Antileishmanial activity of a mixture of *Tridax procumbens* and *Allium sativum* in mice

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Abstract — We tested a mixture of *Tridax procumbens*, known for its direct action against *Leishmania mexicana*, and *Allium sativum*, known for its immunomodulatory effect, as an alternative to treat cutaneous leishmaniasis. Acute oral toxicity was tested with the Up-and-Down Procedure (UDP) using a group of healthy mice administered with either *T. procumbens* or *A. sativum* extracts and compared with a control group. Liver injury and other parameters of toxicity were determined in mice at day 14. The in vivo assay was performed with mice infected with *L. mexicana* promastigotes and treated with either a mixture of *T. procumbens* and *A. sativum* or each extract separately. The thickness of the mice’s footpads was measured weekly. After the 12-week period of infection, blood samples were obtained by cardiac puncture to determine the total IgG, IgG1, and IgG2a immunoglobulins by a noncommercial indirect ELISA. We showed that the mixture of *T. procumbens* and *A. sativum* extracts was better at controlling *L. mexicana* infection while not being toxic when tested in the acute oral toxicity assay in mice. An increase in the ratio of IgG2a/IgG1 indicated a tendency to raise a Th1-type immune response in mice treated with the mixture. The mixture of *T. procumbens* and *A. sativum* extracts is a promising natural treatment for cutaneous leishmaniasis and its healing effects make it a good candidate for a possible new phytomedicine.

Key words: Leishmanicidal activity, *Tridax procumbens*, *Allium sativum*, Acute oral toxicity, Phytomedicine.

Résumé — Activité antileishmaniale d’un mélange de *Tridax procumbens* et *Allium sativum* chez la souris. Nous avons testé un mélange de *Tridax procumbens*, connu pour son action directe contre le parasite *Leishmania mexicana* connue, et d’*Allium sativum*, connu pour son effet immunomodulateur. L’ectoparasite cutanée. La toxicité orale aiguë a été testée avec la procédure Up and Down (UDP) sur un groupe de souris saines auxquelles ont été administrés soit des extraits de *T. procumbens*, soit des extraits de *A. sativum*, comparé à un groupe de contrôle. Les lésions hépatiques et d’autres paramètres de toxicité ont été déterminés au 14ème jour. L’essai in vivo a été réalisé avec des souris infectées avec *L. mexicana* et traitées soit avec un mélange de *T. procumbens* et *A. sativum*, soit avec les 2 extraits séparés. L’épaisseur des cousinets plantaires des souris a été mesurée une fois par semaine. Douze semaines après l’infection, un échantillon de sang a été prélevé par ponction cardiaque et les quantités totales d’immunoglobulines IgG, IgG1, et IgG2 ont été déterminées par ELISA indirect non commercial. Nous avons pu montrer que le mélange d’extraits de *T. procumbens* et *A. sativum* contrôle mieux l’infection tout en n’étant pas toxique lors de l’essai de toxicité orale aiguë chez la souris. L’augmentation du ratio IgG2a/IgG1 a permis d’indiquer une tendance à augmenter une réponse immunitaire de type Th1 dans les souris traitées avec le mélange. Le mélange des extraits de *T. procumbens* et *A. sativum* est un traitement naturel prometteur pour la leishmaniose cutanée et ses effets curatifs en font un bon candidat pour un possible nouveau phytomédocin.

Introduction

The disease caused by infection with *Leishmania* parasites has an important impact worldwide, with up to 200 million new cases per year [3]. In Mexico, the Yucatan peninsula is an endemic area for *Leishmania mexicana*, which causes localized cutaneous lesions, known popularly as “chiclero’s ulcer”, with an incidence of 5/1,000 inhabitants [4, 18], although it can also induce more severe forms of the disease such as the diffuse, mucocutaneous, or visceral forms [8]. Chemotherapy with
Plant collection and extraction

1.5 kg of Tridax procumbens L. (Asteraceae) whole plant were collected in Merida, Yucatan, Mexico, in February 2004; a voucher specimen was authenticated by F. May-Pat and deposited at the herbarium of the CICY under the code number FMay-1955. The whole plant was first dried at room temperature and then in a desiccation stove at 50 °C, powdered, and then submitted to extraction with methanol (MeOH). The MeOH was evaporated to dryness in vacuo and the yield of the resultant crude extract was 150 g (yielding 10%). 1.5 kg of Allium sativum L. (Amaryllidaceae) (garlic) bulbs were peeled and dried in a desiccation stove at 100 °C, and after 48 h they were recovered at constant weight. The dried bulbs were homogenized with a blender in 1 L of water, filtered through Whatman No. 1 filter paper, and centrifuged. The filtrate was lyophilized, dosed and resuspended for treatment.

Parasites

Promastigotes of L. mexicana (strain Hd18-(MHET/MX/97/Hd18) were cultured in Tc 199 medium (Gibco, Invitrogen), supplemented with 10% fetal calf serum (Life Technologies), penicillin (100 U/mL), and streptomycin (100 g/mL). The parasites were harvested in the stationary phase after 8–10 days of culture, centrifuged (1000 g × 5 min), washed twice with Tc 199 medium, counted, and used to inoculate mice.

Mice

Either female BALB/c or CD-1 mice (6–8 weeks old) were fed with standard chow and water ad libitum and kept under standard temperature (26 °C and 60% humidity conditions) and a natural light-darkness cycle. All in vivo experiments with mice were performed according to the established ethical standards of the CIR-UADY Bioethics in Research Committee.

Acute oral toxicity assay

Acute oral toxicity was tested using the Up-and-Down Procedure (UDP) recommended by the United States Environmental Protection Agency (EPA) and adopted by the Organization for Economic Cooperation and Development (OECD). This procedure significantly reduced the number of animals used in comparison with the traditional LD50 test [11].

Sixteen healthy, eight-week-old BALB/c mice were used to perform this test; six mice were used as a control group (saline solution) and ten were used as experimental groups (T. procumbens and A. sativum extracts). Animals were randomly selected and marked to allow individual identification. They were acclimatized and individually kept in their cages for at least five days, and fasted 24 h prior to dosing. Food and water were withheld for 3–4 h after dosing. First, two mice were dosed at 2000 mg/kg of body weight with either T. procumbens or A. sativum extracts using distilled water as a vehicle; as the mice survived, another eight were administered with the same dose of either T. procumbens or A. sativum. The control group was dosed only with the vehicle. Each mouse of the experimental groups was carefully observed in the first 3 h to look for toxicity parameters, such as tremors, convulsions, breathing changes, piloerection, loss of balance, hyperactivity,
and lethargy, then animals were housed for 14 days counted from the dosage day. The parameters observed during these days were body weight, amount of food consumed, and any behavior that could be a sign of toxicity [6]. At day 14, the mice were sedated and blood was taken by heart puncture to determine liver injury. For toxicity assays in mice models, it is a good parameter to determine the increase in glutamate transaminase activities (GOT and GPT), which is a sign of liver injury, thus we determined the aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) using the IFCC Mod. Liqui-UV Test. The mice were killed to remove the liver and spleen and evaluate their appearance and weight [22].

In vivo assay of T. procumbens and A. sativum extracts and their mixture

All mice were infected subcutaneously into the left hind footpad with 10^6 logarithmic phase promastigotes of L. mexicana to a final volume of 50 μL of physiological saline solution. The contralateral right footpad received an identical volume of saline solution without parasites as an internal control.

The in vivo assay was performed using four groups of six mice: (1) the control (saline solution), (2) treatment with T. procumbens, (3) treatment with A. sativum, and (4) treatment with a mixture of T. procumbens and A. sativum. The treatments with T. procumbens and A. sativum extracts started two weeks after infection. The first group was used as infection control receiving saline solution injections with the same schedule as the groups treated with extracts. The other three groups were treated daily, for two weeks, by intraperitoneal (i.p.) injections of 20 mg/kg of either T. procumbens methanol extract or A. sativum aqueous extract, or 40 mg/kg of a mixture of T. procumbens and A. sativum extracts (1:1).

The thickness of infected and uninfected footpads was measured weekly with a vernier caliper, for 12 weeks, and the difference of the lesion. The mice were also regularly examined to detect cutaneous ulcers and secondary lesions. After the 12-week period of the experiment, the mice were anesthetized and a blood sample was obtained by cardiac puncture, and they were euthanized immediately after.

Immunoglobulin detection

The total IgG, IgG1 and IgG2a immunoglobulins were measured by a noncommercial indirect ELISA, using as an antigen a whole parasite lysate and goat anti-mouse immunoglobulin G (IgG)-horseradish peroxidase conjugate (Sigma) at 1:4000 or goat anti-mouse IgG1- or IgG2a-horseradish peroxidase-conjugated antibodies (Southern Biotechnology Associates, Birmingham, Ala.) in a 1:4000 dilution in blocking buffer, as previously described [2, 13].

Statistical analysis

Data are presented as mean and SD, and comparison among multiple groups was performed by an ANOVA test. Comparisons of footpad swelling between T. procumbens and A. sativum groups and saline were performed with Student’s t-test.

Results

Toxicity assay

In the acute oral toxicity study of T. procumbens methanol and A. sativum aqueous extracts, none of the animals died during the assay or showed signs and symptoms of toxicity up to a dose of 2000 mg/kg. These results indicated that the extracts have a high margin of safety. The loss of body weight as a toxicity indicator was within the normal parameters of the growth curve of BALB/c mice at day 14. Mice gained the same weight and there were no significant differences (p < 0.05) in weight gain (at the 14th day minus weight at the 1st day) in the comparison between the control group (2.6 ± 1.1) (mean ± SD) and the mice treated either with T. procumbens methanol extract (5 ± 2.5) or A. sativum aqueous extract (3 ± 0.70). All groups registered an equal amount of food consumed (data not shown). The weight of livers and spleens was similar among the control and experimental groups (p > 0.05) (data not shown), and no macroscopic signs of damage were observed in these organs for either extract.

GOT and GPT levels were measured and the data showed no significant differences in transaminases (p > 0.05) between the control group and the group treated with T. procumbens or A. sativum (Table 1).

Effect of T. procumbens, Allium sativum, and mixed extracts on L. mexicana in vivo

The infection with L. mexicana induced a progressive increase in the size of the footpad of CD-1 mice. Both T. procumbens methanol extract and A. sativum aqueous extract in separate treatments showed a tendency to reduce the development of the cutaneous lesion in mice; however, four out of six mice treated with T. procumbens methanol extract presented minor lesions, while the other two did not respond to the treatment and developed severe lesions (Fig. 1 bottom). This variability can be explained by the genetic diversity of the CD-1 strain. The mixture of both extracts showed the best activity, significantly reducing the development of the lesion caused by L. mexicana (Fig. 1).

In general, lesions observed in mice treated with the mixture of T. procumbens and A. sativum did not present necrosis or cause damage to the skin compared with the control group treated with saline solution after day 12 (Fig. 2).

In order to understand the function of the mixture in humoral immune responses to control the infection, we measured the level of total IgG, IgG1, and IgG2a against L. mexicana in serum of mice treated either with T. procumbens, A. sativum, or the mixture of both compared with mice without treatment (Fig. 3). The means of antibody levels of each group were compared. Previous studies have demonstrated that the immunomodulatory effect of A. sativum extract is due
to an increase in IFN-γ and NO in infected mice [12]. In the present study it was shown that A. sativum extract is also able to promote the production of antibodies (Fig. 3A). The group treated with A. sativum showed the highest total IgG production compared with the control group; however, the highest ratio of IgG2a/IgG1 was observed in the mixture group (Fig. 3B).

Discussion

The results of the toxicity assay showed that the limit dose of 2000 mg/kg was innocuous to mice, and they were consistent with another toxicity study which demonstrated that T. procumbens methanol extract was not toxic at 5 g/kg body weight in rats, confirming a high margin of safety [23]. According to these results, T. procumbens LD50 is located over 2000 mg/kg body weight. Regarding the toxicity of A. sativum extract, previous studies have revealed its curative and protective effects in a nephrotoxicity model [27] and a protective effect in the kidney and liver of rats [17]. Accordingly, the present study shows no toxicity of A. sativum aqueous extract in livers of mice receiving an oral dose of 2000 mg/kg.

The simultaneous application of the mixed extract (T. procumbens and A. sativum extracts) avoided the development of a lesion; to our knowledge, there are no other studies reported using a mixture of two bioactive plant extracts against Leishmania spp. In a previous study, we demonstrated that A. sativum aqueous extract had a visible effect on the reduction of the lesion caused by L. mexicana [12]. This was confirmed by Wabwoba et al., who also concluded that the mechanism of action of A. sativum is apparently immunomodulatory, and that A. sativum compounds should be purified and tried as complementary medicine in the management of leishmaniasis [32]. However, we believe that these components perhaps do not need to be purified to obtain a good treatment, they just have to be standardized [14, 21].

The highest IgG2a/IgG1 ratio in mice treated with mixed extract, which controlled the progress of infection for L. mexicana, suggests that the simultaneous application of T. procumbens and A. sativum extracts redirects the immune response caused by the infection to a Th1-type immune response. This explains why the observed lesions in this group are less than those of the other experimental groups, as this type of response is considered most suitable for the control of infection [2].

There are reports on T. procumbens aqueous extract inducing an immunomodulatory response in mice [30] and immunomodulatory activity in T. procumbens methanol extract has been detected [1]. Phytochemical studies performed on T. procumbens against L. mexicana led to the isolation of an oxylipin, highly selective against promastigotes of L. mexicana [20]. This metabolite was tested in vitro against intracellular amastigotes, showing an IC50 = 0.48 μg/mL. [19]. This suggests that T. procumbens methanol extract could have a direct effect not only on the amastigotes, but also an additive effect with A. sativum in modulating the immune response, so that the simultaneous application of both extracts has a more efficient therapeutic effect. Similar results were obtained in a study which demonstrated an in vivo immunomodulatory effect with direct and indirect actions with a flower extract of Tilia sp. to improve the antitumoral activity [9].

Table 1. Transaminase levels of GOT and GPT (UI) in mice treated with T. procumbens methanol, A. sativum aqueous or saline solution.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>TGO</th>
<th>TGP</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>308.6 ± 168.9</td>
<td>58.9 ± 18.0</td>
</tr>
<tr>
<td>T. procumbens</td>
<td>346.1 ± 228.3</td>
<td>47.7 ± 15.8</td>
</tr>
<tr>
<td>A. sativum</td>
<td>289.7 ± 152.6</td>
<td>62.6 ± 13.0</td>
</tr>
</tbody>
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Each result is mean ± SD of TGO and TGP (UI) for three or six mice samples.

Figure 1. Footpad swelling in CD-1 mice after two weeks of infection with 1 × 10⁶ L. mexicana promastigotes. Top: four groups of mice were treated either with saline, T. procumbens extract, A. sativum extract or the mixture of T. procumbens and A. sativum extracts. The treatments were applied i.p. daily for two weeks. Bottom: the footpad thickness was measured and compared to the noninoculated footpad for each mouse for 12 weeks. Each point represents the average increase of the infected footpad thickness, standard error (n = 6). Comparison of four experimental groups in 12 weeks after infection, each symbol represents each mouse of the group, the horizontal line represents the mean, and * indicates significant differences with the PBS-treatment control group (unpaired t-test, p < 0.05).
Our work suggests that *T. procumbens* and *A. sativum* extracts used together control localized cutaneous leishmaniasis caused by *L. mexicana*.

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**References**


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