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Pharmacological evaluation of 2-angeloyl *ent*-dihydrotucumanoic acid

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ABSTRACT

Context: *Gymnosperma glutinosum* (Spreng.) Less. (Asteraceae) is a bush used for the empirical treatment of pain, fever, and cancer. An *ent*-neo-clerodane diterpene (2-angeloyl *ent*-dihydrotumanoic acid; ADTA) was isolated from *G. glutinosum*.

Objective: This study evaluates the cytotoxic, anti-inflammatory, and antinociceptive effects of ADTA.

Materials and methods: The cytotoxic effects of ADTA (1–350 μM) were evaluated using the MTT assay with human tumorigenic (SW-620, MDA-MB231, SKLU1, SiHa, and PC-3), and non-tumorigenic (HaCaT) cells for 48 h. The *in vitro* anti-inflammatory effects of ADTA (0.23–460 μM) were assessed using murine peritoneal macrophages stimulated with LPS and estimating the levels of pro-inflammatory mediators for 48 h. The antinociceptive effects of ADTA (25–100 mg/kg p.o.) were evaluated using two *in vivo* models of chemical-induced nociception during 1 h.

Results: ADTA lacked cytotoxic activity (IC_{50} > 100 μM) on tumorigenic cells. In non-tumorigenic cells (HaCaT), ADTA exerted low cytotoxic effects (IC_{50} = 273 μM). ADTA, at concentrations of 115 μM or higher, decreased the release of pro-inflammatory mediators. The maximum antinociceptive effects of ADTA in the acetic acid-induced abdominal constrictions by ADTA was found at 100 mg/kg (63%), whereas in the formalin test at phase 1 and phase 2, ADTA (100 mg/kg) decreased the licking time by 47 and 71%, respectively.

Conclusion: The results indicate that ADTA, obtained from *G. glutinosum*, exerts moderate *in vitro* anti-inflammatory and *in vivo* antinociceptive effects, but lacks cytotoxic effects on human cancer cells.

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Gymnosperma glutinosum; antinociceptive; antiinflammatory

Introduction

Gymnosperma glutinosum (Spreng) Less (Asteraceae), commonly known as 'tatalencho', 'escobilla' or 'jarilla' (syn. *Gymnosperma corymbosum* DC, *Gymnosperma multiflorum* DC, and others) is a bush distributed from Southern portion of the United States of America to Central America. This plant is used for the empirical treatment of pain, fever, diarrhea, cancer, rheumatism, wounds, and headache (Martínez 1979; Gomez-Flores et al. 2012). The isolation and characterization of diterpenes, flavonoids, long-chain alkenes, *ent*-labdene-type and *neo*-clerodane-type diterpenes, as well as monoterpenes, have been reported from *G. glutinosum* (Domínguez & Torre 1974; Yu et al. 1988; Martínez et al. 1994; Maldonado et al. 1994; Calderón et al. 2001; Gomez-Flores et al. 2012). The pharmacological effects such as cytotoxicity against cancer cells, antibacterial, and moderate antifungal, spasmolytic and antiprotozoal activities have been reported in *G. glutuinosum* and some of its active compounds (Rojas et al. 1995; Canales et al. 2007; Serrano et al. 2009; Gomez-Flores et al. 2008, 2012; Quintanilla-Licea et al. 2012). Nevertheless, there are no pharmacological studies with the *ent-neo*-clerodane diterpene (2-angeloyl *ent*-dihydrotumanoic acid; ADTA) (Figure 1) isolated from *G. glutinosum*. The aim of this study was to evaluate the cytotoxic, anti-inflammatory and antinociceptive effects of ADTA.

Methods and materials

Chemicals and reagents

Naproxen sodium (NPX) was obtained from Tripharma (Distrito Federal, Mexico), whereas cisplatin (CDDP) was acquired from Accord Farma (Distrito Federal, México) and clonazepam (CNZ) was purchased from Tecnofarma (Mexico City, Mexico). Dimethyl sulfoxide, 3-(4,5-dimetylthiazol-yl)-diphenyl tetrazolium bromide (MTT), LPS (*Escherichia coli* 0111:B4), propidium iodide (PI), sodium nitrite ($NaNO_2$), Griess reagent, dextrose, phenol red, and type I HRP, tramadol (TRD) were purchased from Sigma Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco-BRL (Invitrogen-Gibco-BRL, Grand Island, NY). ELISA assay kits for measuring mouse TNF-α and IL-6 were obtained from Peprotech (London, UK).

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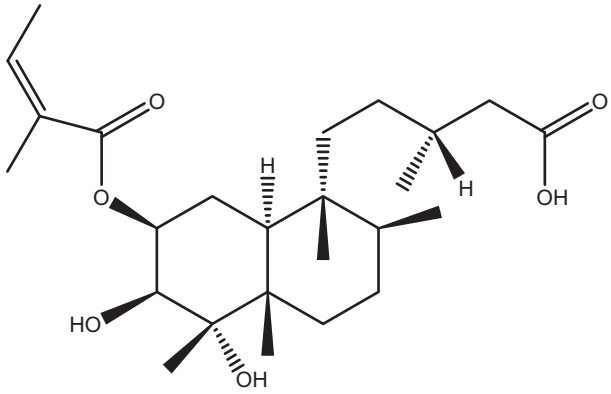


Figure 1. Chemical structure of ADTA.

Isolation and purity analysis of ADTA

The isolation of ADTA was carried out as described previously (Martínez et al. 1994). Briefly, dried and ground aerial parts of *G. glutinosum* were successively extracted at room temperature with hexane. The extract was concentrated *in vacuo*. The purity and the structure of the compound were determined by using NMR spectroscopy and chromatographic methods, respectively. Briefly, 1 mg of ADTA was dissolved in 1 mL of *iso*-octane, and the purity was performed using a gas chromatograph (Agilent 6890N Technology) coupled to a selective mass detector (Model 5973). Then, 0.4 µL of sample was injected into the injection port at 300 °C. For separation of the sample, a capillary column (DB-5HT, 15 m long, 0.25 mm intern diameter, and 0.10 µm of particle size) was used. The temperature program in the oven was 50 °C for 3 min, increasing 15 °C/min until 300 °C for 2 min. The spectra were collected at 71 eV of ionization voltage and the range of analyzed mass was 33–800 m/z. Finally, ADTA was identified by its mass spectrum and its purity was determined based on the area under the curve of all peaks present in the chromatogram. The purity of the compound was 98.5%. The yield was 0.125% dry weight.

Animals

Male Balb/c mice weighing 25 to 30 g, were obtained from the Universidad of Guanajuato animal facility and the Centro de Investigaciones Regionales (CIR) Dr. Hideyo Noguchi of the Universidad Autónoma de Yucatán. Animals were housed in isolated cages at 24 °C under a 12 h light-dark cycle. The animals were supplied with food and water *ad libitum*. The experiments were carried out according to the principles and guidelines of the National Institutes of Health (NIH) Guide for Treatment and Care for Laboratory Animals and the Official Mexican Norm NOM 062-ZOO-1999 (Technical specifications for the production, care, and use of laboratory animals). The research also followed the Guidelines on Ethical Standards for Investigations of Experimental Pain in Animals (Zimmerman 1983).

Cell lines and culture conditions

Cell lines of colorectal adenocarcinoma (SW-620), breast carcinoma (MDA-MB231), lung adenocarcinoma (SKLU1), cervical carcinoma (SiHa), and prostate carcinoma (PC-3), primary murine peritoneal macrophages, isolated as reported by do Rosário et al. (2011), and non-tumorigenic cells (human immortalized keratinocytes HaCaT) were maintained in DMEM supplemented by 7% fetal bovine serum and antibiotics (100 U/mL penicillin and 100 pg/mL streptomycin). All cell lines were obtained from ATCC (Manassas, VA). All cell cultures were grown at 37 °C, in a humidified atmosphere of 5% CO_2.

Cytotoxic activity (MTT assay)

Human cancer cell lines were seeded in 96-well microplates at a density of 5000 cells/well. After 24 h of incubation, ADTA at concentrations ranging 1 to 350 µM were added to the cells. Then, the assay was carried out as described by Jacobo-Salcedo et al. (2011) and optical density (O.D.) was measured at 590 nm using an ELISA reader (Biorad Laboratories, Hercules, CA). The wells without cells were considered as blank. The viability of treated cells was estimated from the relative growth as follows:

$$\text{relative viability} = \frac{\text{control O.D.} - \text{sample O.D.}}{\text{control O.D.}} \times 100$$

The concentration leading to 50% inhibition of viability (IC_{50}) was also calculated by regression analysis (percent survival versus log concentration).

Anti-inflammatory activity

Cell culture

Murine peritoneal macrophages were seeded (1×10^5 cells/well) in 96 well plates. For the phagocytosis assay, macrophages were seeded at 5×10^5 cells/well in 24-well plates. After 24 h of incubation, macrophages were activated with 1 µg/mL LPS of *E. coli* and treated with various concentrations of ADTA (0.23–460 µM), dissolved in 0.1% DMSO, during 48 h following the method proposed by Mueller et al. (2010), with some modifications.

NO production

Nitric oxide (NO) production was determined based on the Griess reaction (Green et al. 1981). Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride/5% H_3PO_4) were mixed and the OD was read on a microplate reader (iMark Microplate Reader, Bio-Rad, Hercules, CA) at 490 nm. The nitrite concentration (µM) was determined by the interpolation of standard curves constructed with known concentrations of $NaNO_2$ (0–50 µM)

Phagocytic activity

The phagocytosis assay was carried out as described previously (Esteban et al. 1998). After 24 h, macrophages were co-cultured with *Saccharomyces cerevisiae* yeasts (5×10^6 yeast/well) and labeled with 100 µg/mL propidium iodide. The non-ingested labeling yeasts were removed and 500 µL of separation buffer (0.5% BSA, 6.29 mM EDTA) was added. After 90 min, non-ingested labelling yeasts were removed and the amount of *Saccharomyces cerevisiae* phagocytosed by macrophages was determined by measuring the cellular fluorescent intensity using a Cell Lab Quanta SC (Beckman Coulter) flow cytometer.

Hydrogen peroxide release

The H_2O_2 release by murine peritoneal macrophages was determined following the protocol of Pick and Mizel (1981). A volume

Table 1. Cytotoxic activity of ADTA on human cancer and non-tumorigenic cells.

IC_{50} (μM)	PC3	SW620	SKLU1	SiHa	MDA-MB231	HaCaT
CDDP	1.9 ± 0.3	2.3 ± 0.6	2.7 ± 0.9	1.7 ± 0.5	3.2 ± 0.5	2.7 ± 0.5
ADTA	>100	>100	>100	>100	>100	273 ± 12.5

of 100 μL of fresh phenol red solution (5.5 mM dextrose, 0.056 g phenol red and 8.5 U/mL Type I HRP in DPBS) was mixed, in a 96-well plate, with 100 μL of cell culture supernatants. The plate was incubated during 3 h in darkness. The reaction was stopped by adding 10 μL of NaOH 1 N solution. The absorbance was measured at 620 nm on a microplate reader and the concentration of H_2O_2 was determined by comparison with a H_2O_2 standard curve (0–50 μM).

Cytokine production

Enzyme-linked immunosorbent assay (ELISA) was used according to the manufacturer's manual (Peprotech, London, UK) to determine the IL-6 and TNF-α concentration in the supernatants of macrophages activated and treated with ADTA. Capture antibody was used at a concentration of 2 μg/mL for IL-6 or 1 μg/mL for TNF-α in PBS. Serial dilutions of recombinant IL-6 (0–4000 pg/mL) or TNF-α (0–2000 pg/mL) were used as standard curve. The absorbance was measured at 490 nm using a microplate reader and the concentration of the cytokines (pg/mL) was determined by extrapolation of the absorbance in the standard curve.

Antinociceptive activity

Acetic acid-induced constrictions

The acetic acid method was carried out as described by Koster et al. (1959). One hour prior to acetic acid injection, mice ($n = 8$ per group) orally received: (a) saline solution (the vehicle group), (b) 100 mg/kg NPX, or (c) ADTA at doses of 25, 50, and 100 mg/kg. Each group was administered (i.p.) with 10 mL/kg body weight of acetic acid (1%). The mice were individually placed in glass cylinders, and the number of abdominal constrictions was counted over a period of 0–30 min. The inhibition of nociception (IN) was calculated as follows: IN = [(Cc-Ct)/Ct ×100] where:

Cc = Mean of contractions in mice treated with different doses of ADTA or naproxen

Ct = Mean of contractions in vehicle group

Formalin

The formalin test was carried out as described by Hunskaar and Hole (1987). One hour prior to formalin injection, mice ($n = 8$ per group) orally received (a) saline solution (the vehicle group), (b) 30 mg/kg TRD i.p., and (c) ADTA at doses of 25, 50, and 100 mg/kg. Mice were injected with 30 μL of 1% formalin (in 0.9% saline) into the subplantar space of the right hind paw and individually placed in glass cylinders. The duration of paw licking was recorded at 0–15 min (first phase) and 15–45 min (second phase) after formalin injection.

Ketamine-induced sleeping time

The effect of ADTA on ketamine-induced sleeping time was measured as described by Mimura et al. (1990). One hour prior to ketamine injection, groups of mice ($n = 8$) were treated orally with ADTA (25–100 mg/kg), vehicle (saline solution), or CNZ (1.5 mg/kg). Thereafter, animals were injected with ketamine (100 mg/kg, i.p.). The interval between the administrations of ketamine until the loss of the righting reflex was recorded as the onset of sleep, whereas the time from the loss to regaining of the righting reflex was recorded as the duration of sleep (Bastidas-Ramírez et al. 1998).

Statistical analysis

All experimental values are expressed as the mean ± the standard deviation of at least two independent experiments. Statistically significant differences from the vehicle group were identified by Student's *t*-test or ANOVA with *post hoc* Tukey test for paired data. The level of $p \leq 0.05$ was used to determine statistical significance. All calculations were performed using the Graph Pad Prism V.3 software system (GraphPad Software, San Diego, CA).

Results

Cytotoxic activity

Cisplatin (CDDP), the positive cytotoxicity control, exerted toxic effects on all human cancer cell lines with IC_{50} values ranging from 1.9 to 3.2 μM (Table 1). ADTA lacked cytotoxic activity ($IC_{50} > 100$ μM) on human cancer cells. In non-tumorigenic cells (HaCaT), ADTA exerted low cytotoxic effects ($IC_{50} = 273 \pm 12.5$ μM), whereas CDDP induced high toxicity ($IC_{50} = 2.7 \pm 0.5$ μM) (Table 1).

Anti-inflammatory activity of ADTA

ADTA decreased, in a concentration dependent manner, the NO production, H_2O_2 release, phagocytic activity, as well as the production of TNF-α and IL-6 in LPS-stimulated murine macrophages. In all the cases, there was a significant ($p \leq 0.05$) decrease in the production of these pro-inflammatory mediators by ADTA at concentrations of 115 μM or higher, compared with the LPS group (Figure 2). The highest inhibitory activity of ADTA was found at 230 and 460 μM: 49–54% (NO production), 51 and 58% (phagocytic activity), 48 and 55% (H_2O_2 release), 58 and 65% (IL-6 production), and 55 and 63% (TNF-α production), respectively (Figure 2).

ADTA exerts antinociceptive effects

ADTA showed antinociceptive activity in the two models of nociception. In the acetic acid test, ADTA decreased the occurrence of writhing significantly ($p \leq 0.05$), compared to the vehicle group (Figure 3(A)). The maximum percentage inhibition of acetic acid-induced abdominal constrictions by ADTA was found at 100 mg/kg (63%), whereas 100 mg/kg NPX showed antinociception activity of 77% (Figure 3(A)).

In the formalin test at phase 1 and phase 2, ADTA decreased the licking time significantly ($p \leq 0.05$), compared to the vehicle group (Figure 3(B)). In phase 1, ADTA exerted antinociceptive

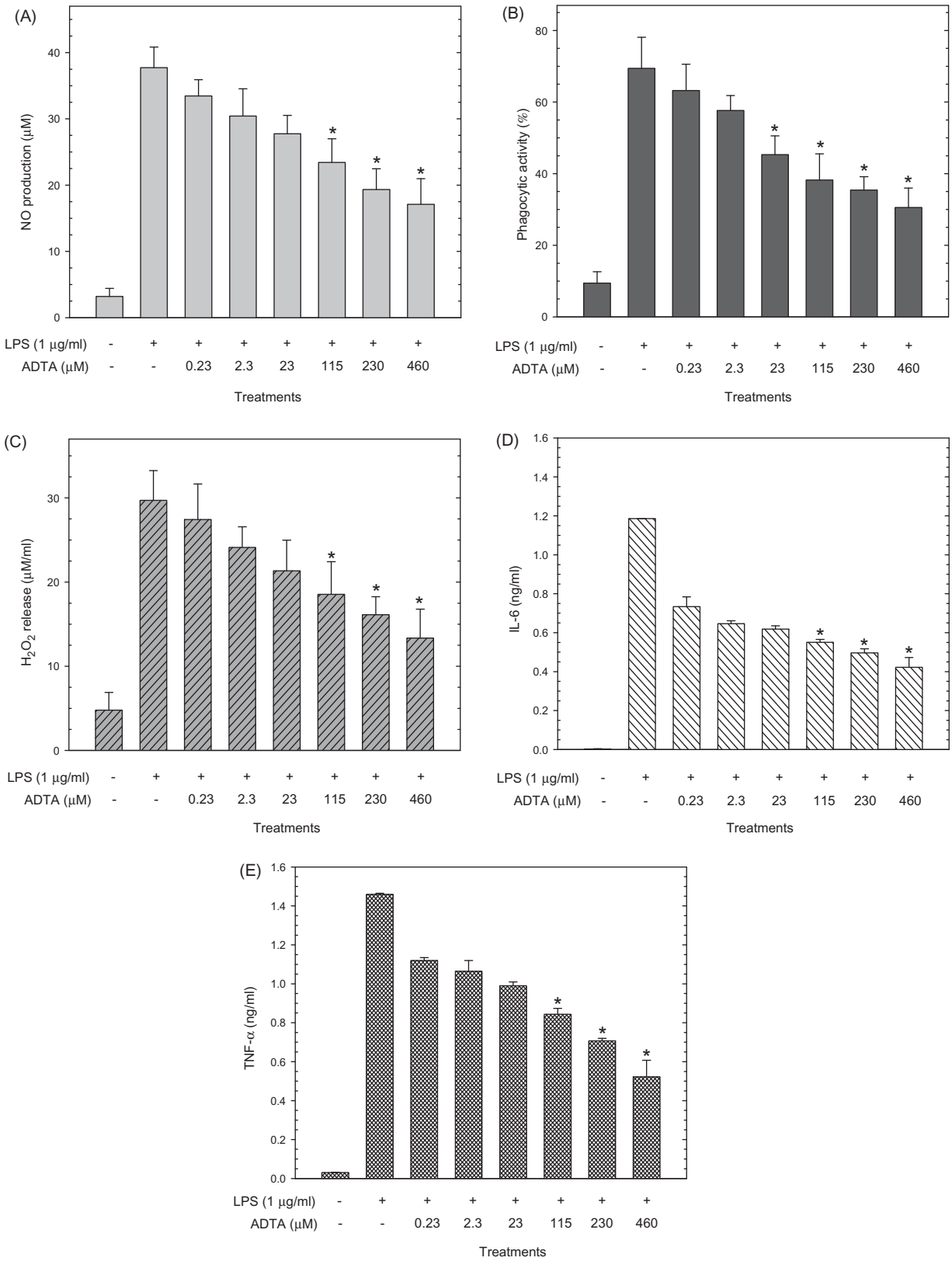


Figure 2. ADTA induces anti-inflammatory effects *in vitro*. The amount of NO production (A), phagocytic activity (B), H_2O_2 release (C) and the production of IL-6 (D) and TNF-α (E) were measured as described in 'Materials and methods' section. Data are representative of three independent experiments in hexaplicate. Results represent the mean ± standard deviation. *denotes $p \leq 0.05$, compared to LPS treatment.

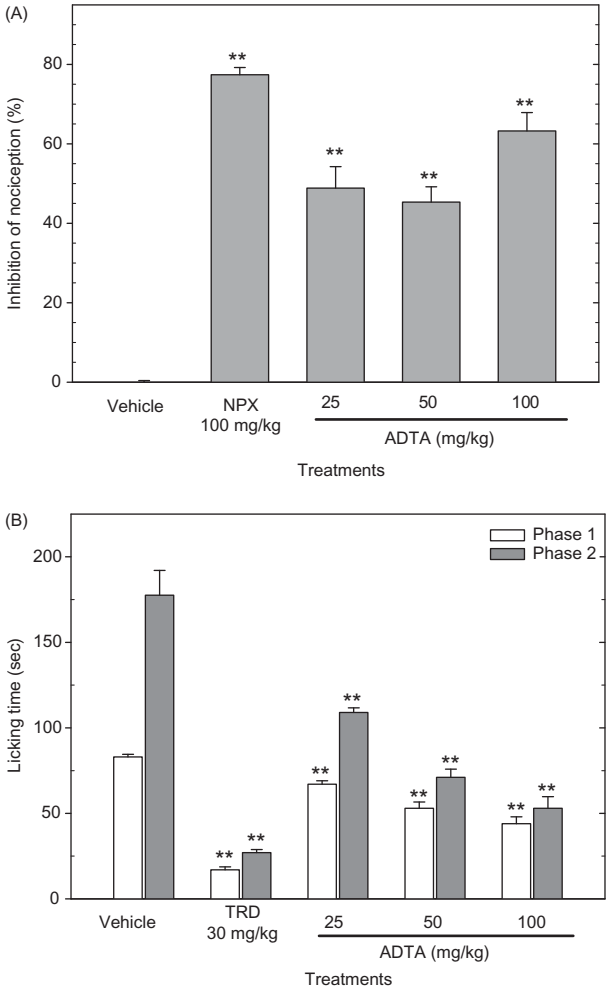


Figure 3. ADTA exerts antinociceptive effects. The antinociceptive effects of ADTA (25–100 mg/kg p.o.) were evaluated using the nociceptive tests acetic acid (A) and formalin (B). Other groups of mice received 100 mg/kg of NPX as the positive control or the vehicle (saline solution). Data are representative of two independent experiments ($n = 8$). Results represent the mean ± standard error (SE). **denotes $p \leq 0.05$, compared to the vehicle group.

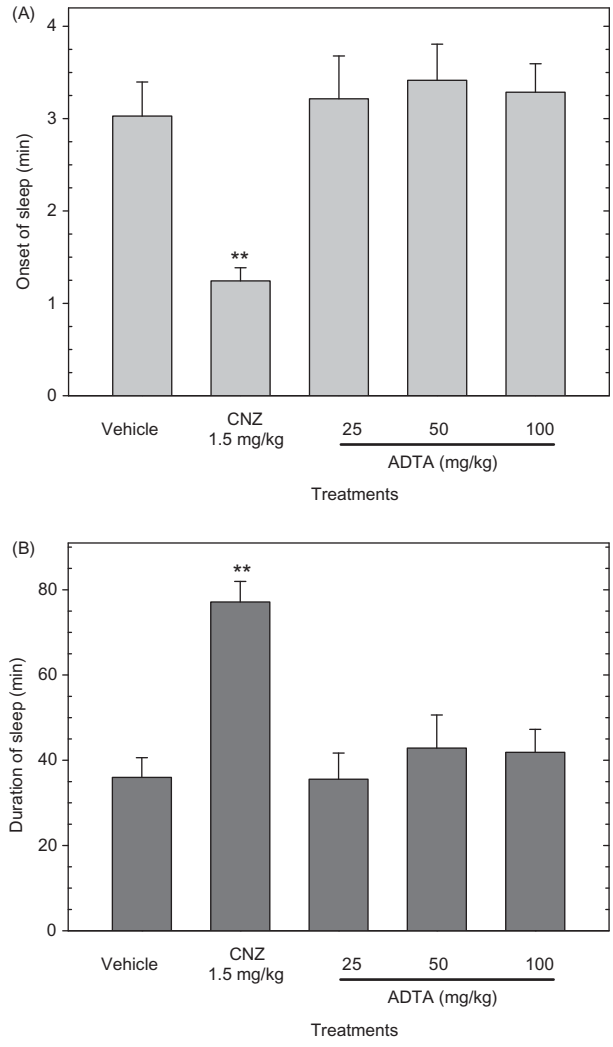


Figure 4. Effects of ADTA on the sedation of mice. The sedative effects of ADTA (25–100 mg/kg p.o.) were evaluated using the ketamine-induced sleeping time test recording the onset of sleep (A) and the duration of sleep (B). Other groups of mice received 1.5 mg/kg of clonazepam (CNZ) as the positive control or the vehicle (saline solution). Data are representative of two independent experiments ($n = 8$). Results represent the mean ± standard error (SE). **denotes $p \leq 0.05$, compared to the vehicle group.

effects by 19% (25 mg/kg), 36% (50 mg/kg), and 47% (100 mg/kg), respectively, whereas in phase 2 the antinociceptive effects of ADTA were 38% (25 mg/kg), 60% (50 mg/kg), and 71% (100 mg/kg) (Figure 3(B)). The antinociceptive effects of 30 mg/kg TRD were 79% (phase 1) and 85% (phase 2) (Figure 3(B)).

Effects of ADTA on sedative activity

The positive control 1.5 mg/kg CNZ decreased the onset of sleep by 56%, and significantly ($p \leq 0.05$) increased (2.2-fold) the duration of sleep, compared to the vehicle group. On the contrary, ADTA did not affect the onset of sleep or the length of sleep (Figure 4).

Discussion

According to our knowledge, this is the first report that shows the pharmacological effects of ADTA, an isolated compound from *G. glutuinosum*.

Previously, it was reported that *G. glutuinosum* showed low toxicity using an *in vivo* assay (Canales et al. 2007). In this study, ADTA exerted low cytotoxic effects ($IC_{50} = 273 \pm 12.5$ µM) in non-tumorigenic cells (HaCaT). The results corroborate the findings reported in *G. glutuinosum*. Nevertheless, further toxicological studies, including genotoxicity, mutagenicity, teratogenic, among others, should be performed with ADTA to analyze its safety for use in long-term studies.

Macrophages are the main target for the action of LPS and participate in host defence. The activation of macrophages plays a critical role in the inflammatory process by releasing a variety of inflammatory mediators including NO, H_2O_2, IL-6, and TNF-α, among others (Fujiwara & Kobayashi 2005). The results indicated that ADTA, decreased in a concentration dependent manner, the production of NO, H_2O_2, IL-6, TNF-α, as well as the phagocytic activity in LPS-activated macrophages. ADTA exerts the *in vitro* anti-inflammatory actions by the inhibition of the release of pro-inflammatory mediators. The molecular mechanisms by which ADTA exerts the *in vitro* anti-inflammatory effects remain to be studied. Some of the molecular mechanisms that will be assessed include the participation of NF-κB, p38 MAPK protein, among others, which are elements involved in the inflammatory process (Hoare et al. 1999). Further *in vivo*

studies should be performed with ADTA to test its anti-inflammatory activity.

Two models of chemical-induced nociception were used: the acetic acid test evaluates peripherally and centrally acting antinociceptive agents, whereas the formalin test evaluates peripherally acting antinociceptive drugs (Le Bars et al. 2001). The nociception induced by acetic acid directly stimulates nociceptive fibres and promotes the release of pro-inflammatory mediators including bradykinin, serotonin, among others (Chau 1989). In the formalin test, the first phase (0–15 min) reflects neurogenic pain and the release of pro-inflammatory mediators such as substance P, bradykinin, histamine, among others, whereas the second phase corresponds (15–45 min) pain response due to inflammation associated to prostaglandins, glutamate, tachynins, etc. (Beirith et al. 2002). The results suggest that ADTA might exert peripheral antinociceptive effects. The role of nitrergic pathway in the antinociceptive effect of ADTA will be assessed by using L-NAME (a selective nitric oxide synthase inhibitor) (Turner et al. 1996).

The ability of drugs to prolong ketamine-induced sleeping time is an indicative of sedative activity. Ketamine acts as an antagonist on *N*-methyl-D-aspartate (NMDA) receptors, by increasing the concentration of GABA in brain (Mimura et al. 1990). The antinociception caused by ADTA is not related to sedation since the mice tested in the ketamine-induced sleeping time test showed no significant effect on this behaviour.

Other neo-clerodane diterpenes such as ajugacumbin J, obtained from *Ajuga decumbens* Thunb (Lamiaceae), and tahuanin D, obtained from *Salvia herbacea* Benth (Lamiaceae) exerted *in vitro* anti-inflammatory activities (Bautista et al. 2012; Lv et al. 2014). This suggests that neo-clerodane diterpenes might be good option as anti-inflammatory agents. The related compound trans-dehydrocrotonin, a 19-nor-clerodane diterpene, showed *in vivo* anti-inflammatory and antinociceptive effects (Carvalho et al. 1996). Nevertheless, similarly to our study, tahuin D was reported to lack cytotoxic effects on cancer cells (Bautista et al. 2012).

In summary, the results indicate that ADTA, obtained from *G. glutinosum*, exerts moderate *in vitro* anti-inflammatory and *in vivo* antinociceptive effects, but lacks cytotoxic effects on human cancer cells.

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Disclosure statement

The authors declare that there are no conflicts of interest.

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