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

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ABSTRACT
Introduction: The estragole (EST) is a phenylpropanoid present 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 400 mg/kg) significantly decreased the prostaglandin E2 levels in the inflamed paw tissues four hours after stimuli. However, EST was inactive against 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 interfering 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 present 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. Then, the understanding of its biological and toxic effects has a great significance.

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

The acute inflammation is characterized by swelling, heat, redness, and pain and is an important defense mechanism against invading pathogens. 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. 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.
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\textsuperscript{®}, 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\(_{50}\))

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.

Some researchers have suggested that the anti-inflammatory activity of EST is related to its ability to reduce the biosynthesis of prostaglandins by inhibition of COX enzymes \(^2,^{6}\). 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.

![Fig. 1: Chemical structure of the phenylpropanoid estragole](image-url)
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 the 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 E₂ levels in inflamed paw of mice
The effect of EST on the PGE₂ 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 PGE₂ levels. EST at 200 and 400mg/kg significantly decreased the PGE₂ 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 lipooxygenase 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. Then, it validates the assays conducted 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 E₂ (PGE₂) levels in inflamed paw of mice. Values represent mean values ± standard error of the mean for each group (n = 6). * 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

<table>
<thead>
<tr>
<th>Compound</th>
<th>COX-1 IC₅₀ (μM)*</th>
<th>COX-2 IC₅₀ (μM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estragole</td>
<td>Inactive</td>
<td>Inactive</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>3.12</td>
<td>1.56</td>
</tr>
</tbody>
</table>

* Concentration required to produce 50% inhibition of COX-1 or COX-2. The result (IC₅₀, μM) is 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. These mediators induce vasodilatation, increasing vascular permeability and recruiting leukocytes to the injury site.

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, 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, 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. 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-inflammatory drugs (NSADs), as previously...
observed. The most important side effect, the gastric damage, caused by the NSADs is related to their ability to inhibit both isofoms 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. 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.

ACKNOWLEDGEMENTS

The study was supported by the grant BEX 11387/13-0, CAPES Foundation, Ministry of Education of Brazil, Brasilia – DF 70.040-020, Brazil.

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