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Analgesic effect of leaf extract from *Ageratina glabrata* in the hot plate test

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Abstract: *Ageratina glabrata* (Kunth) R.M. King & H. Rob., Asteraceae (syn. *Eupatorium glabratum* Kunth) is widely distributed throughout Mexico and popularly known as "chamizo blanco" and "hierba del golpe" for its traditional use as external analgesic remedy. Though *glabrata* species has been chemically studied, there are no experimentally asserted reports about possible analgesic effects which can be inferred from its genus *Ageratina*. To fill the gap, we evaluated *A. glabrata* extracts in an animal model of nociception exploiting thermal stimuli. NMR and mass analyses identified a new thymol derivative, 10-benzoiloxy-6,8,9-trihydroxy-thymol isobutyrate (**1**), which was computationally converted into a ring-closed structure to explain interaction with the COX-2 enzyme in a ligand-receptor docking study. The resulting docked pose is in line with reported crystal complexes of COX-2 with chromene ligands. Based on the present results of dichloromethane extracts from its dried leaves, it is safe to utter that the plant possesses analgesic effects in animal tests which are mediated through inhibition of COX-2 enzyme.

Introduction

Pain is a subjective experience that is the result of the perception of an injurious stimulus and includes an emotional component that requires that the individual is conscious so that this happens. In 1986, the International Association for the Study of Pain (IASP) defined pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (Merskey & Bogduk, 1994). But, the pain has a physiological component, which is called nociception. That is the process by which intense thermal, mechanical or chemical stimuli are detected by a subpopulation of peripheral nerve fibers, called nociceptors (Basbaum & Jessell, 2000). The information is processed via specialized central nervous system pathways (Danneman, 1997).

The animal models for the study of the pain measure the latency time and/or the threshold of the escape responses evaluating the effect of several drugs in the nociceptive responses that are those associated with the injurious stimulation perceived by the nociceptors (Danneman, 1997). The models of acute pain by application of intense stimuli of short duration induce changes in motor reflexes or behavior such as retirement reflexes, vasomotor changes and vocalization, besides not requiring the accomplishment of a previous injury in the animal (González-Darder, 2000). Therefore, nociception is susceptible to a pharmacological blockade for the treatment of the pain and the common analgesic drugs are opioids and non-opioids drugs. But these drugs have disadvantages in the dosage, gastrointestinal problems, dependence and others. Different species of plants are used in folk medicine as an alternative for treatment of diseases and the traditional uses of plants as herbal remedies have played a vital role in the discovery of new molecules as therapeutic agents.

In general, species of the genus *Ageratina* possess therapeutic properties, such as the concerned analgesic effect which in turn was reported by Mandal et al. (2005) and Chakravarty et al. (2010). The species belongs to the well-known plant family of Asteraceae, and *Ageratina* is a member of the tribe Eupatorieae. *Ageratina* constitutes a medium-sized shrub growing from 1 to 2 m with dense branches. The leaves are opposite, lamina narrowly deltoid to elliptical and the capitula densely corymbose. The genus comprises nearly 146 species according to King &

Robinson (1970). It is found in the tropical and temperate regions of Europe and America, especially Mexico.

The plant under investigation, *Ageratina glabrata* (Kunth) R.M. King & H. Rob. (syn. *Eupatorium glabratum* Kunth) (King & Robinson, 1970), is popularly known as "chamizo blanco" or "hierba del golpe" which means "white greasewood" and "contusion herb". The rural population recognizes its pain - relief effects in external remedies. Reports from the literature describe flavones, thymol derivatives and other phenolic terpenoids (Romo de Vivar et al., 1971; Bohlmann et al., 1977; Guerrero et al., 1978). Though *A. glabrata* has been chemically studied, there are no scientific reports about its analgesic effects. Therefore, we studied this Mexican plant extract in a hot plate model of nociception exploiting distinct thermal stimuli.

Materials and Methods

General

NMR measurements, including DEPT experiments, were performed at 400 MHz for ^{1}H and 100 MHz for ^{13}C on a Varian Mercury Plus 400 spectrometer, using $CDCl_3$ as the solvent and TMS as internal reference. Mass spectra were recorded at 20 eV on a Hewlett-Packard 5989B Series II Plus spectrometer adapted to a HP 5890 gas chromatography. Column chromatography was carried out on Merck silica gel grade 60 (70-230 mesh).

Plant material

Specimens of *Ageratina glabrata* (Kunth) R.M. King & H. Rob., Asteraceae, were collected on January 4, 2006 near km 191 of México-Morelia state road No.15, in the municipality of San José de la Cumbre, State of Michoacán, México (N 19°40'859'', W 100°50'423'' and 2,234 meters above sea level). A voucher specimen (No. 188319) is deposited at the Herbarium of the Instituto de Ecología, A.C., Centro Regional del Bajío, Pátzcuaro, Michoacán, Mexico, where Prof. Jerzy Rzedowski kindly identified the plant material (Figure 1).

Figure 1. Photos of *A. glabrata* by Dr. Rosa E. del Río. The rhomboid-shaped leaves and flower grouping are clearly visible.

Preparation of plant extract

Air-dried leaves of *A. glabrata* (200 g) were extracted with CH_2Cl_2 (1.5 L) at room temperature for three days, three consecutive times. Filtration and evaporation of the extract afforded green viscous oil (19 g).

*Isolation of the thymol derivative (**1**) from dichloromethane extract*

The dichloromethane extract (0.5 g) was chromatographed on silica gel column using *n*-hexane and ethyl acetate mixture of increasing polarity, and finally pure ethyl acetate. Elution with hexane:EtOAc (9:1) afforded a mixture of thymol derivatives. The sub-fraction 30-32 (100 mg) was subjected to rechromatography on silica gel (5 g). Elution with *n*-hexane-AcOEt (8:2) afforded pure 10-benzoiloxy-6,8,9-trihydroxy-thymol isobutyrate (**1**) (20 mg). Identification was supported by spectroscopic analyses.

Animals

Female Sprague Dawley rats (180-200 g) were used for the study. The animals were housed in rooms maintained at 24±2 °C, twelve-hours-light-darkness cycle and maintained with *ad libitum* access to Harlan Tekland Global Diets® and bottled water. The animals were handled 3 min a day for four weeks until the experiment day. All animal experiments complied with recommendations of the Regulation of the Committee for the Care and Use of Laboratory Animals of the Benemérita Universidad Autónoma de Puebla in 2007, which provides for compliance with Mexican Official Norm (NOM-062-ZOO-1999) appendix A, about the "Specifications for the production, care and use of laboratory animals" (Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación; 2001).

Hot plate test

The extract was dissolved in sesame oil and administered to animals intraperitoneally at doses of 100 mg/Kg (n=9) and 150 mg/kg (n=10). The control group (n=8) was intraperitoneally exposed to a vehicle (0.7 mL) of pure sesame oil. In addition, independent groups of rats received, meloxicam intraperitoneally (n=8) at a dose of 4 mg/kg and nalbuphine (n=8) at a dose of 5 mg/kg. The control group (n=7) received isotonic saline solution (SSI, 0.4 mL). In the hot plate test, all animals were placed on a hot plate maintained at a temperature of 40 °C. The antinociceptive effect was quantified by measuring latency times for kicking or licking of hind paw. In addition, prior to the aforementioned verum and

control test, all animals had just been exposed to the hot plate in order to observe the so-called pre-injection-latency times, of which three measurements were taken. Each animal was repeatedly placed on the hot plate in time intervals of 10, 20, 30, 40, 50, 60, 70, 80 and 90 min after injection. The observation series of each animal was continued in order to collect data for every other hour until reaching the defined limit of 12 h evaluation time.

Docking simulations

The chromene derivative computationally modified and the control structure meloxicam (Engelhardt et al., 1995) were docked manually into the COX-2 catalytic site. This study was performed with the Sybyl®X software suite (Tripos International, 2010).

Vaginal smears

The vaginal smears were used to detect the phase of the estrous cycle during the hot plate test. We used this method as a control to determine whether the estrous cycle has influence on the latency times in the hot plate test.

Autopsy

The rats were observed for ten days after the nociception test in order to determine possible changes in the intake of food and water. On day eleven, animals were randomized and sacrificed using an overdose of sodium pentobarbital (55 mg/kg intraperitoneally). At autopsy the organs (heart, liver, kidneys, lungs, spleen, intestine and adjacent tissue to the administration area of *A. glabrata*) were given a macroscopic examination about their size, shape, color and texture.

Statistical analysis

Data were analyzed by one way ANOVA and were expressed as the means and respective standard deviation. This analysis allows checking as to whether there are any statistically significant differences ($p<0.05$) between the mean latency times of the groups. To determine which groups are different, the data were analyzed by Tukey test. In addition, Dunnett test was used to compare the experimental groups versus respective control group using Statistica program.

Results and Discussion

The dichloromethane extract of *A. glabrata* leaves was fractionated by column chromatography affording a pure compound 10-benzoiloxy-6,8,9-trihydroxy-thymol isobutyrate (**1**). The mass spectrum indicated a molecular ion at *m/z* 388 in agreement with the molecular formula $C_{21}H_{24}O_7$, *m/z* 370 (12%) $[M\text{-}H_2O]^+$, 178 (46%), 165 (43%), 150 (15%), 137 (5 %), 122 (23%), 105 (100%), 91 (5%), 71 (40%). The ^{1}H NMR spectrum (Figure 2) indicates the presence of a benzoate group at 7.98, 7.55 and 7.40 ppm; singlets at 6.65 and 6.58 ppm for 2 and 5 aromatic hydrogens, respectively. The signals for the isopropyl group at 2.52 and 1.09 ppm. A singlet at 4.64 ppm for two hydrogens of methylene group in the 10-position of the molecule and the hydrogens of methylene group in C-9 were observed as a doublet each at 4.56 and 4.49 ppm. The ^{13}C NMR spectrum (Table 1) was in agreement with the proposed structure. Eleven thymol derivatives have been obtained by Bohlmann et al 1977 from *A. glabrata*, but all of them differ from the new thymol derivative **1**.

Table 1. NMR data for compound **1** in $CDCl_3$.

Position	^{13}C	DEPT	^{1}H
1	149.2	C	-
2	120.0	CH	6.65 (s)
3	129.2	C	-
4	146.9	C	-
5	112.8	CH	6.58 (s)
6	126.0	C	-
7	15.7	CH_3	2.16 (s)
8	78.2	C	-
9	67.9	CH_2	4.64 (s)
10	67.3	CH_2	4.56 (d), *J*=12.0 Hz 4.49 (d), *J*=12.0 Hz
1’	177.7	C	-
2’	33.9	CH	2.52 (sep), *J*=7.0Hz
3’	18.8	CH_3	1.09 (d), *J*=7.0 Hz
4’	18.8	CH_3	1.09 (d), *J*=7.0 Hz
1”	166.9	C	-
2”	130.12	C	-
3”	129.8	CH	7.98 (dd), *J*=7.0 and 1.3 Hz
4”	128.5	CH	7.40 (dd), *J*=7.9 and 7.7 Hz
5”	133.5	CH	7.55 (dddd), *J*=7.9, 7.7, 1.3, 1.3 Hz
6”	128.5	CH	7.40 (dd), *J*=7.9 and 7.7 Hz
7”	129.8	CH	7.98 (dd), *J*=7.9 and 1.3 Hz

The results obtained from this study reflect an analgesic effect for *A. glabrata* (100 mg/kg), meloxicam (4 mg/kg) and nalbuphine (5 mg/kg). The analysis of the data has revealed that a significant difference exists between control (vehicle and SSI) and experimental (verum) groups at doses of 100 mg/kg ($p<0.05$). The injected extract produced a significant increase in reaction times in all verum animals upon exposure to the thermal stimuli (Figure 3). The apex of this increase was found

during the eighth hour after injection at a dose level of 100 mg/kg. Intriguingly, dose augmentation did not enhance protective effects (a higher level of 150 mg/kg).

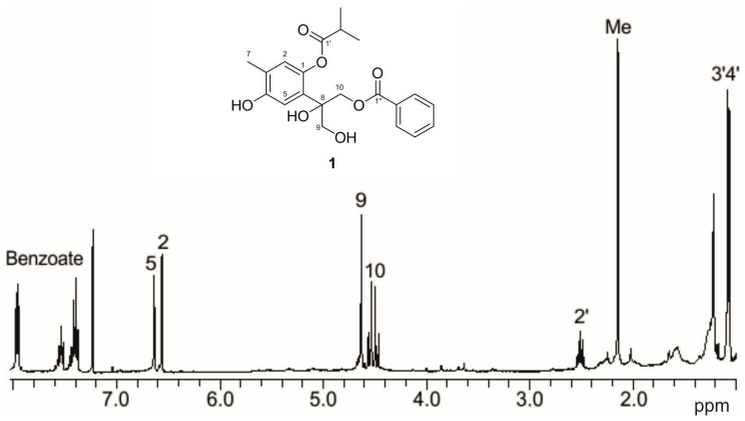

Figure 2. 400 MHz ^{1}H-NMR spectrum of 10-benzoiloxy-6,8,9-trihydroxy-thymol isobutyrate (**1**).

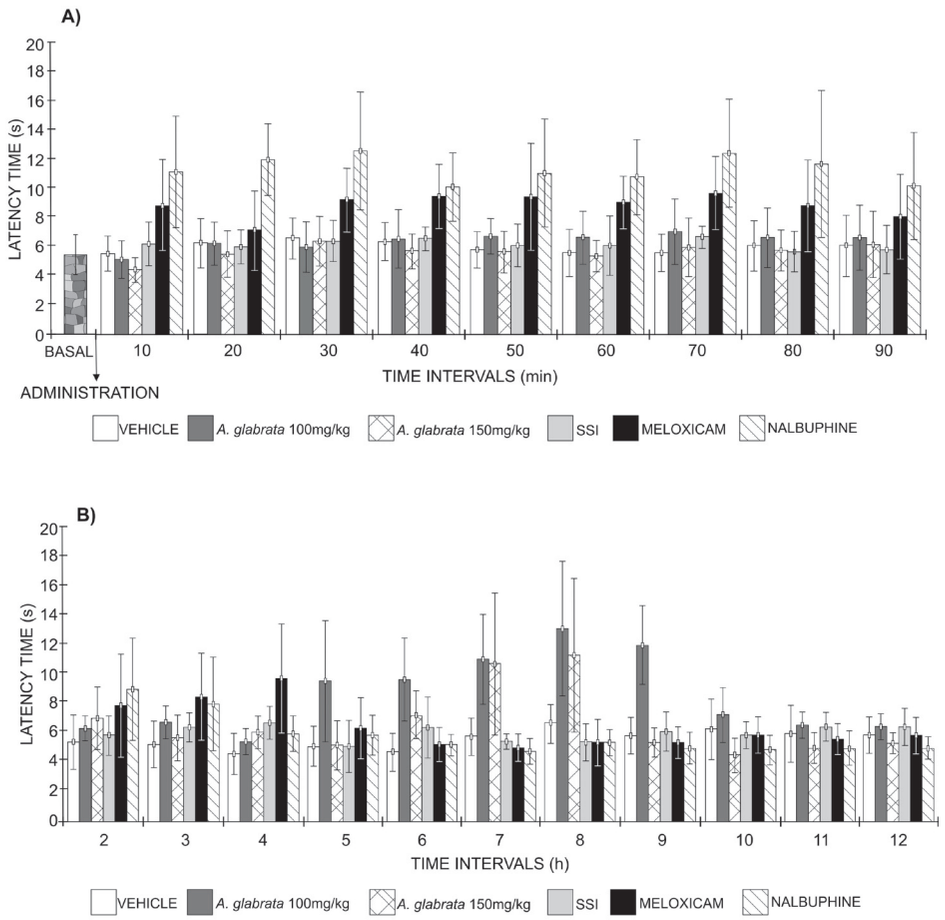

Figure 3. The latency times of the substances and analgesics during the hot plate test for A. the first 90 minutes and B. continuous hourly sampling until reaching 12 h evaluation time. Each point represents the mean values of the latency time group and respective standard deviation. One way ANOVA tests show statistically significant differences (p<0.05) between all groups. On the other hand, the Tukey test finds no statistically significant difference between the verum group of *A. glabrata* (100 mg/kg), and both positive controls (meloxicam and nalbuphine) but in contrast to the *A. glabrata* 150 mg/kg group which does show a statistically significant difference with both controls (logically, vehicle and SSI do show). The basal first sample point in A. represents the latency time means for all groups before the intraperitoneally administration. In addition, Dunnett's test was applied for each time interval, comparing vehicle versus *A. glabrata* 100 and 150 mg/kg and SSI versus meloxicam and nalbuphine (*p<0.05).

The analgesia of the non steroidal anti-inflammatory drugs (NSAID) like meloxicam which was used as positive control, is longer than that seen under opioid analgesic drugs application (positive control nalbuphine). NSAID inhibit the synthesis of prostaglandins by cyclooxygenase (COX) enzymes. Certain prostaglandins mediate pain processes (Vanegas & Schaible, 2001). Therefore, the observed analgesic effect of the leaf extract from *A. glabrata* suggests that the underlying molecular mechanism is mediated via COX because of its duration and pain-suppressing character.

The COX enzyme is divided into two isoforms. COX-1 is expressed constitutively throughout the body while the expression of COX-2 is inducible. However in spinal cord and brain, particularly in hippocampus, cortex and amygdala, both isoforms are expressed constitutively. This finding supports the idea that NSAID also act in the spinal cord by inhibiting prostaglandin synthesis and their effect is demonstrated in models of supraspinal analgesia as the hot plate test (Vanegas & Schaible, 2001).

Contemporary research and development for potential new analgesics focus on selective COX-2 inhibition, in order to lower the incidence of gastrointestinal and renal side effects ascribed to COX-1 inhibition (Gajraj, 2003). Hence a docking study was conducted to dock meloxicam and the proposed chromene derivative into the active site of COX-2 target. The chromene structure was hypothetically designed based on the extracted thymol derivative **1** which was identified through NMR analyses.

Based on theoretical grounds and known literature the thymol derivative **1** is converted into a bicyclical system via a ring closure reaction of condensation type (Paredes & Clemente, 2005; Tenorio et al., 2006; Chemler et al., 2009). This occurs on the original thymol derivative **1** which was identified through NMR by our group. We postulate that it is chemically converted into a compound similar to positive control meloxicam which itself is a known COX-2 inhibitor (Engelhardt et al., 1995, Gris et al., 2009). To determine whether this plausible compound (Figure 4) could also block the enzyme COX-2 a docking study was conducted by Sybyl® X software suite (Tripos International, 2010) to position the chromene derivative into the binding pocket of meloxicam, exploring the interacting amino acids and complementary shape information (Wang et al., 2007). The target structure is a human COX-2 enzyme, also called prostaglandin synthase-2 (Padhye et al., 2009). The target receptor was downloaded from the Protein Data Bank site (Berman et al., 2000). Its entry code is 3LN0 (Wang et al., 2010). As a result, both the chromene derivative and the meloxicam ligands were successfully docked into the same orientation and position as seen on the experimental chromene-COX-2 complex. The crystal chromene ligand, meloxicam and our ring-closed chromene derivative have an analogous bicyclical system in common (Figure 4). Hence, the orientation and binding mode of our computed models are in good agreement with the related crystallographic structure (Figure 5).

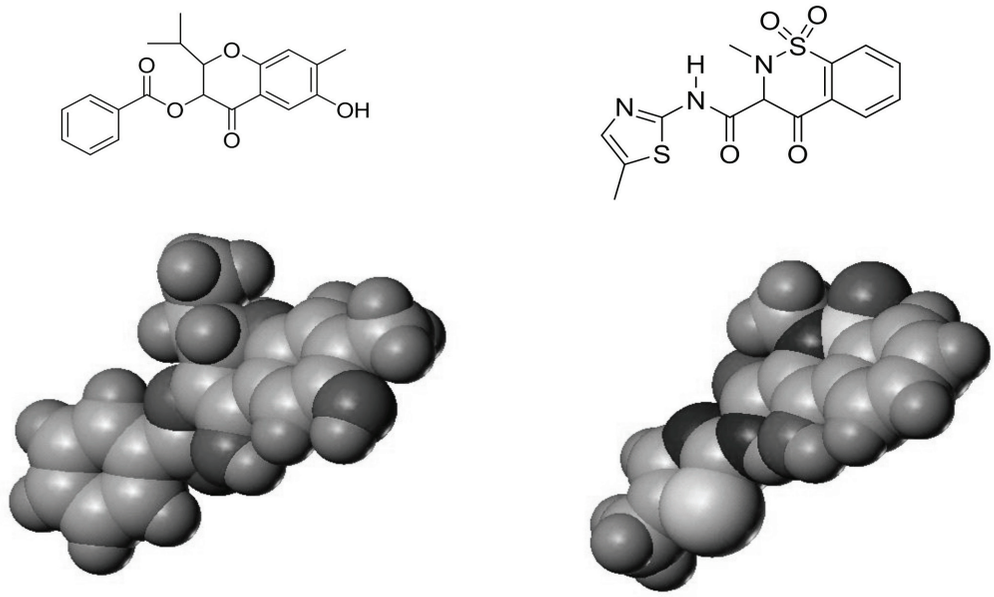

Figure 4. The drawing show structures of chromene derivative and meloxicam in plane molecules (above) and in 3D (below) display. Although both molecules present different functional groups, there is a spatial and structural similarity.

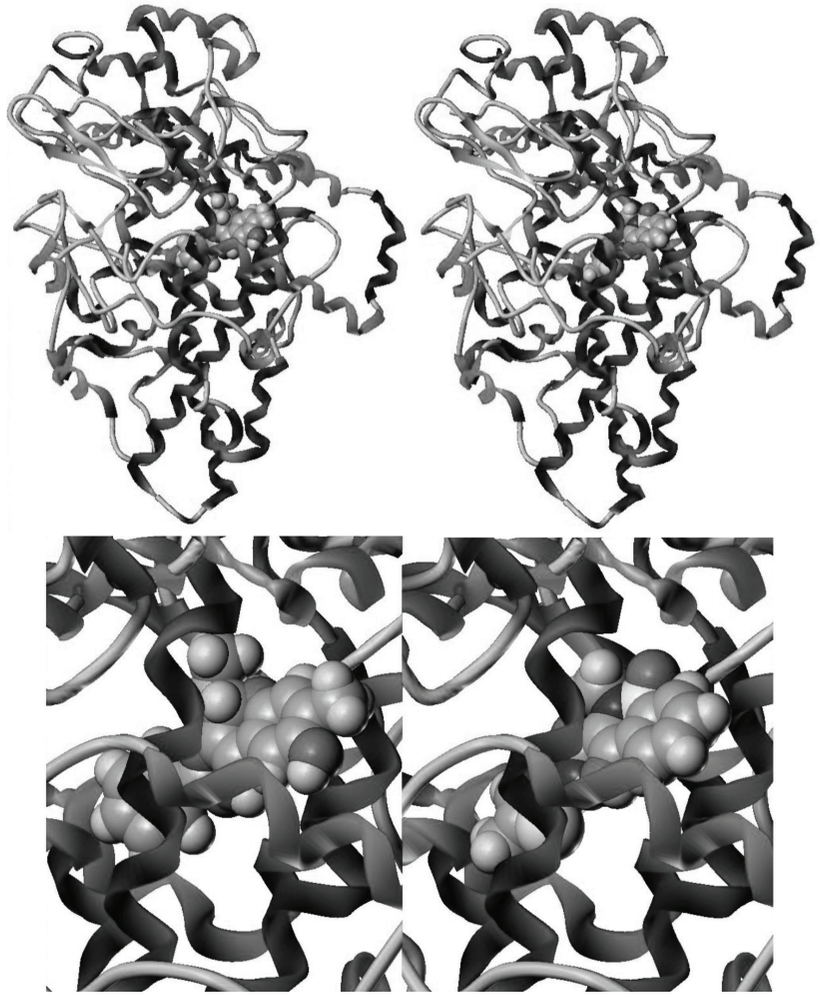

Figure 5. Computational model of chromene derivative (left) and meloxicam (right) docked into the active site of prostaglandin synthase-2 (COX-2). The parallel view allows the comparison and conclusion that the ligands' orientation and binding mode is the same because of the highly similar overall shape and electronic properties in space. The target enzyme is displayed in ribbon mode with its helical parts and beta strands. The entry to the ligand cavity is from the right toward center in each protein model. The entire enzyme is depicted above and the detail of the cavity is displayed below.

On the other hand, there is controversy concerning the use of female rats in models of pain with regard to missing data about the hormonal action mechanism (Gordon & Soliman, 1996; Vinogradova et al., 2003; Kuba et al. 2006). The estrous cycle lasts on average four days. It consists of four phases which are proestrus, estrus, metaestrus and diestrus. In each stage variable hormone concentrations are found such as prolactin, LH, FSH, estradiol, or progesterone. Each phase of the estrous cycle is determined by a ratio of three types of cells obtained by vaginal smears (Marcondes, 2002): epithelial cells, cornified cells and leukocytes (Figure 6A-D).

The influence of the phases of estrous cycle on the latency times in hot plate test, vaginal smears was performed in rats of the negative controls (vehicle and SSI). During the hot plate test vaginal smears were performed, one at ninety minutes and again at seven hours. The statistical analysis shows no significant difference between the latency times and the phases of the estrous cycle (proestrus, estrus, and diestrus metaestrus). There is no correlation or trend between values of latency times and the phases of the estrous cycle of female rats. Hence, the latency time values of four groups with either *A. glabrata* 100 mg/kg, 150 mg/kg, meloxicam or nalbuphine treatments are not influenced by hormonal stages but only due to full drug effects either through the extract itself or the positive controls (Figure 6E, F). Our study design allows similar interpretation about "no hormonal influence" as earlier reported by Ratnasooriya et al. (2002) and Kiasalari et al. (2010).

At autopsy there were no injuries during the

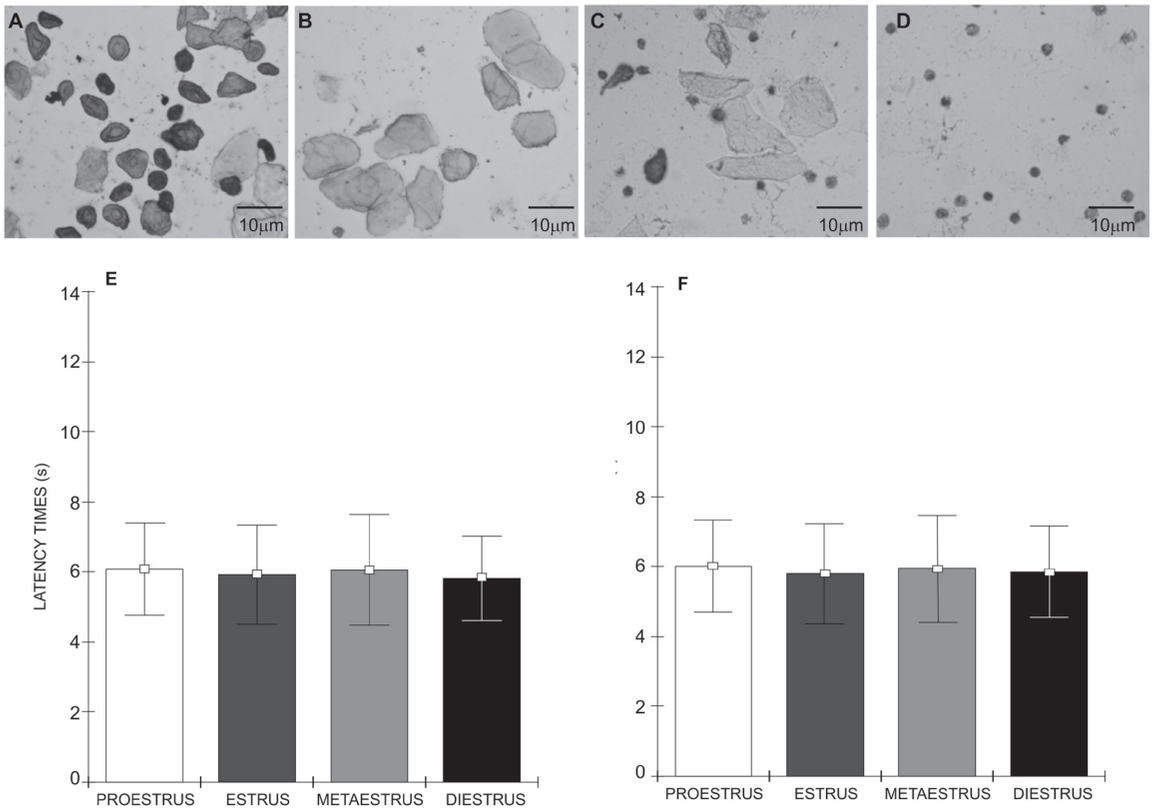

Figure 6. The phases of the estrous cycle: A. proestrus (n=4); B. estrus (n=4); C. metaestrus (n=4) and D. diestrus (n=3). Samples were prepared with hematoxylin and eosin stain under 40-fold microscopic magnification. The histogram bars show the mean and respective standard deviation values of the latency times for the control groups (vehicle and SSI) according to the phase of the estrus cycle at 90 minutes (E) and 7 h (F). Statistical analysis (one way ANOVA) does not show statistically significant difference between the groups (p<0.05).

macroscopic examination in the heart, liver, kidneys, lungs, spleen, intestine and adjacent tissue to the administration area of leaves extract of *A. glabrata* or vehicle. This reflects that the extract formulation is well-tolerated and administration is well performed. In addition, during the hot plate test, all animals did not show specific signs of pain such as piloerection, aggression, hunched posture, abdominal writhing, squeals on handling or pressure on administration area. They are exhaustively described by the Committee on Recognition and Alleviation of Distress in Laboratory Animals and Institute for Laboratory Animal Research (2008). Moreover, no animal died during or after treatment.

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