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Novel bisabolane derivative from "arnica-da-serra" (*Vernonieae*: Asteraceae) reduces pro-nociceptive cytokines levels in LPS-stimulated rat macrophages

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ABSTRACT

Ethnopharmacology relevance: Hydro alcoholic leaves extracts (HALE) of *Lychnophora ericoides* Mart. ("false arnica" or "arnica-da-serra") had been popularly used against pain and inflammatory process.
Aim: The present work aimed to look for possible active volatile compounds that could be found in HALE of *Lychnophora ericoides* among the non volatile anti-inflammatory and analgesic compounds previously reported.
Methods: Harvests were performed during the end of the wet summer season (April) when scented branches were instantly collected and frozen. HALE's were simulated at the lab by following the procedures lectured by the locals. Mass Spectrometry experiments suggested structural information when using both EI–MS and ESI–MS/MS. After isolation through classical thin layer chromatography (TLC) procedures, the NMR experiments and signals assignments were carried out. The effects on the cytokines or nitric oxide (NO) production were assessed at *in vitro* assays that had monitored the levels of these substances on the supernatant of LPS-stimulated macrophage primary cell culture.
Results: The major metabolite from HALE was isolated from the essential oil and the major compound had its molecular formulae established by Mass Spectrometry (High Resolution) and its structure by NMR. Literature-based investigation enables us to define the structure of the new metabolite as 6-methyl-2-(4-methylcyclohex-4-enyl-2-acetyloxy) hept-5-en-2-ol and its name as *orto*-acetoxy-bisabolol. *In vitro* assay of interleukins release inhibition was carried out using rat peritoneal macrophages cultures. IL-1β and TNF-α levels were significantly reduced when cells were previously treated with low doses of *orto*-acetoxy-bisabolol, but neither IL-6 nor NO levels have their levels reduced. Results suggest that ethnical knowledge of anti-inflammatory and analgesic effects of the "arnica-da-serra" HALE may be associated to the *orto*-acetoxy-bisabolol ability on synthesis inhibition of the key inflammatory/hypernociceptive mediators.
Conclusions: Phytochemical investigation of the volatile active compounds in *Lychnophora ericoides* HALE allows us to isolate a new bisabolane derivative (*orto*-acetoxy-bisabolol) and to infer that this compound inhibits the synthesis of TNF-α and IL-1β, two important inflammatory mediators in the hypernociception. Our present data, in addition to literature's data, furnish scientific support to folk's use of *Lychnophora ericoides* as an endemic wound healer.

1. Introduction

Brazil is the host of a huge biodiversity which had long been the basis of handcrafted folk medicines for the treatment of ailments and diseases. Thus, several species had undergone human selection to afford today's Brazilian medicinal plants of popular use. Moreover, some plants have a different history of use. The founding fathers naturalists who studied Brazilian medicinal flora have never reported pharmacological activities for *Lychnophora* species (Semir et al., 2011). Its use as a medicinal plant appeared with the landing of Caucasians in southern Brazil during the late decades of nineteenth century for coffee farming. The immigrants, mainly Italian people,

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have felt the lack of Mediterranean medicinal plants like *Arnica montana* L. (*Heliantheae*: Asteraceae) in Brazil (Lopes, 2000; Semir et al., 2011). That may have pushed them to look for other pecies for topic applications to treat inflammatory process and alleviate pain. This "trial" on *Lychnophora* spp. was based on the similarity of the smell of its leaves. Therefore, *Lychnophora ericoides* was named "falsa-arnica" or "arnica-da-serra" in a true case of vernacular name for *Arnica montana* (Semir et al., 2011). We shall draw attention here for the fact that *Arnica montana* exhibit analgesic and anti-inflammatory effects, as well as a genomic control of inflammation mediated by the sesquiterpene lactone (STLs) helenalin which has the ability of interfer on NF-kappa B expression (Lyss et al., 1997). Early phytochemical investigations of *Lychnophora ericoides* revealed the presence of four STLs that display *in vitro* anti-inflammatory activity as well (Borella et al., 1998; Zidorn et al., 1999). Reinvestigation of two *Lychnophora ericoides* sites of collection occurring in distinct types of savannas (or Brazilian "Cerrado") afforded isolation of STLs through classical phytochemical approach (Sakamoto et al., 2003; Gobbo-Neto et al., 2010). However, further examination showed that within typical Habitat the major compounds are flavonoid derivatives and chlorogenic acid derivatives instead of STLs earlier founded in plants from different areas (Gobbo-Neto and Lopes, 2008). Recent *in vivo* experiments displayed the anti-inflammatory action of one of these flavonoid types compounds (Gobbo-Neto et al., 2005). Furthermore, the anti-inflammatory, analgesic and antipyretic effect of chlorogenic acid derivatives from *Lychnophora* sp were also reported (Santos et al., 2006, 2010). Roots of *Lychnophora ericoides* do accumulate chlorogenic acid derivatives, as well, among lignans that show analgesic effects (Borsato et al., 2000). Despite this late effort to understand pharmacological action of *Lychnophora ericoides*, the early Italians' testimonial of the plant use highlights the importance of harvesting the plant at the time its scent is stronger in the field (Lopes, 2000; Semir et al., 2011). Such information suggests the existence of a possible active compound in the volatile fraction. Current literature of *Lychnophora ericoides* volatiles concerns the essential oils of plants occurring in Goiás state and have encountered α-bisabolol as a major compound, which is a bisabolane derivative well characterized as anti-inflammatory (Costa et al., 2008; Curado et al., 2006; Kamatou and Viljoen, 2010). On the course of our early approaches, the analysis of locally handcrafted hydro-alcoholic preparation, acquired in southern Minas Gerais state, showed presence of volatile constituents but α-bisabolol was absent. In view of this mentioned issue, we aimed to carry out a phytochemical investigation looking for the major metabolite of the volatile fraction yield in folk preparations and to investigate its possible antiinflammatory effects. This present contribution brings the results achieved from this investigation as well as a brief discussion of its pharmacology relevance. Figs. 1 and 2

2. Material and methods

2.1. Plant material and HALEs preparation

Harvest was done during the morning in the month of April, 2012. Using a Garmin "Etrex" we georeferenced the collection site as S 20°38,316′, W 046°15,318′. Botanical voucher was kept in UEC —Universidade Estadual de Campinas (Unicamp) under the entry code 221. The identification was done by Prof. Dr. João Semir from botany department of Unicamp's Biology Institute. Aerial parts (branches) were harvested, instantly frozen using dry ice and immediately brought back to laboratory. Field expeditions and plant material collection were both carried out under the allowance of both CNPq's bureau for "Genetic Heritage" of Brazil (License 010143/2011-4), and IBAMA (License 26801-4).

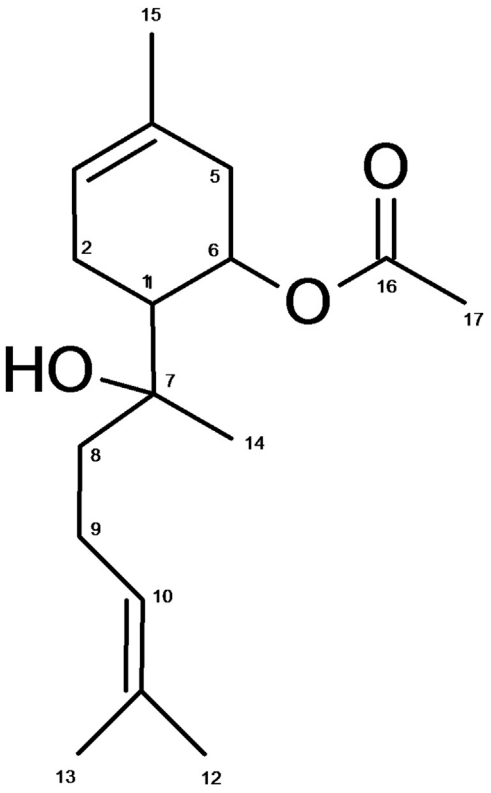

Fig. 1. Structure of 6-methyl-2-(4-methylcyclohex-4-enyl-2-acetyloxy)hept-5-en-2-ol, the *orto*-acetoxy-bisabolol.

2.2. HALEs simulation and hydrodestillation

Simulations of HALEs were done by using branches of *Lychnophora ericoides* harvested in April 2012. Plant material was immersed in enough volume of hydrated ethanol [70% (v/v)] right after the harvest. The preparation went through a 15 days maturation period, as lectured by locals. Lipophilic fraction was concentrated by liquid–liquid partition with *n*-hexanes (3 times; 150 mL each time). Hexanic layer was concentrated and analyzed in thin layer chromatography (TLC) sheets ($CHCl_3$;Vanillin). The major compound detected in HALE was isolated from essential oils.

For essential oil yielding, 600 g of frozen leaves of *Lychnophora ericoides* were grounded, using $N_{2(l)}$ and a mortar, and kept frozen. The plant material was placed in a Clevenger apparatus for hydrodestillation. After the complete process ending the oil was recovered from the glassware using drops of hexane. This oil was dried by direct contact with Na_2SO_4 and under flow of $N_{2(g)}$. Afterwards, the oil was stored under freezing until the analysis by Gas Chromatography and Mass Spectrometry GC–MS (Shimadzu QP 2010, Tokyo, Japan).

2.3. Mass spectrometry analysis

We have analyzed the samples of our investigation using Electron Ionization Mass Spectrometry (EI–MS). Detector has operated under settled parameters as 70 eV ionization energy, source temperature of 250 °C, scanning ratio of 0.50 scan/s for a method with total ion chromatogram (T.I.C.) analyzer mode switched on, within a mass weight frame corresponding to the interval of 40*m/z* up to 500*m/z* mass charge ratio. Chromatographic resolution was guaranteed by the use of a split injector (240 °C) operating at a ratio of 1/10 injection quantity towards the column. One Db-5MS (30 m × 0.25 mm × 0.25 μm) column (Agilent J&W, Folsom, USA) was employed and Helium was used as a carrier gas (flow: 1.33 mL/min.; settled pressure: 81.5 kPa). Programmed heating (3 °C/min) was used to

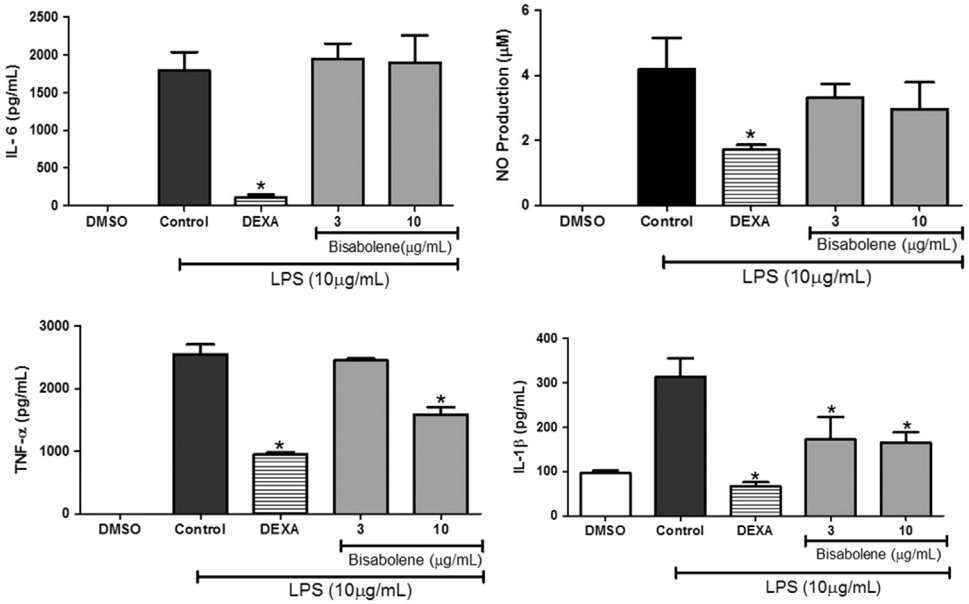

Fig. 2. Effects of 6-methyl-2-(4-methylcyclohex-4-enyl-2-acetyloxy)hept-5-en-2-ol, the *orto*-acetoxy-bisabolol in measured levels of IL-6 (top-left), NO (top-right), TNF-α (bottom-left) and IL-1β (bottom-right) released *in vitro* by isolated macrophage cells of Wistar rats previously stimulated by LPS contact. Cells were cultured in a concentration of 4×10^5 cells/well and exposed to 3 or 10 μg/mL of *orto*-acetoxy-bisabolol for 30 min. After that LPS or DMSO (Negative control) was added to the culture in a 4 h time-frame. Supernatant was, at last, recovered and cytokine levels were assessed through ELISA assays. NO production was determined by the Griess method. Control refers to cells treated with LPS (10 μg/mL). "DEXA" refers to cells treated with dexametasone (9 μg/mL). Results are drawn as average ± standard deviation. $^*p < 0{,}05$ when DEXA or *orto*-acetoxy-bisabolol treated groups are compared with the control group (LPS).

elute the compounds out of the column orderly and clearly. Starting temperature was at 60 °C and finishing temperature was 240 °C.

Orto-acetoxy-bisabolol was used purified to acquire high resolution mass spectra. Spectrometric data were furnished by MS and MS/MS analysis on an UltrOTOF Bruker–Daltonics instrument (Billarica, USA) equipped with an ESI ion source and operating in positive mode High Resolution Electrospray Ionization Mass Spectrometry (HR–ESI–MS). Samples were dissolved in methanol and pumped into the ESI source at a flow-rate of 5 mL/min, by using a Harvard Apparatus model 1746 (Holliston, USA) syringe pump.

2.4. Compounds isolation and NMR analysis

An amount of 200 μL of essential oil was equally distributed and applied on 8 plates of TLC. Plates were allowed to elute by using Hexane/Etila acetate 9:1 as mobile phase. We took this only chromatography step during the isolation.

After elution we used UV lights and partial revelation with H_2SO_4 (10%v/v) and Vanillin to reveal the spots of interest. The major compound, which had the most intense spot on the plates, was recovered from silica by using filtration with ethyl acetate (100%). The recovered solution was carefully concentrated under room temperature.

NMR experiments: ^1H and ^{13}C spectra were acquired under 500 and 125 MHz field, respectively, using a Bruker DRX-500 equipment (Bruker BioSpin GmBH, Rheinstetten,Germany). Heteronuclear Multiple-Quantum Correlation (HMQC) and Heteronuclear Multiple-Bond Correlation (HMBC) and correlation maps were also acquired in order to provide more detailed information.

2.5. Measurements of cytokines and nitric oxide release from rat macrophages

2.5.1. Animals

Male Wistar rats (180–200 g) were housed at a temperature-controlled room (27 ± 2 °C) with a 12-h light/dark cycle and *ad libitum* access to water and food. All rats were sacrificed shortly to minimize suffering of the animal. The study was conducted in compliance with the ethical guidelines of the University of São Paulo Animal Care and Use Committee (Protocol number 149/2005).

2.5.2. Isolation of peritoneal macrophage cells

To yield peritoneal macrophage, the rats were treated with 10 mL of 3% thioglycolate (i.p.) during four days. Afterwards, the animals were sacrificed by cervical dislocation and the peritoneal cavity was washed with 10 mL of ice-cold phosphate-buffered saline (PBS) twice. The peritoneal fluid was collected and the cells were washed twice with RPMI medium using centrifugation at 100*g* for 5 min. After centrifugation the supernatant were discarded and cell pellet was re-suspended in 1 mL RPMI medium. Cells number was determined with trypan blue exclusion. The cells were plated (4×10^5 cells/well) in 24 well culture plates in RPMI medium for 24 h at 37 °C under 5% CO_2 in the humidified incubator. After cell adhesion, the non-adhered macrophages were removed by three successive washes with (PBS). The macrophage monolayer was cultured in the RPMI medium and kept at 37 °C in a 5% CO_2 atmosphere until the following experiments procedures.

2.5.3. Determination of production of pro-inflammatory cytokines (TNF-α; IL-6 and IL-1β).

All measured doses of *orto*-acetoxy-bisabolol were prepared by dissolving the compound in DMSO (< 1% final volume well) and diluting with RPMI. Solution was placed separately in contact with peritoneal macrophage monolayer cultured in 24 well plates. Final doses were of 3 and 10 μg/mL and the pre-treatment were done by incubation for 30 min. After this treatment, the cells were stimulated with lipopolysaccharide (LPS) for 4 h at 37 °C in a 5% CO_2 atmosphere. Dexamethasone (DEXA—9 μg/mL) treatment of the cells was used as a positive anti-inflammatory control. After incubation, the supernatant was collected and TNF-α; IL-6 and IL-1β were measured by using an enzyme-linked immunosorbent

assay (ELISA) as described before (Verri et al., 2006). The cytotoxicity of *orto*-acetoxy-bisabolol was determined by MTT assay.

2.5.4. Determination of NO production

The production of nitric oxide (NO) was assessed for nitrite concentration (NO_2^-) in the supernatants through the Griess method (Green et al., 1882). Briefly, the adherent macrophages were treated with *orto*-acetoxy-bisabolol as describe above. After treatment, 50 μL of the supernatants were incubated with an equal volume of the Griess reagent (0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride, 1% sulfanilamide, 2.5% phosphoric acid and incubated for 10 min at room temperature (light protected). The absorbance of the resulting mixture was measured at 540 nm on an automated SpectraMAX 340. The concentration of NO_2^- was calculated with a calibration curve using sodium nitrite ($NaNO_2$) as a standard. The results were expressed as micromoles of nitrite.

2.5.5. Statistical analysis

All results were expressed as mean ± S.D. of cytokines or NO concentration ($n=3$). Statistical evaluation of data was carried out using analysis of variance (ANOVA one way followed by Bonferroni's test. A p value of less than 0.05 was considered statistically significant.

3. Results and discussion

Our simulation of HALE was 3 times extracted with hexane and the non-polar fractions were analyzed by TLC. One major spot was observed [Retardation factor (Rf)=0,08 (Hexane/Etila acetate 9:1 as mobile phase)] and comparison with the essential oil, obtained from plants from the same regions displayed the same spot in addition to other components. A preliminary biological assay indicated that compound with Rf=0.08 may be responsible for the expected activity. Supported by this data the essential oil yielding was carried out by starting with frozen leaves and after 7 h of hydrodestilation, an aromatic and greenish-yellow oil, weight≅200 mg, was recovered using drops of hexane, dried with Na_2SO_4 and $N_{2(g)}$, and readily stored under freezing. The crude oil was fractionated by preparative TLC (in 10 plates) affording 27 mg of a pure compound. Initial GC–MS analysis showed a tiny molecular ion at *m/z* 262, generated by electron ionization while the base peak (*m/z* 43) was observed suggesting a functionalized sesquiterpene (Supplementary material). Intense ion on *m/z* 69 is displayed in MS possibly due to the formation of a dimethyl aril radical ion (C_5H_9). Such hypothesis would afford molecular formulae of $C_{17}H_{26}O_2$. However, HR–ESI–MS acquisition had displayed ions like the protonated molecule *m/z* 281.2103 (Supplementary material). Accepting this first formulae hypothesis, an ion at *m/z* 281 would suggest the occurrence of an hydroxyl group that would not be noticed at EI however confirmed in ESI full scan detection of ion *m/z* 263 ($[M+H-H_2O]^+$). In order to confirm the OH presence, fragmentation of ion *m/z* 281 (MS/MS) was induced under collision energy shifting (from 0 up to 15 eV) (Supplementary material). It was clearly shown the abundance of product ion (*m/z* 263) rising under precursor ion (*m/z* 281) abundance depletion while energy rising. Such experiment proved the hydroxyl presence on the structure once the accurate mass error for $C_{17}H_{28}O_3$ was around 3 ppm. The molecular formulae in addition of the carbon signals observed in ^{13}C NMR (two possible olefins at 124.6/132.2 ppm and 121/130 ppm and one ester carbonyl group 171.7) would suggested a possible diunsaturated cyclic compound. Such information is in agreement with previous bisabolane skeleton reported for *Lychnophora ericoides*. Heteronuclear correlation maps and DEPT spectrum (Distortionless Enhancement by Polarization Transfer; Supplementary material) gives the connectivity of the olefinic and quaternary carbon **11** to **2** methyl groups. Supported by the previous report of bisabolane derivatives in *Lychnophora ericoides* leaves volatile fractions (Costa et al., 2008; Curado et al., 2006) we tested the hypothesis of an acetoxy cyclohexane ring. HMBC made possible to distinguish methyl groups at 1.68 (**12**) and 1.61 (**13**) that correlates to olefin carbons **10** and **11**. This double bond forms a dimethyl aril chain that displays a deshielded proton at 5.11 (**10**) correlating with the linear chain, confirming the 2-hydroxyl-6-methyl-heptene moiety of sesquiterpene. Signal for carbon **9** is deshielded likely a β-carbinolic carbon and proton correlates (maps) with carbon **7**. Deshilded signals suggests hydroxyl group presence, what goes along with the tertiary alcohol trait of showing high abundance of $[M–H_2O]$ ions during Mass Spectrometry experiments. Correlation of carbon **7** with protons from methyl group (**14**) confirms the aliphatic chain identity. NMR data acquired for *orto*-acetoxy-bisabolol was compared with bisabolane derivatives data available in literature. The tri substituted cyclohexene was displaying confirmatory signals like the correlations of cyclic olefin with methyl group C**15**. High similarity was observed and a trimethylacetoxybiasabolene carbon skeleton was confirmed.

Carbonyl signals for carbon **16** and methyl group **17** were assigned to ester function due to chemical shifts and correlations observed in contours maps (Supplementary material).

Confirmation of carboxyl group in position **2** came from correlation as well as by noticing compatible chemical shift (δ 69.1 ppm) and multiplicity of proton NMR (heptet due to position **6** protons coupling in position **1** and **2** protons in HMBC).

By hanging all these data together we were able to confirm the structure as 6-methyl-2-(4-methylcyclohex-4-enyl-2-acetyloxy) hept-5-en-2-ol and to the best of our knowledge, this contribution is the first report of this compound in current literature. The structural identity of this novel bisabolane derivative suggests similarity with α-bisabolol structure. Based on the classical rules stated by American Chemical Society (Grafflin, 1955) we named the compound *orto*-acetoxy-bisabolol.

In order to investigate the potential of this new bisabolane derivative on inhibiting the production of important inflammatory mediators involved in the inflammatory hypernociception, we investigated the effect of the compound on TNF-α IL-6, IL-1β and NO production in LPS-stimulated primary macrophage cell cultures. We found that *orto*-acetoxy-bisabolol inhibits the TNF-α and IL-1β production by LPS stimulated macrophages cultures. On the other hand, *orto*-acetoxy-bisabolol was not able to reducing the synthesis of IL-6 or NO by these stimulated cells.

Sensorial neurons do express TNF-α and IL-1β (Verri et al., 2006) transmembranic receptors, what suggests a central role of these cytokines on the nociceptive sensitization during inflammation (Copray et al., 2001 *apud* Verri et al., 2006; Parada et al., 2003b *apud* Verri et al., 2006). Furthermore, exogenous TNF-α deflagrates, *in vivo* action potential of peripheral neurons while simultaneously enhance sensibility to mechanical and chemical stimulus (Sorkin et al.,1997 *apud* Verri et al., 2006; Nicol et al., 1997a *apud* Junger and Sorkin, 2000; Verri et al., 2006 *apud* Verri et al., 2006). Regarding neural sensitization, it should be stated that IL-1β is reported to acts when heat stimulus are present (Obreja et al., 2002 *apud* Verri et al., 2006). The reduction of TNF-α and IL-1β concentration induced by *orto*-acetoxy-bisabolol treatment may well suggest this compound as a candidate in the treatment of the inflammatory and painful process.

IL-6 is known to be a very important pro-inflammatory mediator, as TNF-α and IL-1β. In our experimental conditions the action of bisabolene in inhibiting TNF-α and IL-1β production was not seen to the former. It has suggested that TNF-α, IL-6 and IL-1β constitute a cascade pathway during inflammation induction (Cunha, et al., 1992). Therefore those cytokines have been

considered targets to the development of new antiinflamatory drugs. Nevertheless, some studies have demonstrated that IL-1β and TNF can play a role in an inflammatory cascade without the participation of IL-6 (Cunha et al., 2005). Similar results in cell cultures were found by Drazan et al. (1996) where the TNF-alpha and IL-1 beta, but not IL-6 are suppressed in Kupffer cell response to LPS.

The treatment with the *orto*-acetoxy-bisabolol did not induce any change on the nitric oxide (NO) levels induced by LPS on the culture supernants. It is known that NO is involved in many physiological processes and NO plays a dual effect in the nociceptive system and pain modulation. Nitric oxide is an important neurotransmitter involved in the nociceptive process and, in the dorsal horn of the spinal cord, it contributes to the development of central sensitization (Cury, et al., 2011).

Conversely, experimental data have also demonstrated that NO inhibits nociception in the peripheral and also in the central nervous system. These analgesic effects are dependent of the activation of the NO-cyclic GMP-protein kinase G-ATP-sensitive potassium channel signaling pathway (Cunha et al., 2010). Many drugs like Non-Steroidal Anti-inflammatory Drugs (NSAIDs), opioids and anti-inflammatory steroids depend of NO to induce analgesic effect (Ferreira et al., 1991; Lozano-Cuenca et al., 2005; Rhen; Cidlowski, 2005).

Because *orto*-acetoxy-bisabolol reduced TNF-α and IL-1β levels with no changes on NO levels we can suppose that the analgesic effect of this compound can be related to the reduction of cytokines without interference of the dual role of NO signaling on the nociception.

The noticeable compound's majority on TLC analysis of hexanic fractions concentrated from HALE's liquid–liquid partition not only highlights the antialgic activity but also contribute to better understand the mechanism of action supporting the popular usage of this natural medicine.

Concluding, this work allowed us to isolate the new bisabolane derivative 6-methyl-2-(4-methylcyclohex-4-enyl-2-acetyloxy) hept-5-en-2-ol, named *orto*-acetoxy-bisabolol and to confirm that this compound inhibits the synthesis of TNF-α and IL-1β, important mediators of inflammatory nociception. Therefore, the data found in the present study can help to explain the different analgesic and anti-inflammatory mechanism of actions displayed by the substances from *Lychnophora ericoides*, by associating with the activity of the other compounds from this folk medicine. Furthermore we believe that present results are relevant to discuss the role of Cerrado's flora in World Health Organization Strategy for traditional medicine (WHO, 2005) once this very well known specie is once more displaying results that goes along with folk medicine lessons, however it is still listed as an endangered species (MMA, 2008).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2013.05.003.

References

Borella, J.C., Lopes, J.L.C., Vichnewski, W., Cunha, W.R., Herz, W., 1998. Sesquiterpene lactones, triterpenes and flavones from *Lychnophora ericoides* and *Lychnophora pseudovillosissima*. Biochemical Systematic and Ecology 26, 671–676.

Borsato, M.L.C., Grael, C.F.F., Souza, G.E.P., Lopes, N.P., 2000. Analgesic activity of the lignans from *Lychnophora ericoides*. Phytochemistry 55, 809–813.

Costa, M.A.C., Jesus, J.G., Farias, J.G., Nogueira, J.C.M., Oliveira, A.L.R., Ferri, P.H., 2008. Variação estacional do óleo essencial em arnica (*Lychnofora ericoides* Mart.) [Season time scale shifts on arnica's essential oil (*Lychnofora ericoides* Mart.)]. Revista de Biologia Neotropical 5, 53–65.

Cunha, F.Q., Poole, S., Lorenzetti, B.B., Ferreira, S.H., 1992. The pivotal role of tumor necrosis factor alfa in the Development of Inflammatory Hyperalgesia. British Journal of Pharmacology 107, 660–664.

Cunha, T.M., Verri Jr, W.A., Silva, J.S., Poole, S., Cunha, F.Q., Ferreira, S.H., 2005. A cascade of cytokines mediates mechanical inflammatory hypernociception in mice. Proceedings of National Academy of Science USA 102, 1755–1760.

Cunha, T.M., Roman-Campos, D., Lotufo, C.M., Duarte, H.L., Souza, G.R., Verri, W.A.J.R., Funez, M.I., Dias, Q.M., Schivo, I.R., Domingues, A.C., Sachs, D., Chiavegatto, S., Teixeira, M.M., Hothersall, J.S., Cruz, J.S., Cunha, F.Q., Ferreira, S.H., 2010. Morphine peripheral analgesia depends on activation of the PI3Kgamma/AKT/nNOS/NO/KATP signaling pathway. Proceedings of National Academy of Science USA 107, 4442–4447.

Curado, M.A., Oliveira, C.B.A., Jesus, J.B., Santos, S.C., Seraphin, J.C., Ferri, P.H., 2006. Environmental factors influence on chemical polymorphism of the essential oils of *Lychnophora ericoides*. Phytochemistry 67, 2363–2369.

Cury, Y., Picolo, G., Gutierrez, V.P., Ferreira, S.H, 2011. Pain and analgesia: The dual effect of nitric oxide in the nociceptive system. Nitric Oxide 25, 243–254.

Drazan, K.E., Wu, L., Bullington, D., Shaked, A.J., 1996. Viral IL-10 gene therapy inhibits TNF-alpha and IL-1 beta, not IL-6, in the new born endotoxemic mouse. Journal of Pediatric Surgery 31, 411–414.

Ferreira, S.H., Duarte, I.D., Lorenzetti, B.B., 1991. The molecular mechanism of action of peripheral morphine analgesia: stimulation of the cGMP system via nitric oxide release. European Journal of Pharmacology 201, 121–122.

Gobbo-Neto, L., Santos, M.D., Kanashiro, A., Almeida, M.C., Lucisano-Valim, Y.M., Lopes, J.L.C., Souza, G.E.P., Lopes, N.P., 2005. Evaluation of the anti-inflammatory and antioxidant activities C-glucosylflavones from *Lychnophora ericoides* (Asteraceae). Planta Medica 71, 3–6.

Gobbo-Neto, L., Lopes, N.P., 2008. On-line identification of chlorogenic acids, sesquiterpene lactones and flavonoids in the Brazilian arnica *Lychnophora ericoides* Mart. (Asteraceae) leaves by HPLC–DAD–MS and -MS/MS and a validated HPLC–DAD method for their simultaneous analysis. Journal of Agricultural and Food Chemistry 56, 1193–1204.

Gobbo-Neto, L., Guaratini, T., Pessoa, C., Moraes, M.O., Costa-Lotufo, L.V., Vieira, R.F., Colepicolo, P., Lopes, N.P., 2010. Differential metabolic and biological profiles of *Lychnophora ericoides* mart. (Asteraceae) from different localities in the Brazilian campos rupestres. Journal of Brazilian Chemical Society 21, 750–759.

Grafflin, M.W. (1955) System of Nomenclature for the Terpene Hydrocarbons. In: Advances in Chemistry, vol. 14. Chapter 1 pp.1–11.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S. R., 1882. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Analytical Biochemistry 126, 131–138.

Junger, H., Sorkin, L.S., 2000. Nociceptive and inflammatory effects of subcutaneous TNF alpha. Pain 85, 145–151.

Kamatou, G.P.P., Viljoen, A.M., 2010. A Review of the application and pharmacological properties of α-bisabolol and α-bisabolol-rich oils. Journal of American Oil Chemists' Society 87, 1–7.

Lopes, N.P., 2000. A essência da arnica. [Essentials of arnica] FAPESP—Pesquisa, 64, 42–44.

Lozano-Cuenca, J., Castaneda-Hernandez, G., Granados-Soto, V., 2005. Peripheral and spinal mechanisms of antinociceptive action of lumiracoxib. European Journal of Pharmacology 513, 81–91.

Lyss, G., Schmidt, T.J., Merfort, I., Pahl, H.L., 1997. Helenalin, an anti-inflammatory sesquiterpene lactone from *Arnica*, selectively inhibits transcription factor NF-*K*B. Biological Chemistry. 378, 951–961.

MMA. BRASIL. Ministério do Meio Ambiente [Brazilian Environment Ministry]. Lista oficial das espécies da flora Brasileira ameaçadas de extinção. [Official Rank of Brazilian Flora Endangered Species] Instrução normativa N° 6, de 23 de setembro de 2008 [Regulatory Paperwork #6, September 23th, 2008].

Rhen, T., Cidlowski, J.A., 2005. Anti-inflammatory action of glucocorticoids—new mechanisms for old drugs. The New England Journal of Medicine 353, 1711–1723.

Santos, M.D., Almeida, M.C., Lopes, N.P., Souza, G.E.P., 2006. Evaluation of the anti-inflammatory, analgesic and antipyretic activities of the natural polyphenol chlorogenic acid. Chemical and Pharmaceutical Bulletin 29, 2236–2240.

Santos, M.D., Chen, G., Almeida, M.C., Soares, D.M., Souza, G.E.P., Lopes, N.P., Lantz, R.C., 2010. Effects of caffeoylquinic acid derivatives and C-Flavonoid from *Lychnophora ericoides* on *in vitro* inflammatory mediator production. Natural Product Communications 5, 733–740.

Sakamoto, H.T., Flausino, D., Castellano, E.E., Stark, C.B.W., Gates, P.J., Lopes, N.P., 2003. Sesquiterpene Lactones from *Lychnophora ericoides*. Journal of Natural Products. 66, 693–695.

Semir, J., Rezende, A.R., Borges, M., Lopes, N.P., 2011. As Arnicas Endêmicas das Serras do Brasil [The endemic Arnicas of Brazilian Hill tops]. Editora UFOP, First Edt., Ouro Preto.

Verri, W.A.J.R., Cunha, T.M., Parada, C.A., Poole, S., Cunha, F.Q., Ferreira, S.H., 2006. Hypernociceptive role of cytokines and chemokines: targets for analgesic drug development? Pharmacology & Therapeutics 112, 116–138.

WHO (World Health Organization) (2005). WHO Traditional Medicine Strategy 2002–2005. Geneva, World Health Organization (Document Reference: WHO/EDM/TRM/2002.1).

Zidorn, C., Dirsch, V.M., Rüngeler, P., Sosa, S., Della Loggia, R., Merfort, I., Pahl, H.L., Vollmar, A.M., Stuppner, H., 1999. Anti-inflammatory activities of hypocretenolides from *Leontodon hispidus*. Planta Medica. 65, 704–708.