compounds in mice.

2. Material and methods

2.1. Chemicals and reagents

Xanthine, potassium oxonate, allopurinol, indometacin, luteolin and apigenin were purchased from Sigma–Aldrich. Uric acid assay kit was purchased from Bioclin (Minas Gerais, Brazil). Monosodium urate (MSU) crystals were prepared according to previously described method (Rasool and Varalakshmi, 2006; Sabina et al., 2011). Sephadex LH-20 (70–100 μm) was purchased from GE Healthcare. Silica gel 60 (40–63 μm or 63–200 μm) was purchased from Merck. Methanol, ethanol, ethyl acetate, hexane dimethylsulphoxide (DMSO) and Tween 80 were of analytical grade.

2.2. Plant material

Aerial parts of *Lychnophora trichocarpha* Spreng. were collected in Minas Gerais, Brazil, in October, 2006. A voucher specimen (20635) is deposited at the herbarium of Instituto de Ciências Exatas e Biológicas – UFOP, Ouro Preto, Brazil.

2.3. Preparation of plant extract and fractions

Plant material was air-dried and ground. 2.2 kg of the obtained powder was exhaustively extracted with ethanol at room temperature. Solvent was removed under reduced pressure, at 40 °C, yielding 149.0 g of dried crude ethanolic extract (LTE). Part of LTE (130.0 g) was submitted to liquid chromatography on silica gel (63–200 μm), using a step gradient of hexane (3.5 L), ethyl acetate (16.0 L) and methanol (6.5 L), to yield the hexane (LTH, 0.3 g), ethyl acetate (LTA, 53.0 g) and methanolic (LTM, 70.0 g) fractions, respectively.

2.4. Isolation and identification of terpenes and steroid

Part of dried LTA (20.0 g) was fractionated by columns chromatographic on silica gel using hexane, ethyl acetate and methanol gradient as solvent. The fractions eluted with hexane: ethyl acetate (85:15) yielded β-sitosterol (200 mg, white solid, mp 136–137 °C) and lupeol (30 mg, white solid, mp 193–196 °C). The fractions eluted with hexane: ethyl acetate (80:20) and hexane: ethyl acetate (50:50) yielded lychnopholide (7 mg, colorless solid, mp 128–129 °C, ethanol) and eremantholide C (500 mg, colorless solid, mp 194–195 °C, ethyl acetate), respectively.

2.5. Isolation and identification of flavonoids

Part of dried LTA (10.0 g) was resuspended with methanol (20 mL) and submitted to partition with hexane (8 × 30 mL) in order to remove low polarity constituents. The methanol-soluble fraction was chromatographed over a Sephadex LH-20 column (1.5 × 100 cm) eluted with methanol. Fractions 67–90 (335–450 mL) were combined and chromatographed over silica gel, eluted with CH_2Cl_2, ethyl acetate and methanol gradient. Fractions 6–8, eluted with CH_2Cl_2: ethyl acetate (70:30), yielded apigenin (4 mg, light yellow solid, mp > 300 °C, acetone). Fractions 12–16, eluted with CH_2Cl_2: ethyl acetate (50:50 and 40:60), yielded luteolin (10 mg, yellow solid, mp > 300 °C, acetone). Both compounds were identified by NMR spectroscopy and by comparison with spectral literature data (Deng et al., 2004; Özgen et al., 2011).

2.6. Animals

Male albino Swiss mice (25–30 g) were supplied by Universidade Federal de Ouro Preto. Animals were divided into experimental groups ($n=6$), housed in plastic cages and maintained on a 12-h light/12-h dark cycle. They were given standard chow and water ad libitum. All experimental procedures were approved by the Ethical Committee of Universidade Federal de Ouro Preto, Brazil (no. 2010/58).

2.7. Anti-hyperuricemic effects in oxonate-induced hyperuricemic mice and inhibition of liver XOD activity

2.7.1. Animal model of hyperuricemia in mice

An experimental animal model of hyperuricemia induced by potassium oxonate, uricase inhibitor, has been used in order to evaluate the anti-hyperuricemic activity of *Lychnophora trichocarpha* extract (LTE), ethyl acetate fraction (LTA) and pure compounds, as described elsewhere (Haidari et al., 2008; Hall et al., 1990; Zhu et al., 2004). Briefly, potassium oxonate (250 mg/kg) dissolved in 0.9% saline solution was administrated intraperitoneally to each animal, except those of normal control group, 1 h before oral administration of test compounds, once a day, for 3 days of the experiment. Mice were anesthetized with ketamine and xylasine (100 and 20 mg/kg, respectively), 1 h after the final drug administration, in order to allow blood collection from abdominal aorta. The blood was allowed to clot for approximately 1 h at room temperature and then centrifuged at 2500 × g for 10 min. Sera were separated and stored at −20 °C until assay for uric acid quantification.

2.7.2. Study design and drug administration

LTE, LTA and pure compounds were solubilized in DMSO: Tween:water (1:1:8). Animals were divided into 13 experimental groups ($n=6$). Animals were fasted 2 h before drug administration. Mice of goups 1 and 2 (normal control and hyperuricemic control) received only vehicle by oral route. In group 3 (positive control), animals were treated with allopurinol (10 mg/kg body weight, P.O.). Animals of remaining groups were orally treated with LTE (125 and 250 mg/kg body weight), LTA (62.5 and 125 mg/kg body weight), luteolin, apigenin, eremantholide C, lychnopholide, lupeol or β-sitosterol (25 mg/kg body weight). Treatments were administrated once a day, for three consecutive days.

2.7.3. Uric acid assay

Serum uric acid concentration was determined by enzymatic-colorimetric method, using a standard diagnostic kit (Bioclin, Brazil), according to manufacturer's instructions.

2.7.4. Liver sample preparation

Mice livers were excised immediately after blood collection, washed in 0.9% cold saline and rapidly stored at −80 °C until further handling. Enzyme extraction has been performed as described elsewhere (Haidari et al., 2008; Zhu et al., 2004). Briefly, livers were homogenized in 5 mL of 80 mM sodium phosphate buffer (pH 7.4) and, then, the homogenate was centrifuged at 3000 × g for 10 min at 4 °C. Lipid layer was carefully removed, and supernatant was further centrifuged at 10,000 × g for 60 min at 4 °C. The final supernatant was used for enzyme assays.

2.7.5. Liver XOD activity assay

XOD activity was assayed spectrophotometrically by monitoring uric acid formation from xanthine, according to a previously described method (Hall et al., 1990), with modification. The reaction mixtures consisted of 50 mM phosphate buffer (pH 7.4), 50 μL liver homogenate, and 1 mM potassium allantoxanate, to avoid oxidation of uric acid to allantoin, in a final volume of 1.65 mL. After preincubation for 15 min at 37 °C, the reaction was initiated by the addition of 350 μL of 250 μM xanthine. The reaction was stopped after 0 and 30 min by adding 0.15 mL of 0.6 M HCl to the reaction medium. Solutions were then centrifuged at 3000 × g for 5 min. The supernatant were separated and the absorbance measured at 295 nm using a Varian 50Bio UV/VIS spectrophotometer. Protein concentration was determined spectrophotometrically by the method of Bradford (1976) using BSA as standard. XOD activity was expressed as nanomoles of uric acid formed per minute per milligram protein.

2.8. Effects on monosodium urate crystal-induced inflammation in mice

2.8.1. Experimental model of gouty arthritis

An experimental model of gouty arthritis was used in order to evaluate the anti-inflammatory activities of LTE, LTA and pure compounds, as described previously (Rasool and Varalakshmi, 2006; Sabina et al., 2008; 2011), with modifications. Monosodium urate (MSU) crystals were suspended in 0.9% sterile saline (40 mg/mL) prior to use. Inflammation was induced, on the first day of the experiment, by intradermal injection of 0.1 mL (4 mg) of MSU suspension into the mice right hind paw. The left paw was injected with vehicle (negative control).

2.8.2. Study design and drug administration

L. trichocarpha extract (LTE), its ethyl acetate fraction (LTA) and pure compounds were solubilized in DMSO: Tween:water (1:1:8). Animals were divided into 12 groups ($n=6$). Treatments were administered 1 h before MSU injection and repeated daily for 2 more days. Mice of goup 1 were orally treated with vehicle and served as MSU-induced control. Mice of group 2 were orally treated with the standard non-steroidal anti-inflammatory drug indometacin (3 mg/kg, P.O.). Animals of remaining groups were orally treated with LTE (125 and 250 mg/kg), LTA (125 and 250 mg/kg), luteolin, apigenin, eremantholide C, lychnopholide, lupeol or β-sitosterol (25 mg/kg).

Paw thickness was measured with a caliper rule (150 mm–6 in, Vonder, China) at 0, 4, 24 and 48 h after MSU injection. Inflammatory swelling was expressed as thickness variation (Δ).

2.9. Statistical analysis

Results of in vivo assays were presented as mean values ± S.E.M. Experimental data were analyzed using GraphPad Prism 5.0 Software (Inc., San Diego, CA, U.S.A.). One-way analysis of variance (ANOVA) was used, followed by Student's Newman–Keul's test. P values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Effects of LTE, LTA and pure compounds on serum urate levels in hyperuricemic mice

Treatment with the uricase inhibitor potassium oxonate significantly increased serum urate levels compared to normal control group. Allopurinol (10 mg/kg), as a positive control, was able to reduce serum urate levels of hyperuricemic mice to values lower than that found in normal animals. A three-day treatment with LTE at the dose of 250 mg/kg, but not at 125 mg/kg, significantly reduced serum urate levels compared to hyperuricemic control group. Fraction LTA showed significant anti-hyperuricemic activity at doses

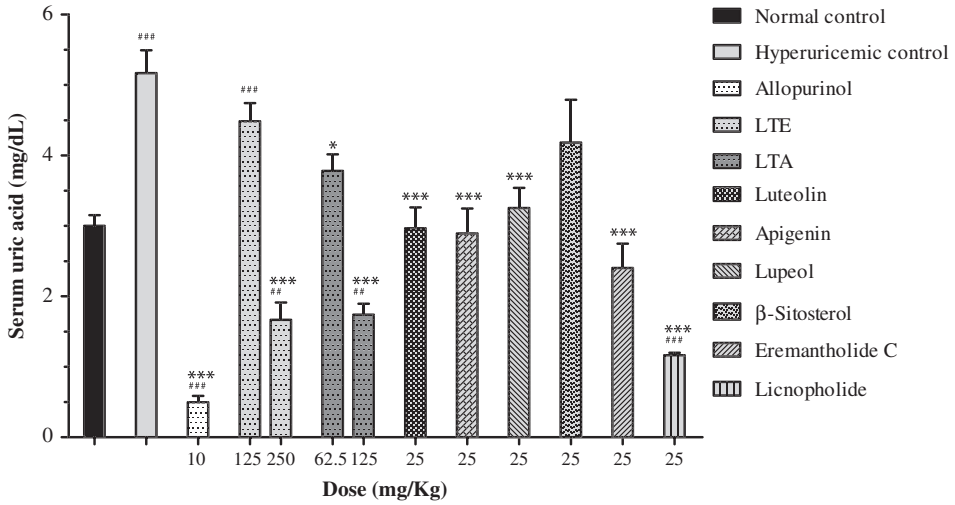

Fig. 1. Anti-hyperuricemic effects of *L. trichocarpha* ethanolic extract (LTE), ethyl acetate fraction (LTA) and pure compounds in mice pretreated with potassium oxonate. Experiments were performed as described in Section 2. Data represent mean ± S.E.M. of 6 animals. One-way ANOVA followed by Student's Newman–Keul's test was used for statistical significance.*$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with hyperuricemic control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ compared to normal control group.

higher than 62.5 mg/kg. Luteolin, apigenin, lupeol, lychnopholide and eremantholide C were able to significantly reduce serum urate levels at the dose of 25 mg/kg (Fig. 1).

3.2. Effects of LTE, LTA and pure compounds on XOD activity in mice liver

Treatment with LTE at the dose of 250 mg/kg was able to inhibit liver XOD activity by 34.8% when compared to hyperuricemic control group. Apigenin also caused significant inhibition of liver XOD activity at the dose of 25 mg/kg (38.4%). Allopurinol inhibited XOD activity by 85.64% (Table 1).

Table 1.
Effects of *L. trichocarpha* ethanolic extract (LTE), ethyl acetate fraction (LTA) and pure compounds on xanthine oxidase activity in mouse liver in vivo.

Treatment	Dose (mg/kg)	XOD activity (*U*/mg protein)	Inhibition (%)
Hyperuricemic control	-	13.16 ± 1.39	–
Allopurinol	25	1.91 ± 0.12***	85.5
LTE	125	10.89 ± 0.72	–
	250	8.58 ± 1.07**	34.8
LTA	62,5	13.03 ± 0.73	–
	125	10.28 ± 0.32	–
Luteolin	25	12.04 ± 0.37	–
Apigenin	25	8.10 ± 0.91**	38.4
Lupeol	25	14.54 ± 0.54	–
β-Sitosterol	25	12.70 ± 0.91	–
Eremantholide C	25	12.82 ± 0.56	–
Lychnopholide	25	10.89 ± 1.07	–

Data represent mean ± S.E.M. of six animals. One-way ANOVA followed by Student's Newman–Keul's test was used for statistical significance.

** $P < 0.01$,.

*** $P < 0.001$ compared to hyperuricemic control group. *U*= nanomole uric acid per minute.

3.3. Effects of LTE, LTA and pure compounds on monosodium urate crystal-induced inflammation in mice

MSU crystals injection caused a significant increase in paw thickness when compared to negative control (left paw). Paw swelling was found to be reduced in mice treated with LTE and LTA both at 125 and 250 mg/kg (Fig. 2). Lupeol, β-sitosterol, luteolin, apigenin, eremantholide C and lychnopholide, at 25 mg/kg, were also able to reduce the paw swelling induced by MSU crystals injection. Indometacin (3 mg/kg) has also shown a significant anti-inflammatory activity in this study.

4. Discussion

The sesquiterpene lactones lychnopholide and eremantholide C, the pentacyclic triterpene lupeol and the steroid β-sitosterol have been isolated from *L. trichocarpha* in previous studies (Oliveira et al., 1996; Saúde et al., 1998). In the present study, we have isolated luteolin and apigenin from the active LTA fraction. As far as we know, this is the first report of the occurrence of these flavones in *L. trichocarpha*.

Anti-inflammatory and anti-hyperuricemic properties are convenient for compounds intended to treat gouty arthritis (Ahmad et al., 2008), but none of the clinically available medicines has both effects at the same time (Liu et al., 2008). A previous study (Ferraz-Filha et al., 2006) demonstrated that ethyl acetate and ethanolic extracts of *L. trichocarpha* inhibit XOD in vitro, showing IC_{50}=6.2 and 28.8 μg/mL, respectively, which justifies the study of this species in order to identify its active compounds and to

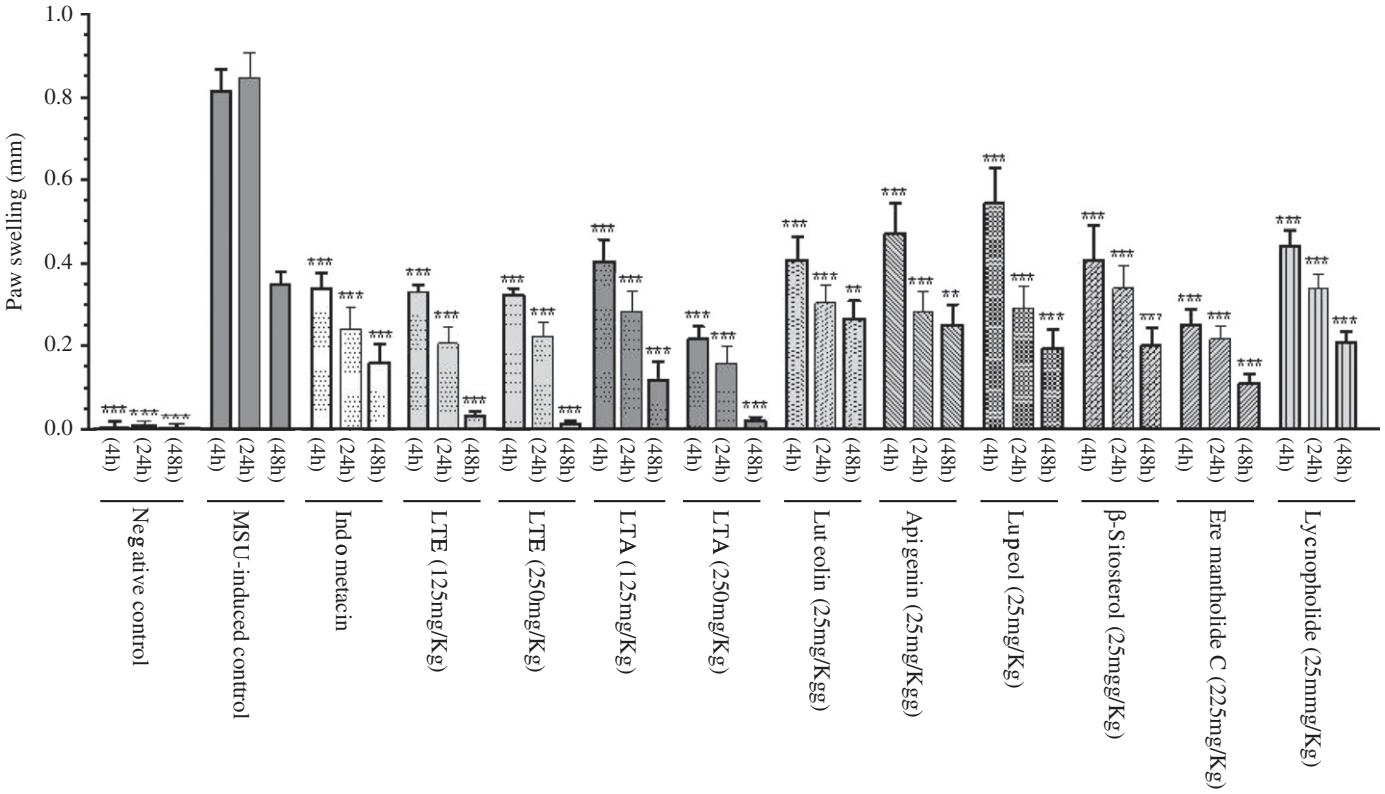

Fig. 2. Effects of *L. trichocarpha* ethanolic extract (LTE), ethyl acetate fraction (LTA) and pure compounds on MSU crystal-induced paw oedema in mice. Experiments were performed as described in Section 2. Data represent mean ± S.E.M. of six animals. One-way ANOVA followed by Student's Newman–Keul's test was used for statistical significance. **$P < 0.01$, ***$P < 0.001$ compared with with paw swelling after MSU crystals injection in vehicle-treated mice.

evaluate their anti-hyperuricemic effects in vivo. Moreover, *L. trichocarpha* anti-inflammatory property has been demonstrated in a preliminary screening study (Guzzo et al., 2008), as suggested by its popular use. Thus, the present study aimed at investigating the effects of this species and its main known constituents in an animal model of gouty arthritis.

LTE and LTA have shown dose-dependent anti-hyperuricemic effects on oxonate-pretreated mice in vivo. LTA seemed to be more potent in reducing urate levels than LTE, which, a priori, was consistent with results reported by Ferraz-Filha et al. (2006) regarding the XOD inhibition in vitro. However, LTA was not able to significantly inhibit mice liver XOD activity in vivo at doses up to 125 mg/Kg, which indicates that this fraction may accomplish its anti-hyperuricemic effect mainly through other action mechanisms.

Lychnopholide, eremantholide C, lupeol, apigenin and luteolin are the main responsible for LTA urate-lowering effect. Eremantholide C, lychnopholide, lupeol and luteolin did not shown significant effect towards liver XOD activity. Thus, the urate-lowering effects of those compounds are probably due to other mechanisms which must be further investigated. Apigenin, in turn, was able to moderately inhibit liver XOD activity, which was accompanied by a reduction in serum urate levels. Apigenin has been shown to be a potent competitive XOD inhibitor in previous studies (Lin et al., 2002; Van-Hoorn et al., 2002), showing an IC_{50} value of 0.75 μM (Van-Hoorn et al., 2002). Therefore, this mechanism is likely to contribute for the urate-lowering effects of this compound in vivo.

MSU crystal-induced inflammation is characterized by infiltration of neutrophils and subsequent release of damage-causing oxygen derived free radicals, lysosomal enzymes, as well as prostaglandin E2, leukotrienes and interleukin-1 (Liu et al., 2008; Sabina et al., 2011). Results of our study suggest that LTE and its fraction LTA can limit MSU-induced acute inflammatory response. Such activity is probably due to the combined effects of lupeol, β-sitosterol, lychnopholide, eremantholide C, luteolin and apigenin.

Previous studies have shown that topical treatment with lupeol or β-sitosterol inhibit neutrophil migration in 12-*O*-tetradecanoyl-phorbol acetate (TPA) induced ear oedema model (Fernández et al., 2001; Navarro et al., 2001). Lupeol was also shown to inhibit IL-1, TNF-α and PGE_2 release by macrophages in vitro (Fernández et al., 2001) and to modulate NF-κB transcription factor pathway (Saleem et al., 2004). Thus, the inhibitory effect of those compounds on MSU-induced paw oedema is probably mediated by the above-mentioned mechanisms.

Luteolin and apigenin have been found to modulate COX-2 and iNOS expression and to down regulate the release of NO and TNF-α (Kim et al., 2004; López-Lázaro, 2009; Mattace Raso et al., 2001). Apigenin was shown to inhibit NF-κB expression as well (Kim et al., 2004). Moreover, these flavones are known to possess strong antioxidant and free radical scavenging properties (Gomes et al., 2008), and could thus reduce cellular damage caused by superoxide and other reactive oxygen species that are involved in acute inflammation in gout.

Although some sesquiterpene lactones structurally related to lychnopholide and eremantholide C have been shown to inhibit the DNA binding of NF-κB (Rüngeler et al., 1999), the exact action mechanism by which these two compounds exert their anti-inflammatory activity has not been well elucidated so far.

5. Conclusion

The ethanolic extract of *L. trichocarpha* (LTE) and its ethyl acetate fraction (LTA) were able to reduce serum urate levels in hyperuricemic mice. Anti-hyperuricemic activities of LTA and LTE are, in part, due to the synergistic actions of the apigenin, luteolin, lupeol, lychnopholide and eremantholide C. The anti-hyperuricemic activity of the apigenin seems to be mediated by XOD inhibition, while eremantholide C, lupeol, luteolin and lychnopholide are likely to possess other action mechanisms. Furthermore, LTE, LTA and its pure constituents proved to be effective in reducing MSU-induced paw oedema in mice. Therefore, *L. trichocarpha* and some of its active compounds may be promising agents for the treatment of gouty arthritis.

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