Evaluation of immunomodulatory activity of \textit{trans}-anethole and estragole, and protective effect against cyclophosphamide-induced suppression of immunity in Swiss albino mice

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Summary. The positional isomers \textit{trans}-anethole and estragole are present in essential oils extracted from many aromatic plants used in traditional medicine, and have largely been used as flavoring agents in the food industry. There are many studies describing the biological activities of these essential oils; however, few studies have been performed to determine the biological effects of these substances as neat compounds. Furthermore, their immunomodulatory activity remains elusive. In this paper, we evaluated the immunogenic activity of both \textit{trans}-anethole and estragole as pure substances. In this regard, a series of experiments was conducted to determine the effect of the test compounds on cell- and humoral-mediated immune responses in mice. Our data showed that \textit{trans}-anethole and estragole promote some changes in the immune system by reducing the delayed-type hypersensitivity response, and increasing the number of leucocytes in peripheral blood in mice. Also, we observed that \textit{trans}-anethole improved the humoral response. Finally, these compounds promoted a protective effect against cyclophosphamide-induced suppression of immunity. In conclusion, we propose that the immunological effects exerted by \textit{trans}-anethole and estragole are promising and merit further investigations.

Industrial relevance. According to recent studies, essential oils containing \textit{trans}-anethole or estragole, as the major natural products, exert a wide variety of biological activities. These two compounds have a wide variety of applications in the food, pharmaceutical, and cosmetic industry and, consequently, the study of their biological activities has promising commercial use. In this study, we conducted a series of experiments with the purpose of evaluating the potential immunogenic activity exerted by \textit{trans}-anethole, and estragole, on cell- and humoral-mediated immune response, using an \textit{in vivo} murine model. The screening of the biological activity of these phenylpropanes may be useful to develop new plant-based products with immunomodulatory properties, which are unlikely to produce the undesirable side-effects of current chemothrapeutical agents.

Keywords. Immunomodulatory activity; \textit{trans}-anethole; estragole

INTRODUCTION

The phenylpropanoids \textit{trans}-anethole (ANE) and estragole (EST) are positional isomers contained in essential oils extracted from plants which are commonly used in traditional medicine; some examples of these plants are \textit{Croton zehntneri}, \textit{Illicium anisatum}, \textit{Ocimum basilicum}, \textit{Artemisia dracunculus}, and others (Mota et al., 2012; Ritter et al., 2013). ANE and EST have a wide variety of applications in the food industry, and have been used as precursors in the synthesis of certain compounds used by the pharmaceutical and cosmetic industry (Poon and Freeman, 2006; Polzin et al., 2007). Thus, the study of their biological activities and toxic effects has a great significance.

According to recent studies, essential oils containing ANE as the major natural product exert pharmacological activities including wound healing (Cavalcanti et al., 2012), antioxidant (Senatore et al., 2013), antithrombotic (Tognolini et al., 2007), gastroprotective (Coelho-de-Souza et al., 2013), and antimicrobial (Astani et al., 2011). Essential oils containing EST in relatively high concentrations also exert biological effects such as myorelaxation (Albuquerque et al., 1981), bradycardia (de Siqueira et al., 2006), anticonvulsant (Dallmeier and Carlini, 1981), and antimicrobial (Shahat et al., 2011). To the best of our knowledge, the vast majority of reports describing the effects of both ANE and EST are based on preparations (solutions) in which these compounds are part of an extract, and therefore, mixed with other compounds. In this regard, there are just a few reports describing the biological effects exerted by pure ANE and EST, as shown in Table 1.

Nowadays, there is a growing interest in identifying plant components with potential immunomodulatory effects, because the immunosuppression associated with stress, auto-immune diseases, and nutritional deficiencies, may respond favorably to treatments with natural immunomodulatory agents. Furthermore, plant-based immunomodulatory agents may be utilized as adjuvant therapy to overcome side-effects commonly observed with the use of cytotoxic chemothrapeutical agents (de Costa et al., 2011; Licciardi and Underwood, 2011; Malabadi et al., 2011; Kumar et al., 2012; Pniewski, 2012; Popov and Ovodov, 2012;...
Evaluation of immunomodulatory activity of trans-anethole and estragole

2013). In this regard, recent studies have demonstrated that natural compounds such as wilforlide A (Xue et al., 2010), ginsenoside Rg1 (Kenarova et al., 1990), and cannabidiol (Liu et al., 2010) exhibit a promising immunomodulatory profile. Moreover, the potential immunogenic effect of eugenol and methyleugenol has been reported in the literature (Carrasco et al., 2009; Choi et al., 2010; Kar Mahapatra et al., 2011; Farhath et al., 2013). These two natural compounds present similar chemical structures to those observed in ANE and EST (Figure 1). Consequently, we hypothesized that ANE and EST would be likely to exert an immunomodulatory profile, which on top of other pharmacological effects attributed to these compounds would constitute a promising therapeutic profile for ANE and EST.

Table 1. Sources of trans-anethole or estragole, their percent of purity and biological effects reported in the literature.

<table>
<thead>
<tr>
<th>Source</th>
<th>Major compound (%)</th>
<th>Biological activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Florence fennel</td>
<td>Anethole (59.8–90.4%)</td>
<td>Antioxidant</td>
<td>Senatore et al, 2013.</td>
</tr>
<tr>
<td>Croton zehntneri</td>
<td>Anethole (85.7%)</td>
<td>Gastroprotective</td>
<td>Coelho-de-Souza et al, 2013.</td>
</tr>
<tr>
<td>Croton zehntneri</td>
<td>Anethole (85.7%)</td>
<td>Wound healing activity</td>
<td>Cavalcanti et al., 2012</td>
</tr>
<tr>
<td>Illicium verum</td>
<td>Anethole (80.0%)</td>
<td>Antimicrobial</td>
<td>Astani et al, 2011.</td>
</tr>
<tr>
<td>Foeniculum vulgare</td>
<td>Anethole (75.8%)</td>
<td>Antithrombotic</td>
<td>Tognolini et al, 2007.</td>
</tr>
<tr>
<td>Synthesis</td>
<td>Estragole (99.0%)</td>
<td>Anticonvulsant</td>
<td>Dallmeier and Carlini, 1981.</td>
</tr>
<tr>
<td>Foeniculum vulgare</td>
<td>Estragole (58.0%)</td>
<td>Antimicrobial</td>
<td>Shahat et al, 2011.</td>
</tr>
<tr>
<td>Croton zehntneri</td>
<td>Estragole (45.9%)</td>
<td>Bradycardic</td>
<td>De Siqueira et al, 2006.</td>
</tr>
<tr>
<td>Croton zehntneri</td>
<td>Estragole (60.0%)</td>
<td>Myorelaxant</td>
<td>Albuquerque et al, 1981.</td>
</tr>
</tbody>
</table>

Figure 1. The chemical structures of trans-anethole (A), estragole (B), eugenol (C), and methyleugenol (D).

As part of an interdisciplinary research project aimed at studying the biological effects of natural products, in this paper we describe the immunomodulatory activity observed with ANE and EST. In this regard, we conducted a series of experiments designed to measure the potential immunogenic activity exerted by ANE and EST on cell- and humoral-mediated immune response, using an in vivo murine model.

MATERIALS AND METHODS

Materials. The compounds EST (98% purity), ANE (99% purity), and cyclophosphamide (CYC) were purchased from Sigma (St. Louis, MO, USA). Sterile sheep red blood cells (SRBC) were purchased from Newprov (Pinhais, PR, BR), and levamisole (LEV) was purchased from Janssen-Cilag (São Paulo, SP, BR).

Animals. A total of 96 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 screening assays. The experimental protocol was approved by the Ethical Committee in Animal Experimentation of the State University of Maringá (CEEA/UEM n° 017/2013).

Immunization and immunosuppression procedure. This assay was performed according to a previously described method (Hurtrel et al., 1992; Carrasco et al., 2009). Briefly, mice were randomly divided in two groups: 1) the immunocompetent (n = 48 mice); and 2) immunosuppressed groups (n = 48 mice). On the first day of experimentation, all mice were immunized by injecting 0.1 mL of a freshly prepared SRBC suspension in sterile saline (10⁸ cells/0.1 mL). In the immunosuppressed group, the immunosuppression was induced by administering CYC (50 mg/kg, i.p.) on days 4, 5, and 6 after the initial immunization, one hour after treatment with LEV, ANE, EST or saline.

Dosage and treatment of animals. For this study, we used the dosages for both ANE and EST (125, 250, and 500 mg/kg) that were previously tested in similar animal models (Ritter et al., 2013; Silva-Comar et al., 2014). These dosages were determined based on the oral dose of the test compound that causes death to half the mice (LD₅₀ for ANE = 1250 mg/kg; LD₅₀ for EST = 3050 mg/kg) (Jenner et al., 1964). Consequently, we considered 125-500 mg/kg to be a safe range of concentrations, and therefore, used to dose mice.
The treatment of animals was performed as previously reported (Carrasco et al., 2009). Both groups, immunocompetent and immunosuppressed, were divided into eight subgroups (n = 6) according to their respective treatments. Each subgroup (n = 6) was treated orally with LEV (50 mg/kg), ANE (125, 250 or 500 mg/kg) or EST (125, 250 or 500 mg/kg), once a day, for seven days after immunization. The last group was the control subgroup which received saline orally.

**Delayed-type hypersensitivity response (DTH).** The effect exerted by the compounds ANE, EST, and LEV on DTH was measured seven days after immunization as previously described (Hurtrel et al., 1992; Carrasco et al., 2009). The animals were challenged by injecting 0.05 mL of freshly SRBC suspension in sterile saline (5 x 10⁸ cells/0.05 mL) into the right hind paw. The contralateral paw received an equal volume of saline. The paw volume was measured before, 24, and 48 hours after the challenge with a digital plethysmograph (Ugo Basile®, Italy). The difference between the pre- and post-challenge in paw volume was expressed in μL, and it represents the DTH.

**Humoral antibody response (HA).** To measure antibody levels, we used the hemagglutination method reported previously (Kenarova et al., 1990) with a few minimal modifications. Briefly, seven days after the immunization, blood samples were collected from the caudal vein of each animal; serum was separated, heat inactivated at 56 ºC for 30 minutes, and diluted with phosphate buffered saline (PBS) in 96-wells plates. Equal volumes of freshly SRBC suspension (1%) in sterile saline were added and mixed gently. The plates were incubated at 37ºC for 1 hour and then observed the hemagglutination produced in each well. The agglutination titers were then recorded.

**Total white blood cells count (WBC).** As previously reported (Carrasco et al., 2009), blood samples were collected from the caudal vein of each animal to determine the total leukocytes count, before and seven days after the initial immunization. Then, samples were diluted with Turk liquid, and the number of WBCs was obtained by counting with the aid of a Neubauer chamber. Results were expressed as the number of leukocytes per mm³.

**Statistical analysis.** Data were expressed as mean values ± standard error of the mean (SEM). 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**

**The immune-stimulant activity of levamisole.** LEV, a member of thioimidazole family, was the first chemical agent shown to have an immunomodulatory effect (Renoux, 1986) and has been used as a positive control in this type of immunological assays, in mice (Hadden, 1994; Carrasco et al., 2009). Studies have demonstrated that LEV increases the blastogenic activity of lymphocytes (Babiuk and Misra, 1981), stimulates its proliferation (Mojzisova et al., 2004), and increases leucocyte count in peripheral blood (Holcombe et al., 1998; Carrasco et al., 2009). Moreover, it has been shown...
that LEV enhances the humoral response, and this effect is more pronounced in immuno-compromised animals (Pelletier et al., 1978; Carrasco et al., 2009). In our study, LEV was used as a positive control, and the results represent the positive immune response (stimulant activity) exerted by this compound.

**Effect of trans-anethole and estragole on immunocompetent mice.** As shown in Figure 2, the control subgroup reached maximum inflammation (edema) peak 24 hours after the challenge, and this effect decreased after 48 hours. Treatment with ANE and EST significantly decreased the DTH response, but only at high-doses (250 and 500 mg/kg) compared to control subgroup. In this regard, ANE was more potent than EST. We observed that only the animals treated with ANE (at high-dose) showed a significant increase in antibody production (Figure 3). As shown in Table 1, we observed that ANE and EST markedly increased the leucocyte count on peripheral blood, but EST was more potent than ANE. Furthermore, we also observed that all the effects exerted by both drugs in the immunocompetent mice were dose-dependent.

![Figure 3](image)

**Figure 3.** Effect of trans-anethole (A) and estragole (B) on humoral response in immunocompetent mice. Values represent mean values ± SEM for each subgroup. *a* Significant difference at $p < 0.05$ compared with the control subgroup.

<table>
<thead>
<tr>
<th>Table 2. Effect of trans-anethole and estragole on total white blood cell count in immunocompetent mice.</th>
</tr>
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<tbody>
<tr>
<td>Subgroup</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>CONTROL</td>
</tr>
<tr>
<td>LEV</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>EST</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>ANE</td>
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<td></td>
</tr>
</tbody>
</table>

Values represent mean values ± SEM for each subgroup. *a* Significant difference at $p < 0.05$ compared with the control subgroup.

**Effect of treatment with trans-anethole and estragole on immune response in immunosuppressed mice.** As shown in Figure 4, the control subgroup reached a maximal inflammatory (edema) response 24 hours after the challenge, which decreased after 48 hours. The administration of CYC promoted a perceptible immunosuppressive effect, which decreased the DTH. Treatment with ANE and EST exerted a noticeable reduction of DTH at high doses with a statistically significant difference compared to both, control and CYC subgroups.

The effect of ANE and EST on the HA is presented in the Figure 5; only the highest dose of ANE promoted a significant increase in antibody production, which resulted in antibody levels close to those considered normal; in other words, there was no significant difference compared with the control subgroup. Finally, Table 2 shows that ANE and EST increased the number of leucocytes on peripheral blood, relative to the effect produced in the CYC subgroup, but this effect was not enough to bounce the leucocyte count to normal levels. We observed that EST was more potent than ANE, and all effects produced by the test compounds were dose-dependent.
Figure 4. Effect of trans-anethole (A) and estragole (B) on delayed type hypersensitivity reaction, induced by sheep red blood cells in immunosuppressed mice. Values represent mean values ± SEM for each subgroup. * Significant difference at $p < 0.05$ compared with the control subgroup. ** Significant difference at $p < 0.05$ compared with the CYC subgroup.

Figure 5. Effect of trans-anethole (A) and estragole (B) on humoral response in immunosuppressed mice group. Values represent mean values ± SEM in each subgroup. * Significant difference at $p < 0.05$ compared with the control subgroup. ** Significant difference at $p < 0.05$ compared with the CYC subgroup.


**DISCUSSION**

Background information reported in the literature describe that the DTH has a direct correlation with cell-mediated immunity in vivo. The response peaks around 24 hours after the stimulus, decreases after 48-72 hours, and promotes a significant increase in the levels of several cytokines, as well as a recruitment of macrophages and lymphocytes to the site of reaction. Furthermore, there is an increase in the local vascular permeability, and an improvement in phagocytic activity (Henningsen et al., 1984; Hurtrel et al., 1992). The cell-mediated immunity response is important to develop resistance to infections, and counteract tumors; however, uncontrolled immune reactions may promote a wide variety of diseases such as allergies, autoimmune diseases, transplant rejection, and drug hypersensitivity reactions (Black, 1999; Romano et al., 2011). In these cases, immunosuppressive drugs have been used, but the toxicity associated with their use, and the increased risk of infections, as well as myelosuppression, neutropenia, and lymphopenia, represent major limitations (Bodey et al., 1975; Kang and Park, 2003). Thus, new compounds possessing immunosuppressive effects on DTH without the side effects exerted by classical immunosuppressive agents, represent an attractive alternative.

Our results suggest that ANE and EST exert immunosuppressive effects on SRBC-induced DTH, and these effects are not associated with a reduction of WBC or HA. In this regard, previous studies showed that ANE and EST suppress the release of several cytokines involved in cellular recruitment and migration to the site of injury, such as tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), and interleukin 17 (IL-17) (Chainy et al., 2000; Ponte et al., 2012; Ritter et al., 2013). It has also been observed that treatment with ANE and EST decreased the expression of certain intercellular adhesion molecules, including the intercellular adhesion molecule 1 (ICAM-1) (Hubbard and Rothlein, 2000; Sung and Kim, 2013). Furthermore, administration of ANE has been correlated with an increased level of interleukin 10 (IL-10), a cytokine known to promote differentiation and maturation of B cells after immunization, which leads to a subsequent increase in antibody production (Xu et al., 2004). Nevertheless, we did not observe a similar effect in our experiments when animals were dosed with EST.

Previously, the action of ANE on WBC was evaluated by Freire et al. (Freire et al., 2005) who observed a significant increase in WBC at a dose of 300 mg/kg; however, in our hands this effect was produced only in animals in the subgroup dosed at 500 mg/kg. It has been suggested that the eugenol, a natural compound with a chemical structure similar to both, ANE and EST, might act by activating the hematopoietic system and increasing leukocyte levels (Carrasco et al., 2009). Thus, the data obtained in our experiments suggests that ANE and EST may act similarly to eugenol. Nevertheless, the mechanism(s) of this action should be the subject of future (targeted) studies.

It has been established that CYC elicits a profound suppressive effect in all forms of cell-mediated immunity and antibody production (Balow et al., 1975), and this effect was observed in our experiments. In this regard, treatment with ANE and EST resulted in WBC count close to normal (compared to the non-immunosuppressed control subgroup). Furthermore, the highest dose of ANE (but not that of EST), resulted in HA levels close to normal levels.

Nowadays, the immunosuppression associated with stress, auto-immune diseases, and nutritional deficiencies is increasingly common. In these cases, natural compounds with an immunomodulatory profile such as that exerted by ANE and EST, could be an interesting alternative adjuvant therapy to decrease or counteract the undesired cytotoxic effects exerted by classical chemotherapeutic agents.

Lastly, it is important to note the different profile observed for ANE and EST; ANE proved to be more potent than EST with respect to the reduction of DTH, but EST was more potent than ANE in relation to the increase the WBC; and only ANE was active in the HA assay. This demonstrates the importance of structural differences in similar natural products (in this case, isomers), which results in dramatic changes on their pharmacological and toxic effects.

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**Table 3. Effect of trans-anethole and estragole on the total white blood cell count in immunosuppressed mice.**

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Dosage</th>
<th>Day 6</th>
<th>Day 7</th>
<th>Change(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONT</td>
<td>-</td>
<td>3,025 ± 103</td>
<td>8,607 ± 324</td>
<td>+5.5</td>
</tr>
<tr>
<td>CYC</td>
<td>50 mg/kg</td>
<td>7,058 ± 153</td>
<td>3,242 ± 201</td>
<td>-59.3*</td>
</tr>
<tr>
<td>CYC + LEV</td>
<td>50 mg/kg</td>
<td>6,467 ± 220</td>
<td>6,200 ± 184</td>
<td>-2.6*</td>
</tr>
<tr>
<td>CYC + EST</td>
<td>125 mg/kg</td>
<td>6,642 ± 119</td>
<td>3,922 ± 192</td>
<td>-49.0*</td>
</tr>
<tr>
<td>CYC + EST</td>
<td>250 mg/kg</td>
<td>7,108 ± 173</td>
<td>5,365 ± 172</td>
<td>-29.5 44</td>
</tr>
<tr>
<td>CYC + EST</td>
<td>500 mg/kg</td>
<td>6,533 ± 181</td>
<td>5,825 ± 207</td>
<td>-16.0 43</td>
</tr>
<tr>
<td>CYC + ANE</td>
<td>125 mg/kg</td>
<td>1,367 ± 157</td>
<td>3,032 ± 333</td>
<td>-63.8*</td>
</tr>
<tr>
<td>CYC + ANE</td>
<td>250 mg/kg</td>
<td>1,158 ± 304</td>
<td>3,420 ± 390</td>
<td>-78.2*</td>
</tr>
<tr>
<td>CYC + ANE</td>
<td>500 mg/kg</td>
<td>1,117 ± 189</td>
<td>4,240 ± 241</td>
<td>-47.8 45</td>
</tr>
</tbody>
</table>

Values represent mean values ± SEM for each subgroup. * Significant difference at p < 0.05 compared with the control subgroup. ** Significant difference at p < 0.05 levels compared with the CYC subgroup.
CONCLUSIONS

In conclusion, our data provide evidence that trans-anethole (ANE) and estragole (EST), when administered orally to mice, in a pure (neat) form, exert considerable immunomodulatory activity, by reducing the delayed-type hypersensitivity response (DTH), and increasing white blood cell (WBC) counts; however, only ANE was able to enhance humoral antibody response (HA) in our animal model. It is important to note that these effects were sufficient to increase both, the WBC count and the HA, to normal levels, in mice which were immunosuppressed by cyclophosphamide. This modulation of immunological parameters may offer promising therapeutic benefits exerted by the oral administration of these compounds. Further investigations may elucidate the mechanism of action by which ANE and EST produce the observed changes on the hematopoietic system, and offer additional information on how B cells are activated, with the subsequent increase in antibody production.

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Wiirzler et al.


