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Sesquiterpene lactones from *Lychnophora pohlii*: neutrophil chemiluminescence inhibition and free radical scavenger activity

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Abstract

Excessive production of reactive oxygen species (ROS) by polymorphonuclear leucocytes (PMNLs) is thought to contribute to the pathology of many inflammatory diseases. Sesquiterpene lactones (STLs) seem to be important contributors to the anti-inflammatory activity of many species of *Lychnophora* (Asteraceae), which have been widely used in Brazilian folk medicine because of this pharmacological property. In this study, the inhibitory effects of three STLs isolated from *Lychnophora pohlii* (lychnopholide, centratherin and goyazensolide) on rabbit PMNL oxidative burst were evaluated by the luminol-enhanced chemiluminescence (CL-lum) assay. All STLs tested showed concentration-dependent inhibitory activity on CL-lum but were not cytotoxic to PMNLs (evaluated by lactate dehydrogenase release) under the assessed conditions. Moreover, goyazensolide, the most active STL, had no free radical scavenger property, as assessed by 1,1-diphenyl-2-picrylhydrazyl radical assay, and had no inhibitory effect on the luminol-horseradish peroxidase–hydrogen peroxide chemiluminescence. Taken together, the results of this investigation suggest that the concomitant presence of methacrylate ester and hydroxyl groups contributes to a high inhibitory effect on PMNL oxidative metabolism. This effect was not mediated by free radical scavenger or cytotoxic effects, but probably by inhibition of enzymes involved in the signal transduction pathways of the ROS generation process.

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Introduction

Polymorphonuclear leucocytes (PMNLs) or neutrophils play a crucial role in the defence of mammals against microbial infections (Johnson et al 1992). Recognition of opsonized microbes or particles via complement or antibody receptors triggers a set of effector functions of PMNLs, such as phagocytosis and production of reactive oxygen species (ROS) (Cheung et al 1983; Haas et al 1995). However, excessive release of these ROS can result in undesirable tissue and molecular damage, which have been reported as important participants in a number of inflammatory diseases, including cancer, rheumatoid arthritis, emphysema and acute respiratory distress syndrome (Weiss 1989; Babior 2000). The involvement of PMNL-derived ROS in the physiopathology of various inflammatory illnesses has attracted the interest of many research groups trying to find new compounds to modulate inflammation and to understand the underlying mechanisms of action.

Traditionally, the pharmaceutical industry has used secondary metabolites isolated from higher plants as natural sources of new drugs (Barreiro & Fraga 2001). Plants from the genus *Lychnophora* sp. (Asteraceae) are known as 'arnica' and have been widely used in Brazilian folk medicine due to their anti-inflammatory and analgesic activity (Cerqueira et al 1987; Grael et al 2000; Vieira & Martins 2000). Sesquiterpene lactones (STLs), flavonoids and triterpenes are major compounds occurring in the *Lychnophora* genus (Bohlmann & Jakupovic 1990). STLs display a wide variety of pharmacological and biological properties and are known to have considerable

anti-inflammatory activity, determined by in-vitro and in-vivo assays (Hall et al 1979, 1980). Rungeler et al (1999) observed that these natural products inhibit arachidonic acid pathway enzymes, such as cyclooxygenase type 1 and phospholipase A_2, and the activation of nuclear transcription factor-kappa B (NF-κB). Recently, Siedle et al (2002) also reported inhibition of the catalytic activity and release of human PMNL elastase by low concentrations of STLs. Previous investigations reported a ROS scavenger effect in-vitro (Seitembetova et al 1995; Jodynis-Liebert et al 1999) and inhibition of PMNL respiratory burst associated with the inflammatory process (Pérez-Garcia et al 1996; Nuñez et al 2003).

In this study we evaluated the effect of lychnopholide (STL1), centratherin (STL2) and goyazensolide (STL3), isolated from *Lychnophora pohlii*, on the PMNL oxidative burst and some aspects related to their mechanism of action. In addition, we investigated the relationship between chemical structure and biological activity of these compounds.

Material and Methods

Chemicals

Isolation and identification of STLs from *Lychnophora pohlii* has been reported previously (Grael et al 2005). Quercetin (3′,4′,3,5,7-pentahydroxyflavone), luminol (5-amino-2,3-dihydro-1,4-phtalazinedione), 1,1-diphenyl-2-picryhydrazyl radical (DPPH), zymosan A (isolated from *Saccharomyces cerevisiae*) and horseradish peroxidase (HRP) type IV-A (E.C.1.11.1.7) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Dimethyl sulfoxide (DMSO; Merck-Schuchardt, Hohenbrunn, Germany), hydrogen peroxide (H_2O_2; Labsynth, Diadema, SP, Brazil) and LDH Liquiform (Labtest Diagnostica, Lagoa Santa, MG, Brazil) were the other chemicals used.

Isolation of polymorphonuclear leucocytes (PMNLs)

Peripheral blood from adult female New Zealand White rabbits (weighing about 3 kg) was collected into Alsever solution (v/v) as anticoagulant. The experimental procedure was approved by the Animal Care and Use Ethics Committee from University of São Paulo, campus of Ribeirão Preto (Ribeirão Preto, SP, Brazil) under the protocol number 05.1.1050.53.9. PMNLs were isolated as described by Kabeya et al (2002) and suspended in Hank's balanced salt solution (HBSS; pH 7.2; Paul 1970) containing 0.1% gelatin (w/v) (HBSS-gel). Cells were >90% viable as determined by Trypan blue exclusion test and 80–90% were PMNLs.

ROS generation by stimulated PMNLs

ROS generation was measured by luminol-enhanced chemiluminescence (CL-lum) using the luminometer Autolumat LB953 (EG&G Bethold, Germany), as previously described by Kanashiro et al (2004). Concentrations of each component in a final reaction volume of 1.0 mL are indicated in parentheses. PMNL suspensions (1×10^6 cells/mL) were added to 10 μL of luminol (0.28 mmol L^{-1}) and 10 μL of each test-compound (2.5–125 μmol L^{-1}) or DMSO for the control tube. Luminol and test compounds were previously dissolved in DMSO.

Reaction tubes were incubated for 3 min at 37°C and then opsonized zymosan (1 mg mL^{-1}) was added (Kanashiro et al 2004). CL-lum produced by the stimulated PMNLs was continuously recorded in photon counts per minute (cpm) for 10 min at 37°C. Values of the area under CL-lum time-course curves (AUC), also called CL-lum integrated areas, were determined. The values represent the total amount of ROS produced by PMNLs in 10 min, and CL-lum inhibition promoted by STLs was expressed as percentages, calculated as follows: [(1 − AUC of test compound)/AUC of control] × 100. Quercetin was used as reference compound.

DPPH scavenging activity

The antioxidant potential of STLs was evaluated by the ability to scavenge the stable free radical DPPH, according to a modified method of Okawa et al (2001). Concentration of each component in a final reaction volume of 2.5 mL is indicated in parentheses. Samples of 10 μL of compound (2.5–125 μmol L^{-1}) or 10 μL of DMSO (control) were mixed with DPPH solution (100 μmol L^{-1}) in ethanol. Absorbances were measured spectrophotometrically at 517 nm. The scavenging activity was expressed as percentage of DPPH discoloration and calculated as follows: [(1 − absorbance of test compound)/absorbance of control] × 100. Quercetin was used as reference compound.

ROS generation by the luminol–HRP–H_2O_2 system

ROS scavenger activity in the enzymatic system was evaluated according to Krol et al (1994) with some modifications. Concentrations of each component in a final reaction volume of 1 mL are indicated in parentheses. Samples (10 μL) of each test compound (0.1–1.0 μmol L^{-1}) or 10 μL of DMSO (control) were mixed with H_2O_2 (50 μmol L^{-1}) and 10 μL of luminol (0.28 mmol L^{-1}). ROS generation was initiated by addition of HRP (0.2 IU mL^{-1}). CL-lum production was measured in a luminometer for 10 min at 27°C. CL-lum inhibition was expressed as percentage, as described for the cellular assay. Luminol and test compounds were prepared in DMSO, whereas H_2O_2 and HRP were prepared in 0.1 mol L^{-1} phosphate buffer pH 7.4. Quercetin was used as reference compound.

Cytotoxicity evaluation

PMNL suspensions in HBSS-gel (1×10^6 cells/mL) were incubated for 15 min at 37°C with DMSO (0.28 mol L^{-1}, solvent control), or HBSS (negative control) or 10 μL of test compounds dissolved in DMSO, at final concentration of 125 μmol L^{-1}. Final reaction volume was 1 mL.

Total cell lysis (positive control) was achieved with 0.2% (v/v) Triton X-100. All tubes were centrifuged at 755 *g* for 10 min at 4°C. The activity of lactate dehydrogenase (LDH) released into the supernatant was measured using the test kit LDH Liquiform. The method is based on NADH absorption decrease at 340 nm, measured in a DU-70 spectrophotometer (Beckman, Fullerton, CA, USA) for 3 min at 37°C.

Statistical analysis

Data were processed and analysed with the Graph Pad Prism Software (version 3.00 for Windows). Statistical analysis was performed by analysis of variance followed by Tukey's test or Student's *t*-test, as indicated in the figure legends. $P < 0.05$ was considered statistically significant.

Results

Evaluation of inhibitory effects of STLs on stimulated-PMNL oxidative metabolism

Production of ROS by stimulated PMNLs was measured in the absence (control) or presence of STLs (Figure 1) at different concentrations (2.5–125 μmol L^{-1}). All compounds tested inhibited CL-lum from stimulated PMNLs in a concentration-dependent manner. The inhibitory effects of STLs were generally lower than that of the reference compound, quercetin (12.5–125 μmol L^{-1}), and STL3 was the most active among the STLs tested (Figure 2) ($P < 0.05$).

Cytotoxicity study

We also investigated whether the decrease of CL-lum was related to a cytotoxic effect of the tested compounds on PMNLs. None of the STLs, at a final concentration of 125 μmol L^{-1}, induced a significant LDH release when compared with the control (DMSO) (Table 1). This suggests that quercetin and the STLs tested were not toxic to rabbit PMNLs under the assessed conditions.

Evaluation of the free radical scavenging activity of STLs

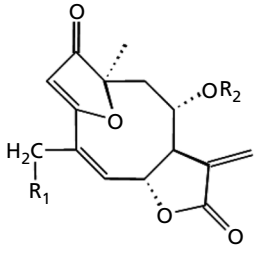
The free radical scavenging activity of STL3, the most active of the molecules tested (Figure 3), was investigated as a possible mechanism underlying PMNL CL-lum inhibition. Under the assessed conditions, STL3 was not able to scavenge the stable free radical DPPH (Figure 3A) or the ROS generated by the luminol–HRP–H_2O_2 system (Figure 3B). On the other hand, the free radical scavenging activity of quercetin on both systems was statistically significant, at the same range of concentrations.

Discussion

Recently, extensive research has been focused on the discovery of novel anti-inflammatory drugs by studying folk medicines and compounds isolated from natural sources (Barreiro & Fraga 2001). Increased knowledge about the mechanisms involved in the development of inflammatory reactions has allowed establishment of experimental parameters to evaluate the activity of new anti-inflammatory compounds, such as synthesis and release of cytokines, development of oedema, increase in vascular permeability, activation of NF-κB transcription factor and release of ROS by phagocytic cells. Among the strategies to attenuate excessive inflammation, a great deal of interest has been paid to the possible modulation of oxidant production by PMNLs. Although the oxidative burst represents an essential physiological response to injury, over-regulated PMNL recruitment and activation, which have been reported to be involved in various autoimmune and chronic inflammatory diseases, result in severe damage to adjacent normal tissues and can contribute to the amplification of inflammation processes (Weiss 1989; Johnson et al 1992; Miesel et al 1996; Babior 2000).

Boughton-Smith et al (1993) reported that leucocyte-derived ROS are important to arachidonic acid metabolism during the development of carrageenan-induced paw oedema in the rat, supporting the hypothesis that control of ROS production might be involved in the maintenance of an inflammatory process. On the other hand, Parij et al (1998) reported that many commercially available anti-inflammatory drugs also inhibit ROS production by stimulated PMNLs, and this mechanism may account for their pharmacological effect.

In this investigation, the in-vitro inhibitory effect of three STLs isolated from *Lychnophora pohlii* on PMNL oxidative metabolism was evaluated. Oxidant production by opsonized zymosan-stimulated PMNLs was measured by the luminol-enhanced chemiluminescence method (CL-lum), which is a highly sensitive assay for monitoring the



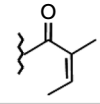
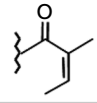
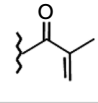
	Lychnopholide (STL1)	Centratherin (STL2)	Goyazensolide (STL3)
R_1	H	OH	OH
R_2			

Figure 1 Chemical structures of the tested sesquiterpene lactones.

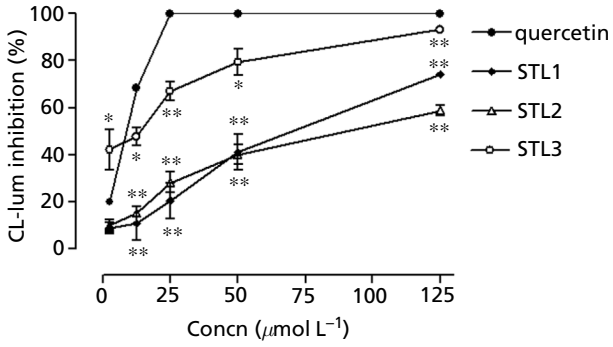


Figure 2 Inhibitory effect of sesquiterpene lactones isolated from *Lychnophora pohlii* on the luminol-enhanced chemiluminescence (CL-lum) of stimulated rabbit PMNLs. Values are shown as percent of CL-lum inhibition (means ± s.e.m.) of three independent experiments with duplicate measurements. $*P < 0.01$, $**P < 0.001$, compared with the reference compound quercetin (analysis of variance and Tukey's post-hoc test).

Table 1 Evaluation of the toxicity of sesquiterpene lactones and quercetin at 125 μmol L^{-1} on rabbit PMNLs

Compound[a]	LDH activity (IU × 1000)[b]
Triton X-100	138.9 ± 5.7
DMSO	17.4 ± 2.5
STL1	20.3 ± 2.0
STL2	16.8 ± 2.3
STL3	21.8 ± 2.1
Quercetin	20.8 ± 0.8

[a]Compound names and structures are shown in Figure 1. DMSO, control tube (dimethyl sulfoxide, 0.28 mol L^{-1}). Triton X-100 at 0.2% (v/v) promoted total (100%) lysis of 1×10^6 PMNLs, and was used as positive control. [b]Data are expressed as mean ± s.e.m. of three independent experiments with duplicate measurements.

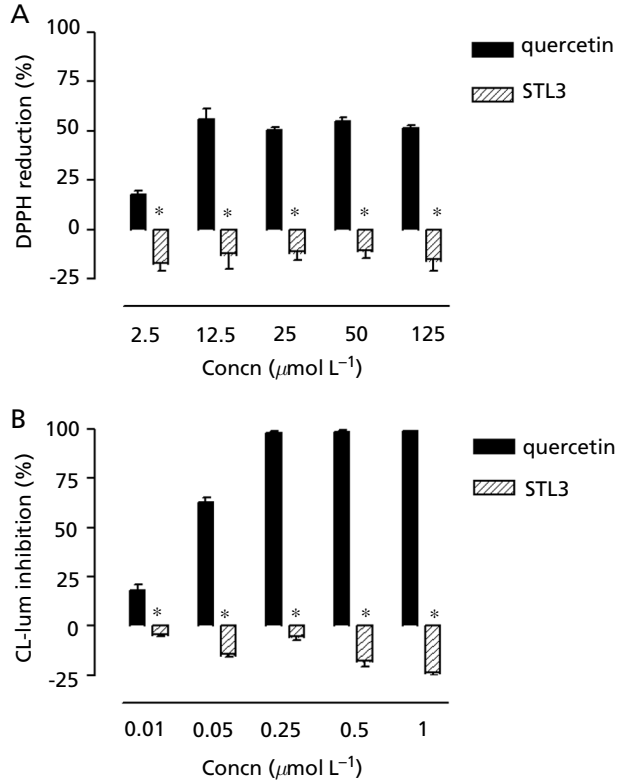


Figure 3 Evaluation of free radical scavenging activity of STL3 and the reference compound quercetin at different concentrations. Values are shown as means ± s.e.m. of three independent experiments with duplicate measurements. A. Scavenging of the stable free radical DPPH, evaluated by absorbance changes at 517 nm. B. Scavenging of reactive species generated by the luminol–HRP–H_2O_2 system, evaluated by CL-lum inhibition. $*P < 0.001$, compared with the reference compound quercetin (Student's t-test).

overall production of ROS by stimulated PMNLs. CL-lum measures all kinds of PMNL-generated ROS, with a higher sensitivity to those produced by the myeloperoxidase–H_2O_2–halide system (Dahlgren & Karlsson 1999).

STL3 was found to be the most effective inhibitor of PMNL oxidative metabolism, followed by STL2 and STL1 (Figure 2). Interestingly, the chemical structures of the three test compounds are closely related, and few modifications led to significant changes in the biological activity. For example, at the highest concentration (125 μmol L^{-1}), the additional presence of a hydroxyl group in STL2 significantly decreased its inhibitory activity when compared with STL1, which lacks this group. Moreover, the substitution of the angelate ester (STL2) by the methacrylate ester (STL3) induced a significant increase in the inhibitory effect of the hydroxylated compounds. It seems that the methacrylate ester and hydroxyl group together contribute to a high inhibitory effect on PMNL oxidative metabolism. The inhibitory activity of SLT3 was lower when compared with the standard compound quercetin, a natural flavonoid that displays high antioxidant properties in various systems in-vitro (Rice-Evans et al 1996), except at the lower concentration tested (2.5 μmol L^{-1}).

Inhibition of stimulated-PMNL CL-lum may be mediated by three main mechanisms: cell death; scavenging of ROS; and inhibition of enzymes involved in the signal transduction pathways of the ROS generation process (Van Dyke & Castranova 1987). The first possibility was initially excluded because the STLs tested had no toxic effects on rabbit PMNLs under the assessed conditions (Table 1).

The second mechanism was assessed by testing the ability of STL3 (the most active STL) to scavenge the stable free radical DPPH and the ROS generated by the luminol-HRP–H_2O_2 system. The DPPH test has been used to evaluate the antioxidant potential of natural products and to provide information on the reactivity of different compounds with free radicals. However, formation of free radical intermediates from oxidized compounds, which could become important propagators of free radical chain reactions, cannot be detected by this assay (Okawa et al 2001). The luminol–HRP–H_2O_2 system, the other experimental model used, generates a variety of ROS and is supposed to catalyse a reaction

similar to the myeloperoxidase–H_2O_2–halide system from PMNL granules (Krol et al 1994). We found that, in contrast to quercetin, STL3 was not able to scavenge DPPH free radicals and the enzymatically generated ROS (Figure 3). These results suggest that this compound does not scavenge free radicals generated during PMNL oxidative burst and probably does not inhibit myeloperoxidase activity. Therefore, PMNL CL-lum inhibition by the tested STLs is probably mediated by inhibition of enzymes involved in the signal transduction pathways of the ROS generation process. This possibility will be assessed in a further study.

STLs have been reported to modulate many inflammatory parameters, such as exocytosis of cathepsin G and acid phosphatase from the azurophilic granules of rat PMNLs, release of histamine from mast cells and serotonin from blood platelets and exocytosis of elastase from human PMNLs (Hall et al 1979, 1980; Klaas et al 2002; Siedle et al 2003). They also possess proapoptotic effects, which can be desirable in eliminating nonfunctional cells in inflammatory tissues, and inhibit the 5-lipoxygenase and leukotriene C4 synthase in human blood cells (Dirsch et al 2001; Tornhamre et al 2001). Furthermore, several STLs inhibited transcription factor NF-κB, which is responsible for regulating expression of various genes involved in inflammatory responses and cellular survival (Rungeler et al 1999; Tak & Firestein 2001).

The various biological actions described for STLs seem to be mediated by a common mechanism of action for all molecules in this class, which is dependent on the presence of an exocyclic methylene group in conjugation with a γ-lactone carbonyl group and an ester side chain moiety. These functional groups have been reported to have strong affinity to nucleophilic groups and to engage in Michael-type additions with amino acids of biomolecules, with a consequent deactivation of functionally vital structures (enzymes, peptides) by covalent bond formation (Picman 1986; Schmidt 1999). Some STL alkylation targets at the macromolecular or subcellular level are nitric oxide synthases, tumour necrosis factor-α, cyclooxygenase-2, proteins of the glutathione system and nuclear transcription factors. These targets might participate in the inflammatory process and ROS production by phagocytic cells (Schmidt 1999).

In summary, the results of this work suggest that modulation of PMNL oxidative metabolism by *Lychnophora pohlii* STLs may be one of the possible mechanisms involved in the anti-inflammatory properties of this plant, which is widely used in Brazilian folk medicine for this purpose, and may be a useful target for therapeutic intervention in many chronic inflammatory diseases.

Conclusion

The three STLs tested herein showed a concentration-dependent inhibitory effect on rabbit PMNL oxidative metabolism, apparently not mediated by free radical scavenging or cytotoxic effects, but most probably by interaction with intracellular enzymes responsible for the activation of the PMNL oxidative burst. The structure–activity relationships observed in this work may contribute to the unravelling of mechanisms underlying the immuno-modulatory activity of STLs.

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