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Evaluation of Anti-inflammatory and Analgesic Activities of *Cymbopogon citratus* in vivo-Polyphenols Contribution

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ABSTRACT

Cymbopogon citratus is one of the most common herbs used in folk medicine due to its anti-inflammatory and antioxidant properties. Taking into account these properties showed on *in vitro* assays, the aim of this study was to evaluate the anti-inflammatory and analgesic activities of *C. citratus* leaves infusion (CcE) and its flavonoid-rich (CcF) and tannin-rich (CcT) fractions. The evaluation of the anti-inflammatory activity was performed in the carrageenan-induced rat paw oedema model. Both central and peripheral analgesic activities were evaluated in mice through the hot plate test and the acetic acid-induced writhing test, respectively. In the acute inflammation model, the statistically significant results obtained in percentage of oedema inhibition were 70.80 and 82.30% for CcE (34.12 and 68.24 mg kg⁻¹, respectively), 59.00% for CcF (7.42 mg kg⁻¹), 61.00% for CcT (5.96 mg kg⁻¹) and 84.00% for positive control group (10 mg kg⁻¹). For the peripheral pain evaluation, statistically significant results showed a pain reduction of 57.00% for CcE (136.48 mg kg⁻¹), 54.60% for CcF (14.8 mg kg⁻¹), 52.20% for CcT (11.92 mg kg⁻¹) and 83.00% for positive control group. This study demonstrates that *C. citratus* infusion compounds are able to reduce inflammation and peripheral pain *in vivo*, with polyphenols showing a significant contribution for these activities.

Key words: *Cymbopogon citratus*, lemongrass, *in vivo*, inflammation, pain, phenolic compounds

INTRODUCTION

Cymbopogon citratus (DC) Stapf, commonly known as lemongrass, is an Indian native herb belonging to the Poaceae, which comprises approximately 500 genus and 8000 herb species (Barbosa *et al.*, 2008). *Cymbopogon citratus* is used in folk medicine with a wide range of indications, such as digestive and nervous disorders, inflammation, pain, fever and diabetes (Lorenzetti *et al.*, 1991). It is also consumed as an aromatic drink and is widely used in traditional

cuisine due to its lemon flavor (Figueirinha *et al.*, 2008). Moreover, *C. citratus* leaves are a source of essential oil used both in the flavor and fragrance industries and for the production of insect repellents and disinfectants (Negrelle and Gomes, 2007; Rauber *et al.*, 2005).

Because of its economical impact, most phytochemical studies are centered in the volatile compounds of *C. citratus* leaves and only essential oil chemical composition is well known (Kasali *et al.*, 2001). However, many of the suggested therapeutic activities of lemongrass have been attributed to the non-volatile phenolic compounds present in leaves, which have gained attention especially by their antioxidant and radical-scavenging activities. This fact has increased both industrial and academic interest on the non-volatile composition of the plant (Figueirinha *et al.*, 2008; Palenzuela *et al.*, 2004). Presently, it has been proven the presence of a great variety of constituents in *C. citratus* leaves, such as phenolic acids, alkaloids, alcohols and more important, flavonoids and tannins, which may be responsible for some therapeutic activities such as anti-inflammatory activity (Figueirinha *et al.*, 2010; Negrelle and Gomes, 2007; Vendruscolo *et al.*, 2005).

Considering that inflammation may lead to various diseases such as rheumatoid arthritis, inflammatory bowel disease or psoriasis and current steroidal and non-steroidal anti-inflammatory drugs used to treat inflammatory disorders can develop many adverse effects, the discovery of new and safer anti-inflammatory agents continue to be an issue of high interest. In this field, plants used in folk medicine become excellent research candidates for their potential content in compounds to lead to effective herbal formulations from standardized active extracts.

To our knowledge, there are no pharmacological studies that evaluate the anti-inflammatory potential of *C. citratus* leaves infusion and its phenolic compounds on *in vivo* models, even if in a previous study of our group it has been reported that, under inflammatory conditions, an essential oil-free infusion of *C. citratus* leaves allowed cell viability and conferred significant reduction of nitric oxide production in dendritic cells stimulated with lipopolysaccharide (Figueirinha *et al.*, 2010). More recently, our group found more evidence of the anti-inflammatory mechanisms behind the beneficial effects of this plant and mainly due to its phenolic compounds *in vitro* (Francisco *et al.*, 2011, 2013, 2014).

Based on these findings, the aim of the present study was to evaluate the anti-inflammatory and analgesic activities of an essential oil-free infusion (CcE), a flavonoid-rich fraction (CcF) and a tannin-rich fraction (CcT) of *C. citratus* leaves on animal models of acute inflammation and pain.

MATERIALS AND METHODS

Plant material and infusion preparation: Dry leaves of *C. citratus* (DC) Stapf. were purchased from ERVITAL® (Mezio, Castro D'Aire, Portugal). The plant was cultivated in the region of Mezio, Castro d'Aire (Portugal), in a greenhouse at 1000 m above sea level and was collected in September 2007. A voucher specimen was deposited in the Herbarium of Aromatic and Medicinal Plants of the Faculty of Pharmacy-University of Coimbra (Figueirinha *et al.*, 2008). The plant identification was confirmed by Dr. J. Paiva (Department of Life Sciences, University of Coimbra, Portugal).

CcE was obtained as previously described by Figueirinha *et al.* (2008). Briefly, the infusion was prepared by adding boiling water to the plant material (30:1 v/w) and left for 15 min. After the extraction, the mixture was filtered under vacuum and it was washed three times with n-hexane to remove the less polar compounds. The aqueous phase was concentrated using a rotary evaporator to a small volume, freeze-dried and kept at -20°C in N₂ atmosphere until use. All subsequent study was performed with this essential oil-free infusion (CcE). For *in vivo* experiments

the infusion was dissolved in distilled water. Administrated doses were calculated based on the traditionally used doses, taking into consideration the yield of the extraction process and the conversion into animal doses, according to the FDA (Food and Drug Administration) guidelines. In order to examine a possible dose-dependent effect, two different doses were used: D'1 and D'2 (dose and double dose for the anti-inflammatory assay) and D''1 and D''2 (dose and double dose for the analgesic assay), according to the animal model used.

Infusion fractionation: The flavonoid and tannin rich fractions were obtained using a reverse phase semipreparative column Lichroprep® RP-18 (310×25 mm, particle sizes 40-63 µm), Merck (Darmstadt, Germany), eluted with water and aqueous methanol solutions. Tannin-rich fraction (CcT), phenolic acids fraction and a mixture of phenolic acids and flavonoids were obtained. Dry residue of that mixture was recovered in 50% aqueous ethanol and fractionated by gel chromatography on a Sephadex® LH-20 (Sigma-Aldrich-Amersham, Sweden) column (85×2.5 cm), using ethanol as mobile phase, as previously described (Figueirinha *et al.*, 2010). A flavonoid-rich fraction (CcF) was obtained. All the fractioning processes described above were monitored by HPLC and thin-layer chromatography.

The tannin and flavonoid-rich fractions were concentrated under reduced pressure at 40°C and freeze-dried. Again, before the *in vivo* experiments, the fractions were dissolved in distilled water and the doses administrated were calculated taking into consideration the yield of the fractioning process, according to Figueirinha *et al.* (2010) and converted into animal doses.

Reagents and drugs: n-Hexane was purchased from Carlo Erba Reagenti SpA (Milan, Italy). Acetic acid was purchased from J.T. Baker (Deventer, Holand). Ketamine (Ketalar, 7.7 mg kg⁻¹) was purchased from Parke-Davis, Pfizer (Seixal, Portugal) Chlorpromazine (Largatil, 2.3 mg kg⁻¹) was purchased from Rhône-Poulenc Rores, Laboratório Vitória SA (Amadora, Portugal). Diclofenac sodium (Voltaren 75 mg/3 mL) was purchased from Novartis Pharmaceuticals SA (Barcelona, Spain). Morphine hydrochloride and carrageenan-6 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were purchased from Merck (Darmstadt, Germany).

Animals: Male Wistar rats weighing 120-220 g and male mice weighing 20-30 g were obtained from Charles River (Barcelona, Spain). The animals were maintained with food and water *ad libitum* and kept at 22±1°C with controlled 12 h light/dark cycle at the Faculty of Pharmacy (University of Coimbra). The animals were allowed to adapt to the laboratory for 7 days before testing.

For evaluation of *C. citratus* anti-inflammatory activity, male Wistar rats were fasted with free access to water at least 24 h prior to experiments and to study the analgesic activities, male mice were fasted with free access to water at least 18 h prior to experiments. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in Directive 2010/63/EU.

Pharmacological assays

Carrageenan-induced rat paw oedema test: The carrageenan-induced rat paw oedema test (Green *et al.*, 1971) was used to evaluate the *in vivo* acute anti-inflammatory activity of the samples of *C. citratus*. Diclofenac sodium was used as reference drug. The animals were divided into eight groups (n = 6): Carrageenan control group received water 0.5 µL g⁻¹ p.o., positive control

group received diclofenac sodium 10 mg kg⁻¹ i.p. and test groups received orally the extract at the doses of 34.12 mg kg⁻¹ (CcE D'1) and 68.24 mg kg⁻¹ (CcE D'2), the flavonoid-rich fraction at the doses of 3.71 mg kg⁻¹ (CcF D'1) and 7.42 mg kg⁻¹ (CcF D'2) and the tannin-rich fraction at the doses of 2.98 mg kg⁻¹ (CcT D'1) and 5.96 mg kg⁻¹ (CcT D'2).

Carrageenan control and test groups were dosed 1 h and positive control group was dosed 30 min before a subplantar injection of 0.1 mL of 1% solution of carrageenan in saline, administered to the right hind footpad of each animal. The paw oedema volume was measured with a digital plethysmometer (model LE7500, Panlab, Barcelona, Spain) after carrageenan injection and then every hour during 6 h. The anti-inflammatory effect was calculated 4 h after carrageenan administration and was expressed as percentage of oedema inhibition for the treated animals with respect to the carrageenan control group. The percentage of oedema inhibition was calculated according to the following equation:

$$\text{Oedema inhibition (\%)} = 1 - \frac{V_t}{V_c} \times 100$$

where, V_t is the mean variation of the paw volume in rats treated with the sample or diclofenac sodium and V_c is the mean variation of the paw volume in the carrageenan control group.

Hot plate test: The hot plate test was used to evaluate the central analgesic activity of the samples by measuring the reaction time according to the method described by Eddy and Leimbach (1953). Morphine was used as reference drug. Mice were placed individually on a hot plate set at 55±1°C (model LE7406, PanLab, Barcelona, Spain). Reaction time was recorded when the animals licked their forepaws, shaken or jumped. The baseline was considered as being the reaction time before treatment. Then, the animals were divided into seven groups (n = 6-8): Positive control group received morphine 10 mg kg⁻¹ i.p. and test groups received orally the extract at the doses of 68.24 mg kg⁻¹ (CcE D''1) and 136.48 mg kg⁻¹ (CcE D''2), the flavonoid-rich fraction at the doses of 7.42 mg kg⁻¹ (CcF D''1) and 14.84 mg kg⁻¹ (CcF D''2) and the tannin-rich fraction at the doses of 5.96 mg kg⁻¹ (CcT D''1) and 11.92 mg kg⁻¹ (CcT D''2). Test groups were dosed 1 h and positive control group was dosed 30 min before placement of the animals on the hot plate for new recording of reaction time to temperature. Analgesic capacity was calculated by using the equation:

$$\text{Analgesic capacity (\%)} = 1 - \frac{T_b}{T_t} \times 100$$

where, T_b is the mean reaction time recorded as baseline and T_t is the mean reaction time recorded after treatment with morphine or the samples.

Acetic acid-induced writhing test: The acetic acid-induced writhing test was carried out according to the method previously described by Collier *et al.* (1968). The test was used to evaluate the peripheral analgesic activity of the samples, employing diclofenac sodium as reference drug. Acetic acid 0.6% in saline was administered i.p., 0.1 mL/10 g b.wt. The number of writhes-a response consisting of abdominal wall contractions followed by hind limb extension-was counted during continuous observation of the animals for 30 min, starting to count 5 min after the injection. The animals were divided into eight groups (n = 6-8): Negative control group received water 0.5 µL g⁻¹ p.o., positive control group received diclofenac sodium 10 mg kg⁻¹ i.p. and test groups

received orally the extract at the doses of 68.24 mg kg⁻¹ (CcE D'1) and 136.48 mg kg⁻¹ (CcE D'2), the flavonoid-rich fraction at the doses of 7.42 mg kg⁻¹ (CcF D'1) and 14.84 mg kg⁻¹ (CcF D'2) and the tannin-rich fraction at the doses of 5.96 mg kg⁻¹ (CcT D'1) and 11.92 mg kg⁻¹ (CcT D'2). Negative control and test groups were dosed 1 h and positive control group was dosed 30 min before the acetic acid injection. A significant reduction in the number of writhes by any drug was considered as a positive analgesic response. Analgesic capacity was calculated by using the equation:

$$\text{Analgesic capacity (\%)} = 1 - \frac{W_t}{W_c} \times 100$$

where, W_t is the average number of writhes in test and positive control groups and W_c is the average number of writhes in the negative control group.

Histological analysis: A histological analysis was carried out in order to evaluate the toxicity of the *C. citratus* leaves essential oil-free infusion (CcE). The animals used in the carrageenan paw edema test were sacrificed and the livers were collected, cut into transverse fragments (5 mm) and immersed in a 10% solution of formaldehyde for 24 h. The pieces were processed into paraffin and stored at -20°C. The fragments were cut in 4 µm pieces in a SHANDON (AS 325) microtome and stained with hematoxylin and eosin. The resulting images were obtained and observed using a NIKON (Eclipse E600) microscope.

Transmission electron microscopy (TEM): Samples (2-3 mm) of liver collected from the untreated rats (control) and the rats treated with CcE at 34.12 mg kg⁻¹ (CcE D'1) and 68.24 mg kg⁻¹ (CcE D'2) were fixed for 3 h at 4°C in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) supplemented with 1 mM calcium chloride. Following rinsing in the same buffer, the samples were postfixed in 1% buffered osmium tetroxide for 1.5 h at 4°C and then in 1% aqueous uranyl acetate for 1 h at room temperature. The samples were further dehydrated in a graded ethanol series (70-100%) and embedded in Spurr's resin. Thin sections were obtained with a LKB Ultratome NOVA, ultramicrotome equipped with a diamond knife, conventionally stained with uranyl acetate and lead citrate and observed in a JEOL JEM-100 SX at 80 kV.

Statistical analysis: Data obtained from animal experiments was expressed as mean and standard error of the mean (Mean±SEM). Comparison between groups was done by one-way ANOVA followed by Bonferroni's multiple comparison test. Values of $p < 0.05$ were considered to be significant.

RESULTS

Carrageenan-induced rat paw oedema test: Subplantar injection of the carrageenan solution into the right hind footpad of each rat led to a time-dependent increase in the paw volume. This increase was observed at 1 h and it was maximal at 4 h after administration.

Carrageenan-induced paw oedema volume was significantly reduced at the fourth hour by the administration of CcE D'1 and CcE D'2, CcF D'2, CcT D'2 and diclofenac sodium. The percentage of oedema reduction was 70.80 and 82.30% for CcE D'1 and CcE D'2, respectively, 59.00% for CcF D'2, 61.00% for CcT D'2 and 84.00% for positive control group. There was no significant difference between CcF D'1 or CcT D'1 and the negative control group (Fig. 1).

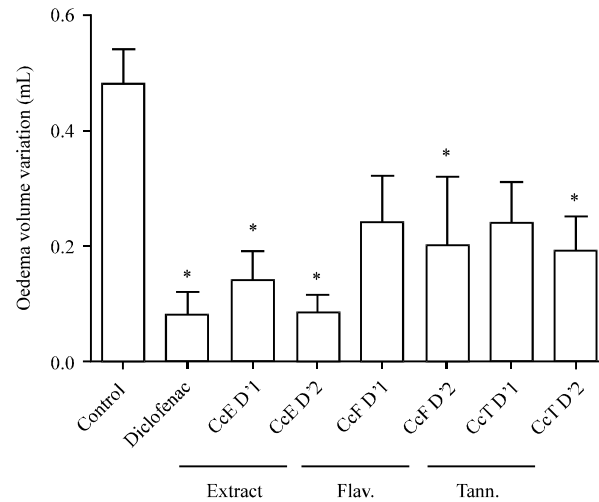


Fig. 1: Results from the carrageenan-induced rat paw oedema test were obtained after administering water $0.5 \mu\text{L g}^{-1}$ p.o. (negative control), diclofenac sodium 10 mg kg^{-1} i.p. (positive control) and *C. citratus* aqueous extract 34.12 mg kg^{-1} (CcE D'1) and 68.24 mg kg^{-1} (CcE D'2), *C. citratus* flavonoid-rich fraction 3.71 mg kg^{-1} (CcF D'1) and 7.42 mg kg^{-1} (CcF D'2) and *C. citratus* tannin-rich fraction 2.98 mg kg^{-1} (CcT D'1) and 5.96 mg kg^{-1} (CcT D'2) p.o. Each value is the Mean \pm SEM of 6 rats. Statistical differences between the treated and the negative control groups were determined by ANOVA followed by Bonferroni test. * $p < 0.05$

Hot plate test: Mice treated with morphine showed a marked increase in the reaction time with a maximum protective effect of 75.9%. However, the results reveal that mice treated with any of the samples did not show any significant increase in the baseline (Fig. 2).

Acetic acid-induced writhing test: Mice treated with CcE D'2 showed a significant inhibition of the writhing response induced by acetic acid. The percent analgesic capacity was 27.5% (34.8 ± 8.1 writhes) and 57.0% (20.6 ± 9.7 writhes) for CcE D'1 and CcE D'2, respectively. Diclofenac sodium showed an analgesic capacity of 83.0% (13.0 ± 3.4 writhes). An important inhibition of the writhing response was also found for CcF D'2 and CcT D'2, with a percent analgesic capacity of 54.6% (21.8 ± 14.9 writhes) and 52.1% (23.0 ± 9.9 writhes), respectively. There was no significant difference between the lowest dose of flavonoids and tannins-rich fractions and the negative control group (48.0 ± 1.8 writhes), corresponding to 22.1% (37.4 ± 11.2 writhes) and 18.55% (39.1 ± 8.9 writhes) of percent analgesic capacity, respectively (Fig. 3).

Histological analysis: In Fig. 4a-c, represented the histological images of liver for control animals, animals treated with CcE single dosage (D'1) and double dosage (D'2), respectively, of the animals used in the carrageenan-induced rat paw edema test.

No differences were observed between the control and test groups, in both tested dosages. The images showed well shaped hepatic cells, with no signs of blood stasis in the extracellular medium or necrotic cells, revealing the absence of toxicity of the *C. citratus* leaves essential oil-free infusion.

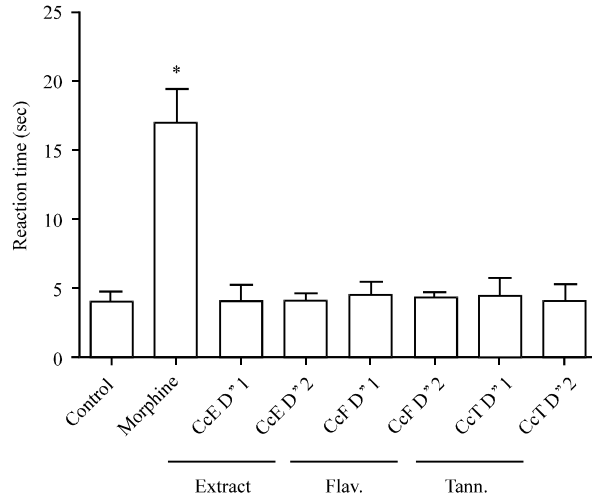


Fig. 2: Results from the hot plate test were obtained before and after administering morphine 10 mg kg⁻¹ i.p. (positive control) and *C. citratus* aqueous extract 68.24 mg kg⁻¹ (CcE D*1) and 136.48 mg kg⁻¹ (CcE D*2), *C. citratus* flavonoid-rich fraction 7.42 mg kg⁻¹ (CcF D*1) and 14.84 mg kg⁻¹ (CcF D*2) and *C. citratus* tannin-rich fraction 5.96 mg kg⁻¹ (CcT D*1) and 11.92 mg kg⁻¹ (CcT D*2) p.o. Each value is the Mean±SEM of 6-8 mice/group. Statistical differences between the treated and the control groups were determined by ANOVA followed by Bonferroni test. *p<0.05

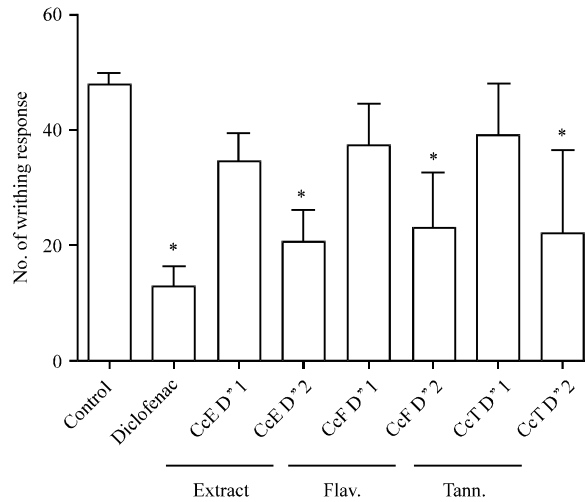


Fig. 3: Results from the acetic acid-induced writhing test were obtained after administering water 0.5 μL g⁻¹ p.o. (negative control), diclofenac sodium 10 mg kg⁻¹ i.p. (positive control) and *C. citratus* aqueous extract 68.24 mg kg⁻¹ (CcE D*1) and 136.48 mg kg⁻¹ (CcE D*2), *C. citratus* flavonoid-rich fraction 7.42 mg kg⁻¹ (CcF D*1) and 14.84 mg kg⁻¹ (CcF D*2) and *C. citratus* tannin-rich fraction 5.96 mg kg⁻¹ (CcT D*1) and 11.92 mg kg⁻¹ (CcT D*2) p.o. Each value is the Mean±SEM of 6-8 mice/group. Statistical differences between the treated and the control groups were determined by ANOVA followed by Bonferroni test. *p<0.05

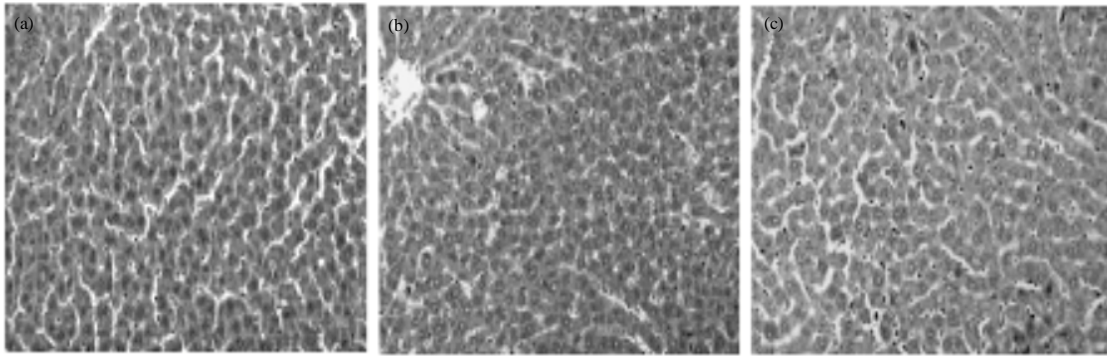


Fig. 4(a-c): Histological study of liver. Staining with hematoxylin (cell nuclei-blue) and eosin (cytoplasm-pink/red), 20x magnification in the original. (a) Negative control group, (b) Animals treated with CcE single dosage D'1 (34.12 mg kgG⁻¹) and (c) Animals treated with CcE double dosage D'2 (68.24 mg kgG⁻¹)

Liver cell ultrastructure: A general view of the ultrastructural organization of the rat liver parenchymal cells (hepatocytes) is shown in Fig. 5a. Briefly, hepatocytes are polygonal in shape with one, or sometimes two, large rounded nuclei containing one or two prominent nucleoli and a few dense masses of heterochromatin. In the whole cytoplasm, the most characteristic feature was the presence of a very high number of mitochondria showing a moderate electron-dense matrix and a reasonable number of electron-dense cristae (Fig. 5a, b). Both the smooth (SER) and rough endoplasmic reticulum (RER) were also abundant, the former appearing as irregularly shaped vesicular structures or short, branching tubules and the latter as single or small groups of flattened cisternae often surrounding closely the mitochondria (Fig. 5b). A few lipid bodies of relatively small size and a reasonable high number of peroxisomes with a homogeneous electron-dense matrix, generally containing a finely granular or crystalline core (nucleoid), were also frequently present (Fig. 5a, b). Secondary lysosomes, golgi bodies and vesicles and glycogen granules were sometimes seen. Adjacent hepatocytes were separated one from each other by a small intercellular space in which junctional complexes were present (Fig. 5a).

The ultrastructure of hepatocytes in rats treated with both CcE D'1 (Fig. 5c) and CcE D'2 (Fig. 5d) did not differ significantly from that of hepatocytes in control rats (Fig. 5a). In general, the integrity of organelles, plasma membrane and junctional complexes remained intact and the organelles retained the features characteristic of the control liver. Only in a few hepatocytes the mitochondria matrix sometimes appeared more electron-transparent (Fig. 5c) and with a more reduced number of cristae and the lipid bodies could be larger (Fig. 5d) and more numerous in the cytosol.

DISCUSSION

Anti-inflammatory and analgesic effects of many herbs have been related to their polyphenolic composition, mostly to flavonoids and tannins. Also, it has been reported that flavonoids such as luteolin produce significant antinociceptive and/or anti-inflammatory activities (Hosseinzadeh and Younesi, 2002). In regard to *C. citratus*, we have previously demonstrated (Figueirinha *et al.*, 2010) that the most active polyphenolic fractions of an essential oil-free infusion are those which held flavonoids and tannins and when tested *in vitro*, CcE showed high anti-inflammatory

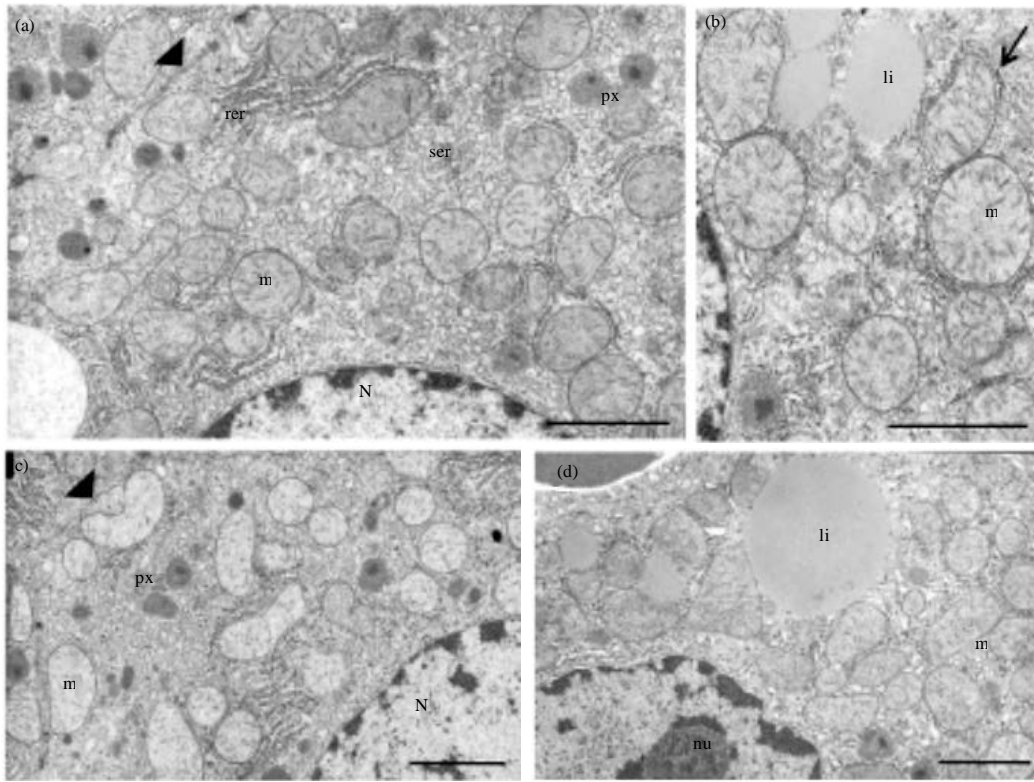


Fig. 5(a-d): Transmission electron micrographs of hepatocytes in (a-b) Control rats, (c) Rats treated with CcE D'1 and (d) CcE D'2. N: Nucleus, nu: Nucleolus, m: Mitochondria, px: Peroxisomes, rer: Rough endoplasmic reticulum, ser: Smooth endoplasmic reticulum, li: Lipid bodies, Arrow: Rer cistern surrounding a mitochondria, Arrowhead: Plasma membranes of two adjacent hepatocytes. Bar = 1 µm

activity, inhibiting the NO production in dendritic cells. In the present study, anti-inflammatory and analgesic activities of an essential oil-free infusion of *C. citratus* leaves were evaluated to verify the claims made by folk medicine. Additionally, its flavonoid and tannin-rich fractions were tested, trying to better characterize their contribution for the pharmacological effects and also to deeper identify the responsible components of *C. citratus* for its traditional uses.

The carrageenan-induced rat paw oedema assay was performed to study the anti-inflammatory effect. This experimental model is commonly used for determining the acute phase of the inflammation because it is related with neutrophil infiltration, as well as the neutrophil-derived free radicals, prostaglandins and cytokines production (Cardoso *et al.*, 2003; Tanas *et al.*, 2010). Diclofenac sodium is widely used as a reference Non-Steroidal Anti-Inflammatory Drug (NSAID), being commonly prescribed due to its anti-inflammatory and analgesic effects. This drug has the ability to reduce inflammation, swelling and pain by inhibiting either the release of arachidonic acid or the prostaglandin synthesis *in vivo* and *in vitro*, as well as by influencing the function of polymorphonuclear leukocytes *in vitro*, decreasing chemotaxis and free radical formation (Mahgoub, 2002; Ojewole, 2004).

Our present results indicate that the *C. citratus* infusion has anti-inflammatory activity, corroborating its traditional use. The observed effect for the highest dose was similar to that obtained by the reference NSAID used. The flavonoid and the tannin-rich fractions also exhibited anti-inflammatory activity at their highest dose, but when compared to the standard and the infusion, a lower inflammation inhibition was observed. Synergistic effects between the different phenolic compounds and/or the presence of other compounds in the extract can probably contribute for the CcE activity.

However, regarding the low doses that were used in the assay with the polyphenols, we may conclude about an important contribution of both flavonoids and tannins for the anti-inflammatory effect. This fact suggests that these polyphenols are significantly responsible for the overall effect observed with CcE. The mechanism behind these results is still unclear, but previous works point that one of the major anti-inflammatory pathways modulated by these compounds might be the inhibition of NF- κ B-regulated cytokines expression, probably from the scavenging capacity of reactive oxygen species, which modulates the activation of NF- κ B pathway (Francisco *et al.*, 2011; Morgan and Liu, 2010). On the other hand, the same reactive species-scavenging ability may be related to the inhibition of polymorphonuclear leukocyte's action, such as neutrophils (Ojewole, 2004; Tanas *et al.*, 2010).

For the potential analgesic activity two different models were used to study both central and peripheral mechanisms of nociception. The hot plate test allows us to verify central analgesic effects while the acetic acid-induced writhing test evaluates peripheral analgesic effects of drugs (De Queiroz *et al.*, 2010; Shang *et al.*, 2010).

The hot plate testis based on the animal behaviour resulting from activation of the primary nociceptors by high temperature and therefore, it is an excellent indicator of potential pharmacological drug effects. In the present study, morphinehydrochloride, a central-acting analgesic drug, which acts by activating opioid receptors (Eddy and Leimbach, 1953; Ruangsang *et al.*, 2010; Silva *et al.*, 2010a), produced a pronounced inhibitory effect on the nociceptive response showed by the increase in mice reaction time in contrast the *C. citratus* infusion, its flavonoid-and tannin-rich fractions showed no antinociceptive activity in this test.

As mentioned above, the acetic acid-induced writhing test is useful to evaluate drugs with analgesic activity at a peripheral level of nociception. It is known that acetic acid acts indirectly by inducing the release of endogenous pro-inflammatory mediators such as histamine, serotonin, bradykinin, acetylcholine, P substance and prostaglandins involved in visceral inflammatory pain, by causing an increased vascular permeability (Ruangsang *et al.*, 2010; Silva *et al.*, 2010b). This model is, therefore, sensitive to the action of NSAIDs (Ahmad *et al.*, 2010; Silva *et al.*, 2010a, b). In this study, diclofenac sodium caused the inhibition of pain induced by acetic acid and CcE, CcF and CcT showed also a significant reduction of the writhing response at the highest dose used. There were no relevant differences in the inhibition of the number of writhes for the three samples, which leads us to conclude that flavonoids and tannins are the compounds that almost exclusively contribute for the *C. citratus* peripheral analgesic activity. This fact corroborates the published data about phenolic compounds exerting an antinociceptive effect *in vivo*, namely for tannins (DalBo *et al.*, 2005; Subarnas and Wagner, 2000) and flavonoids (Gorzalczany *et al.*, 2011; Vidyalakshmi *et al.*, 2010; Valerio *et al.*, 2009).

This study reveals that CcE and its flavonoids and tannins are able to reduce paw oedema induced by carrageenan and the peripheral mechanism of pain, suggesting that their anti-inflammatory and analgesic effects may be related to the inhibition of the synthesis and release of various inflammatory mediators and to its antioxidant activity.

These results suggest that the CcE acts as anti-inflammatory and peripheral analgesic agent, still does not exhibit intrinsic central analgesic activity. Together, it can be inferred that the observed analgesic effect is likely associated with the inflammatory process, flavonoids and tannins having a significant contribution for these activities.

Liver plays a crucial role in the detoxification, modification and excretion of both endogenous and exogenous substances, including a variety of toxic substances. When exposed to toxic substances, hepatocytes show extensive alterations in the number, morphology and/or ultrastructure of organelles, such as mitochondria, endoplasmic reticulum, peroxisomes and lipid bodies (Lude *et al.*, 2007). To investigate the potential for hepatotoxicity of *C. citratus* extracts in experimental animals *in vivo*, the liver tissue and ultrastructural organization of hepatocytes in control rats and in rats treated with CcE D'1 and CcE D'2 were examined. In the control rats, the cellular organization of hepatocytes was found to be very similar to that described by Baratta *et al.* (2009). Following exposure to CcE D'1 and CcE D'2, no significant alterations were found in liver tissue and cell ultrastructure of the hepatocytes when compared with that of the untreated rats (control). Our results show that treatment with effective concentrations of the CcE induced no hepatotoxicity in rats and that, therefore, the administration of CcE can be considered safe under the experimental conditions used.

CONCLUSION

In conclusion, this results support the traditional use of *C. citratus* infusion as an anti-inflammatory agent and suggest that CcE or its polyphenols, namely the flavonoid-and tannin-rich fractions, may be used to obtain a new anti-inflammatory medicine, effective in the treatment of inflammatory-related pathologies.

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