Evaluation of Anti-inflammatory Activity of Estragole by Modulation of Eicosanoids Production

Luiz Alexandre Marques Wiirzler[*], Saulo Euclides Silva-Filho, Rafael Pazinatto Aguiar, Heitor Augusto Otaviano Cavalcante and Roberto Kenji Nakamura Cuman

Laboratory of Inflammation, Department of Pharmacology and Therapeutics, State University of Maringá, Maringá, PR, Brazil.

_____

ABSTRACT

Introduction: The estragole (EST) is a phenylpropanoid presents in many aromatic plants used in the folk medicine. Its anti-inflammatory activity was recently reported in the literature and researchers have suggested that it should be related to the inhibition of prostaglandins biosynthesis. **Objective:** To determine the mechanism of the anti-inflammatory activity exerted by EST. **Materials and Methods:** A series of experiments was conducted to evaluate the effects of the test compound on carrageenan-induced paw edema in mice, on prostaglandin levels in the inflamed paw tissues, and on cyclooxygenase (COX) and lipoxygenase (LOX) activities. **Results:** The treatment with EST (200 and 400 mg/kg) significantly reduced the paw edema after subplantar carrageenan injection. Moreover, EST (200 and 400mg/kg) significantly decreased the prostaglandin E_2 levels in the inflamed paw tissues four hours after stimuli. However, EST was inactive against the both COX and LOX enzymes. **Discussion:** It is known that EST suppresses the translocation of nuclear factor κB (NF-κB) to the nucleus of the cells after inflammatory stimuli. The results suggest that EST only interferes with the synthesis of COX-2 isoform, after inflammatory stimuli, by suppression of NF-κB activity, without interferer with the enzymes activities. **Conclusion:** Our data provide evidence that EST exerts considerable anti-inflammatory activity. The mechanism involved in the anti-inflammatory effect of EST may offer promising therapeutic benefits, since EST does not inhibit the COX-1 enzyme and, consequently, does not promote gastric damage.

Keywords: Anti-inflammatory activity, cyclooxygenases, lipoxygenase, eicosanoids, estragole.

INTRODUCTION

The estragole (EST, Figure 1) is a phenylpropanoid commonly presents in essential oils extracted from many aromatic plants. Some of these plants are extensively used by the population in the folk medicine, such as *Croton zehntneri*, *Illicium anisatum*, *Ocimum basilicum*, and *Artemisia dracunculus* [1,2]. Then, the understanding of its biological and toxic effects has a great significance.

According to the recent studies, EST exerts a wide of pharmacological properties including depression of the central nervous system [3]; anesthetic [4,5], antimicrobial [6], and antioxidant [7] activities; and modulation of the immune responses [1]. Moreover, a very significant activity of EST, the anti-inflammatory activity, was reported in the literature recently [8].

The acute inflammation is characterized by swelling, heat, redness, and pain and is an important defense mechanism against invading pathogens [9]. However, when this important immune response becomes exacerbate and unregulated it may promotes damage to the inflamed tissues and prejudices the organism. Among the many chemical mediators involved, those that arise from the cyclooxygenase (COX) cascade and role of biologically active prostaglandins in the inflammatory process and body homoeostasis have been extensively studied [10]. In this regard, COX inhibitors prevent prostaglandin biosynthesis and are effective anti-inflammatory compounds that reduce the levels of prostaglandins and cardinal signs of inflammation [11].
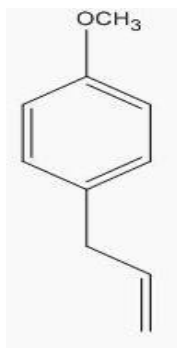
Fig. 1: Chemical structure of the phenylpropanoid estragole

Some researchers have suggested that the anti-inflammatory activity of EST is related to its ability to reduces the biosynthesis of prostaglandins by inhibition of COX enzymes [2,8]. However, to the best of our knowledge, the effect of EST on the COX activity has not been demonstrated and remains to be determined.

In this paper we describe the ability of EST to inhibit the prostaglandin productions. In this regard, we conducted a series of experiments designed to determine this activity, using some *in vivo* and *in vitro* models.

MATERIALS AND METHODS
Compound
The compound EST was purchased from Sigma (St. Louis, USA) with the minimum of 98% purity specified.

In vivo assays
Animals
The male Swiss albino mice (body weight 25 ± 2 g) were obtained from the Central Biotherium of the State University of Maringá. The animals were housed under standard conditions and received food and water *ad libitum* before the experiments. The experimental protocol was approved by the Ethical Committee in Animal Experimentation of the State University of Maringá (CEEA/UEM n° 017/2013).

Dosage and treatment of animals
For this study, we used the dosages for EST (100, 200, and 400 mg/kg) that were previously tested in similar animal models [1,2]. The compound EST was solubilized in saline (vehicle) and the animals were treated (0.2 mL/mouse) one hour before the carrageenan injection. The control groups received only the vehicle orally.

Carrageenan-induced paw edema
As previously reported [8,12], the subplantar injection of carrageenan (100 µg/25 µL/paw) in the right hind paw was given one hour after the treatments. The negative control group received only subplantar injection of sterile saline. The positive control group (Cg) received subplantar injection of carrageenan and only vehicle orally. The paw volume was measured by digital plethysmometer (Ugo Basile®, Italy) prior, 1, 2 and 4 hours after the stimuli. The paw edema, in µL, was calculated by the difference in the paw volume prior and after carrageenan injection. After the last measurement, the animals were euthanized by cervical dislocation and the inflamed hind paws tissues were collected.

Determination of prostaglandin E_2 levels in mice paw tissue
As described in the literature [13], the mice paw tissues were collected 4 hours after inducing the inflammation by carrageenan in extraction buffer containing 0.1% Tween-20 in phosphate-buffered saline (1g/3mL). Tissues were triturated, homogenized, and centrifuged at 6000 g for 15 minutes. The prostaglandin E_2 (PGE_2) levels were determined in the supernatants using a PGE_2 EIA kit (Cayman Chemical Company, USA). The sensitivity of the assays was 9.7 pg/mL.

In vitro assays
Cyclooxygenase inhibition screening assay
EST was evaluated for its ability to *in vitro* inhibit COX-1/COX-2 enzymes using the COX Inhibitor Screening Kit (Cayman Chemical Company, USA) according to the manufacturer instructions. The final concentrations tested range of 0.01 to 100 µM. The concentrations of EST that inhibit 50% of the enzyme activity (IC_{50}) were calculated from the mean of two determinations by linear regression of the dose-response curve. The ability of ibuprofen to inhibit COX-1 and COX-2 was previously reported using the same cyclooxygenase inhibitor screening assay kit [14], then, the ibuprofen was used as positive control for inhibition of COX-1 and COX-2.

Lipoxygenase inhibition screening assay
EST was evaluated for its ability to in vitro inhibit 5-lipoxygenase (5-LOX) enzyme using the LOX Inhibitor Screening Kit (Cayman Chemical Company, USA) according to the manufacturer instructions. The final concentrations tested range of 0.01 to 100 µM. The concentrations of EST that inhibit 50% of the enzyme activity (IC_{50})

were calculated from the mean of two determinations by linear regression of the dose-response curve.

Statistical analysis

Data were expressed as mean values ± standard error of the mean. Statistical significance was tested using one-way analysis of variance (ANOVA), followed by the Tukey's test for comparison between means. The difference was considered significant when the p values were smaller than 0.05.

RESULTS

Effect of the treatment with estragole on carrageenan-induced paw edema in mice

The subcutaneous injection of carrageenan in the mouse paw caused a local inflammatory response with edema peak in the fourth hour after stimuli. As shown in the Figure 2, when compared with de Cg group, the treatment with EST at 100 mg/kg did not significantly reduce the paw edema in any evaluated period. EST at 200 mg/kg significantly decreased the rat paw volume only in the fourth hour (21.1%). EST at 400 mg/kg significantly reduced the development of edema in the second (22.0%) and fourth hour (32.2%) after carrageenan injection. The effect observed here was dose-dependent manner.
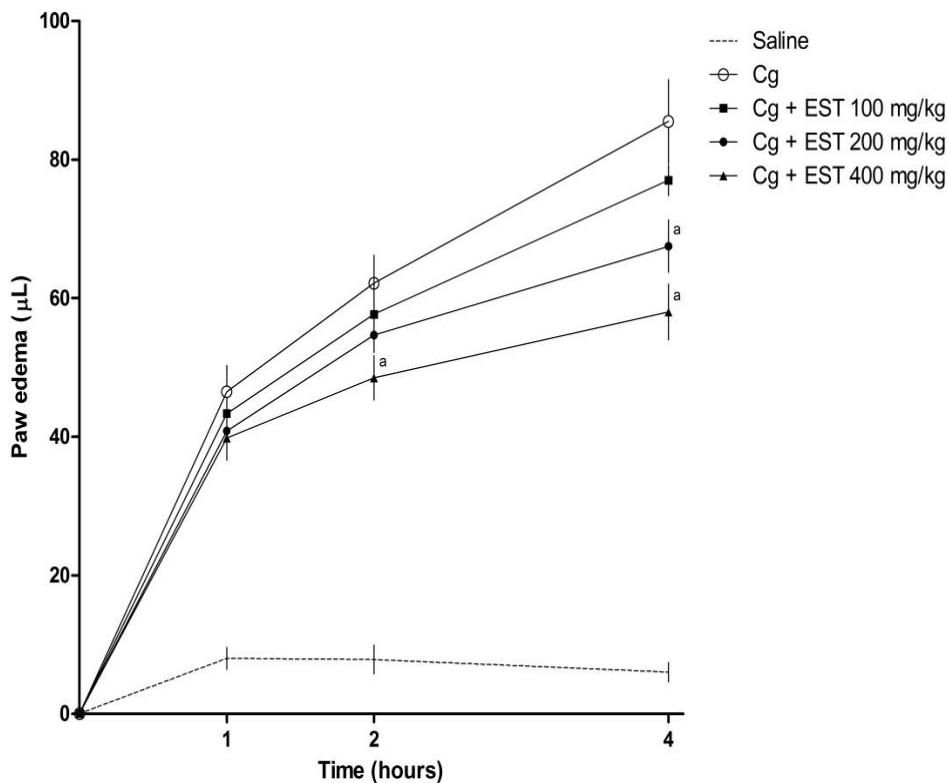

Fig. 2: Effect of the treatment with estragole (EST) on carrageenan-induced paw edema in mice. Values represent mean values ± standard error of the mean for each group (n = 6). [a] Significant difference at $p < 0.05$ compared with the carrageenan (Cg) group.

Effect of the treatment with estragole on prostaglandin E_2 levels in inflamed paw of mice

The effect of EST on the PGE_2 levels in inflamed paws was examined 4 hours after the induction of inflammation. The Figure 3 demonstrates that, when compared with de Cg group, the treatment with EST at 100 mg/kg did not significantly reduce the PGE_2 levels. EST at 200 and 400mg/kg significantly decreased the PGE_2 levels in the inflamed paw tissues 18.5% and 41.8%, respectively .The effect observed here was dose-dependent manner.

Effect of estragole on cyclooxygenase and lipoxygenase activities in vitro

As shown in Table 1, our results demonstrated that EST is inactive against the both COX-1 and COX-2 enzymes. The compound ibuprofen was used as internal control for enzymes inhibition and the results obtained were similar to data described in the literature using the same protocol [14,15]. Then, it validates the assays conduced and our results. Therefore, our results demonstrate that EST is inactive against the 5-LOX enzyme (Figure 4).
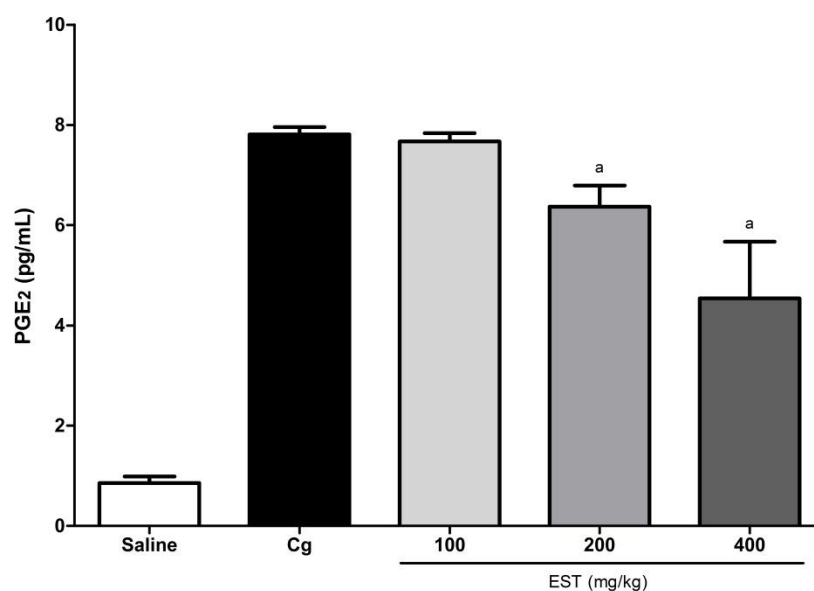

Fig. 3: Effect of the treatment with estragole (EST) on prostaglandin E_2 (PGE_2) levels in inflamed paw of mice. Values represent mean values ± standard error of the mean for each group (n = 6). [a] Significant difference at $p < 0.05$ compared with the carrageenan (Cg) group.

Table 1: Effect of estragole and ibuprofen on COX-1/COX-2 enzymes

Compound	COX-1 IC$_{50}$ (µM) [a]	COX-2 IC$_{50}$ (µM) [a]
Estragole	Inactive	Inactive
Ibuprofen	3.12	1.56

[a] Concentration required to produce 50% inhibition of COX-1 or COX-2. The result (IC$_{50}$, µM) is the mean of two determinations.
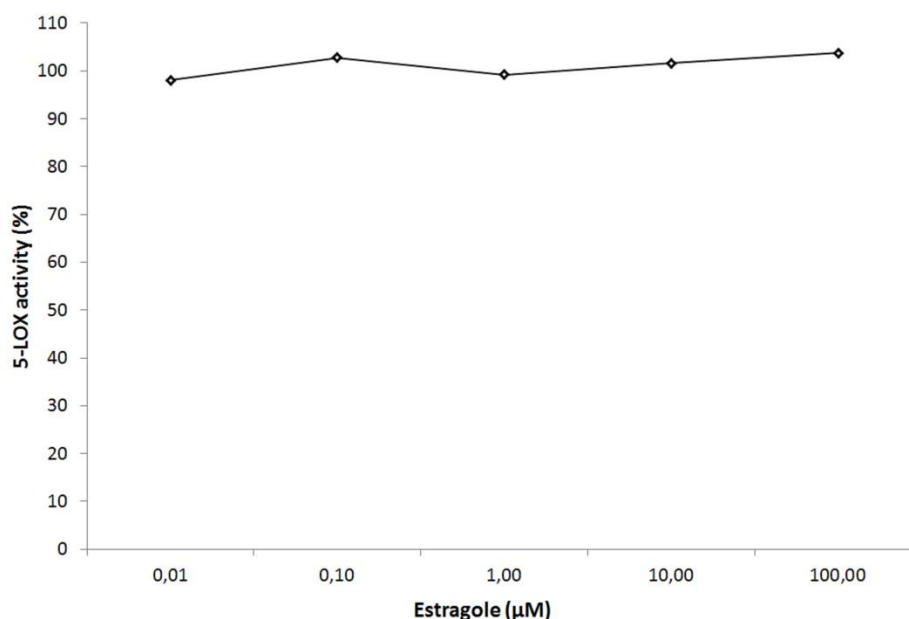
Fig. 4: Effect of estragole on 5-lipoxygenase activity in vitro. Values represent the mean of two determinations

DISCUSSION

One of the most used experimental protocols of acute inflammation is the carrageenan-induced inflammatory response. In this animal model, there is an intense production of many inflammatory mediators after stimuli such as histamine, bradykinin, arachidonic acid derivatives, cytokines, and others [14,16]. These mediators induce vasodilatation, increasing vascular permeability and recruiting leukocytes to the injury site [17].

Our results showed that the treatment with EST promotes significant reduction in the development of inflammatory response, demonstrated by reduction of edema formation after carrageenan injection (Figure 2). There are some biological activities exerted by EST that might be involved in this effect. Previous studies determined that EST downregulates the nitric oxide (NO) production and release [2], a mediator involved in the cellular recruitment and edema development. Therefore, researchers showed the treatment with EST suppresses the releasing of certain pro-inflammatory cytokines [1], such as interleukin-1β (IL-1β) and the tumor necrosis factor-α (TNF-α), that increase the cellular migration to the inflammatory site, production of pro-inflammatory mediators, and releasing of other pro-inflammatory cytokines.

One important mechanism of action certainly involved here is the suppression of prostaglandin production exerted by EST, as shown in the Figure 3. However, the reduction caused by EST on the eicosanoids levels is not related to the inhibition of enzymes involved in their synthesis, since the EST is inactive against COX-1, COX-2, and 5-LOX enzymes isoforms (Table 1 and Figure 4).

Previous studies demonstrated that EST inhibits the translocation of nuclear factor κB (NF-κB) from the cytoplasm to the nucleus of the cells after inflammatory stimuli, *in vitro* and *in vivo* [1,2]. The NF-κB is an important transcription factor involved in the inflammatory process that promotes the overproduction of cytokines, induces the phagocytosis, enhances differentiation and maturation of monocytes/macrophages, and increases the synthesis of eicosanoids by stimulation of production and expression of COX-2 in inflamed cells. Then, we suppose that the reduction of the prostaglandin levels in the inflamed paw tissue is related to suppression of production and expression of COX-2 by downregulation of NF-κB. It explains how the EST promotes anti-inflammatory effect without the side effects exerted by the classical nonsteroidal anti-inflammatories drugs (NSADs) [18], as previously

observed. The most important side effect, the gastric damage, caused by the NSADs is related to their ability to inhibit both isoforms of COX enzymes: the constitutive COX-1 and the inducible COX-2, since the inhibition of COX-1 isoform is the responsible for the loss of gastric protection and consecutive damage to the tissues [19]. As our data suggest, the EST only interferes with the synthesis of COX-2 isoform after the inflammatory stimuli, by suppression of NF-κB activity, without interferer with the COX-1 activity. Then, the final result is the anti-inflammatory activity with no loss of gastric protection.

To confirm our supposition, future studies will be carry out to determine the reduction of COX-2 production and expression in inflamed cells, by western blots.

CONCLUSION

In conclusion, our data provide evidence that estragole (EST), when administered orally to mice, in a pure (neat) form, exerts considerable anti-inflammatory activity, by reducing the carrageenan-induced paw edema and downregulating the prostaglandin production; however, the EST was not able to inhibit the enzymes COX-1, COX-2, and 5-LOX. The mechanism involved in the anti-inflammatory effect of EST may offer promising therapeutic benefits exerted by the oral administration of this compound, since it does not inhibit the COX-1 enzyme and, consequently, does not promote gastric damage. Further investigations may elucidate the mechanism of action by which EST produces the observed changes in the prostaglandin levels.

REFERENCES

1. Wiirzler LAM, Silva-Comar FM de S, Silva-Filho SE, Oliveira MJA de, Bersani-Amado CA and Cuman RKN. Evaluation of immunomodulatory activity of trans-anethole and estragole, and protective effect against cyclophosphamide-induced suppression of immunity in Swiss albino mice. Int J Appl Res Nat Prod. 2015; 8(1): 26–33.
2. Silva-Comar FM de S, Wiirzler LAM, Silva-Filho SE, Kummer R, Pedroso RB, Spironello RA et al. Effect of estragole on leukocyte behavior and phagocytic activity of macrophages. Evid Based Complement Alternat Med. 2014; Jan: 784689.
3. Romano A, Torres MJ, Castells M, Sanz ML and Blanca M. Diagnosis and management of drug hypersensitivity reactions. J Allergy Clin Immunol. Elsevier Ltd. 2011; 127(3 Suppl): S67–73.
4. Albuquerque AA, Sorenson AL and Leal-Cardoso JH. Effects of essential oil of Croton zehntneri, and of anethole and estragole on skeletal muscles. J Ethnopharmacol. 1995; 49(1): 41–9.
5. Dallmeier K and Carlini EA. Anesthetic, hypothermic, myorelaxant and anticonvulsant effects of synthetic eugenol derivatives and natural analogues. Pharmacology. 1981; 22(2): 113–27.
6. Andrade TCB, De Lima SG, Freitas RM, Rocha MS, Islam T, Da Silva TG et al. Isolation, characterization and evaluation of antimicrobial and cytotoxic activity of estragole, obtained from the essential oil of Croton zehntneri (Euphorbiaceae). An Acad Bras Cienc. 2015; 87(1): 173–82.
7. Donati M, Mondin A, Chen Z, Miranda FM, do Nascimento BB, Schirato G et al. Radical scavenging and antimicrobial activities of Croton zehntneri, Pterodon emarginatus and Schinopsis brasiliensis essential oils and their major constituents: estragole, trans-anethole, β-caryophyllene and myrcene. Nat Prod Res. 2015; 29(10): 939–46.
8. Ponte EL, Sousa PL, Rocha MVAP, Soares PMG, Coelho-de-Souza AN, Leal-Cardoso JH et al. Comparative study of the anti-edematogenic effects of anethole and estragole. Pharmacol Rep. 2012; 64(4): 984–90.
9. Rang HP, Dale JM, Ritter JM, Flower RJ. Rang and Dale's Pharmacology. 7th ed. New York: Churchill Livingstone. 2012: 792.
10. Serhan CN, Ward PA and Gilroy DW. Fundamentals of Inflammation. 1st ed. Cambridge: University Press. 2010: 473.
11. Sherwood ER and Toliver-Kinsky T. Mechanisms of the inflammatory response. Best Pract Res Clin Anaesthesiol. 2004; 18(3): 385–405.
12. Ritter AM, Domiciano TP, Verri WA,

Zarpelon AC, da Silva LG, Barbosa CP et al. Antihypernociceptive activity of anethole in experimental inflammatory pain. Inflammopharmacology. 2013; 21(2): 187–97.

13. Banani A, Maleki-Dizaji N, Garjani A, Soraya H, Mostafalou S and Ziaee M. Clavulanic acid exhibits anti-inflammatory effects on carrageenan-induced paw edema model of inflammation in rats. Ann Biol Res. 2012; 3(7): 3312–20.

14. Wisniewski-Rebecca ES, da Rocha BA, Wiirzler LAM, Cuman RKN, Velazquez-Martinez CA and Bersani-Amado CA. Synergistic effects of anethole and ibuprofen in acute inflammatory response. Chem Biol Interact. 2015; 16.

15. Velázquez CA, Rao PNP and Knaus EE. Novel nonsteroidal antiinflammatory drugs possessing a nitric oxide donor diazen-1-ium-1,2-diolate moiety: design, synthesis, biological evaluation, and nitric oxide release studies. J Med Chem. 2005; 48: 4061–7.

16. Silva-Filho SE, Silva-Comar FMS, Wiirzler LAM, Pinho RJ, Grespan R, Bersani-Amado CA et al. Effect of Camphor on the Behavior of Leukocytes In vitro and In vivo in Acute Inflammatory Response. Trop J Pharm Res. 2014; 13(12): 2031–7.

17. Medzhitov R. Inflammation: new adventures of an old flame. Cell. 2010; 140(6): 771–6.

18. De Vincenzi M, Silano M, Maialetti F and Scazzocchio B. Constituents of aromatic plants: II. Estragole. Fitoterapia. 2000; 71(6): 725–9.

19. Scrivo R, Vasile M, Bartosiewicz I and Valesini G. Inflammation as common soil of the multifactorial diseases. Autoimmun Rev. 2011; 10(7): 369–74.