Antileishmanial Activity of a Linalool-Rich Essential Oil from Croton cajucara

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The in vitro leishmanicidal effects of a linalool-rich essential oil from the leaves of Croton cajucara against Leishmania amazonensis were investigated. Morphological changes in L. amazonensis promastigotes treated with 15 ng of essential oil per ml were observed by transmission electron microscopy; leishmanicidal nuclear and kinetoplast chromatin destruction, followed by cell lysis, was observed within 1 h. Pretreatment of mouse peritoneal macrophages with 15 ng of essential oil per ml reduced by 50% the interaction between these macrophages and L. amazonensis, with a concomitant increase by 220% in the level of nitric oxide production by the infected macrophages. Treatment of preinfected macrophages with 15 ng of essential oil per ml reduced by 50% the interaction between these cells and the parasites, which led to a 60% increase in the amount of nitric oxide produced by the preinfected macrophages. These results provide new perspectives on the development of drugs with activities against Leishmania, as linalool-rich essential oil is a strikingly potent leishmanicidal plant extract (50% lethal doses, 8.3 ng/ml for promastigotes and 8.7 ng/ml for amastigotes) which inhibited the growth of L. amazonensis promastigotes at very low concentrations (MIC, 85.0 pg/ml) and which presented no cytotoxic effects against mammalian cells.

Parasites of the genus Leishmania are transmitted by the bite of sand flies and infect cells of the mononuclear phagocyte lineage of their vertebrate hosts (2, 21). Depending both on the virulence factors of the parasite itself and on the immune response established by the host, a spectrum of diseases known as leishmaniasis can appear, and these can be cutaneous and/or visceral (30). Approximately 350 million people live in areas of active transmission of Leishmania, with 12 million people throughout Africa, Asia, Europe, and the Americas directly affected by leishmaniasis. More than 90% of the cutaneous cases appear in Afghanistan, Saudi Arabia, Algeria, Brazil, Iran, Iraq, Syria, and Sudan (13). Cutaneous leishmaniasis either can resolve spontaneously after a few months or, depending on the causative Leishmania species, can evolve into diffuse cutaneous, relapsing cutaneous, or mucocutaneous leishmaniasis, while untreated visceral leishmaniasis leads to death in the majority of patients (2). Leishmania amazonensis is one of the principal agents of diffuse cutaneous leishmaniasis, which is usually unresponsive to all treatments known to date (31). Also, visceralization of Leishmania strains that are classically restricted to cutaneous leishmaniasis has often been observed in patients with Leishmania-human immunodeficiency virus coinfection (3).

The control of leishmaniasis remains a problem because no vaccines exist and the available chemotherapy still relies on the potentially toxic pentavalent antimonials, which cause serious side effects and require long-term treatment (38). The rise in the rates of in vitro antimonial resistance due to intermittent drug exposure (15, 16), the isolation of antimonial-resistant Leishmania strains from patients with unresponsive cutaneous leishmaniasis (2, 9), and recently, the numerous cases of visceral leishmaniasis among patients infected with the human immunodeficiency virus (3) make the search for new agents for the treatment of leishmaniasis urgent. Extensive studies of new drugs with antileishmanial activities, including both natural products and synthetic compounds, have been undertaken worldwide (9), although problems with the side effects of the chemotherapies used at present have not yet been solved.

In recent years, there has been growing interest in alternative therapies and the use of natural products, especially those derived from plants (39). The bark of Croton cajucara is used in Brazilian folk medicine as an infusion to treat gastrointestinal disorders (22). Experimental studies with laboratory animals have identified potential applications for some purified substances from C. cajucara bark extracts, which present anti-inflammatory, antiulcerogenic, antitumorigenic, antimutagenic, antiestrogenic, hypoglycemic, and triglyceride-lowering effects (1, 6–8, 14, 20, 22–24, 33, 34, 40).

Linalool, a terpenic alcohol (Fig. 1), is the principal component of rosewood (Aniba rosaeodora var. amazonica Ducke syn Aniba duccei Kostermans) and Ho tree (Cinamomon camphora) oils (37). It is also obtained as a by-product in the industrial synthesis of vitamin E (37). The antimicrobial and anesthetic activities of linalool-containing essential oil have...
been reported (4, 17, 35). Recently, the linalool-rich essential oil from the leaves of *C. cajucara* has been purified, and its contents have been analyzed by gas chromatography-mass spectrometry (GC-MS) (32). Based upon the facts that the essential oil extracted from the bark of *C. cajucara* presents anti-inflammatory activity in rodents (22, 23) and that linalool-rich essential oil extracted from other plants presents antimicrobial properties (35), in this work we investigated the effects of essential oil extracted from the leaves of *C. cajucara* on *L. amazonensis* parasites, on the interaction of these flagellates with mouse peritoneal macrophages, and on nitric oxide production by the infected macrophages.

### MATERIALS AND METHODS

**Plant material.** Plant material from *C. cajucara* Benth was obtained from Embrapa Experimental Farm, Amazonas, Brazil. A voucher specimen was deposited at the Embrapa Occidental Amazon Herbarium (registry no. IAN 165013).

**Essential oil extraction.** Leaves of *C. cajucara* were dried at room temperature and coarsely powdered. The oil was obtained by hydrodistillation (5 h) of *C. cajucara* leaves with a modified Clevenger apparatus (18); the yield was 0.40% (on a dry weight basis).

**Linalool purification.** Linalool was purified and identified by GC and GC coupled with MS (GC-MS). GC was performed with a Hewlett-Packard (HP) 6890 gas chromatograph fitted with a BP-5 fused-silica capillary column (25 m by 0.33 mm; film thickness, 0.5 μm), with helium used as the carrier gas (1 ml/min). Retention indices were obtained by injection of a series of n-alkanes and were compared with those in the literature. The GC-MS system used was an HP 5973 MSD system coupled with an HP 6890 gas chromatograph; helium was used as the carrier gas, and the same column and conditions described above were used (32). Results were compared with the Wiley library of spectra.

**Parasite culture.** Promastigote forms of *L. amazonensis* (Raimundo strain, MHOM/BR/76/Ma-5) were maintained by weekly transfers in brain heart infusion medium (BHI) supplemented with 10% fetal bovine serum (FBS) at 26°C. The infectivities of the parasites were maintained by periodic inoculation into hamster footpads.

**MIC evaluation.** *L. amazonensis* promastigotes (10⁶ parasites/ml) were incubated at 26°C for 120 h in fresh medium (brain heart infusion medium) supplemented with 10% FBS in the absence or presence of several concentrations (1 pg/ml to 1 mg/ml) of essential oil or purified linalool (cell growth was determined daily by assessment of visible turbidity) in order to evaluate the MIC, as described previously (36). Cell morphology evaluation was performed with fresh as well as Giemsa-stained preparations, as described previously (31). The essential oil and the purified linalool were diluted in dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, Mo.) at 100 mg/ml and then in RPMI. In all tests, 1% DMSO (the same concentration present in the highest dose of the compounds) and medium alone were used as controls.

**Electron microscopy.** *L. amazonensis* promastigotes were incubated in the absence or presence of 1 ng of linalool-rich essential oil per ml for 5, 10, 15, 20, 25, or 30 min. The parasites were washed twice in Ringer’s solution (0.9% NaCl, 5.0% KCl, 5.0% CaCl₂) and fixed in a solution containing 2.5% glutaraldehyde, 4% formaldehyde, and 3.7% sucrose in 0.1 M phosphate buffer (pH 7.2) at room temperature. The parasites were then washed in 0.1 M cacodylate buffer (pH 7.2), and the parasites were gently scraped off with a rubber policeman and postfixed in a solution containing 1% OsO₄, 0.8% potassium ferricyanide, and 5 mM calcium chloride in 0.1 M cacodylate buffer (pH 7.2) for 1 h at room temperature in the dark. The parasites were then rinsed in cacodylate buffer, dehydrated in acetone, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and were examined in a transmission electron microscope (90; Carl Zeiss, Oberkochen, Germany) operated at 80 kV.

**Infection of macrophages and nitric oxide production.** Mouse peritoneal macrophages were obtained from Swiss mice (age, 6 to 8 weeks) were collected in 0.85% saline solution (0.9% NaCl, 5.0% KCl, 5.0% CaCl₂) and fixed in a solution containing 2.5% glutaraldehyde, 4% formaldehyde, and 3.7% sucrose in 0.1 M phosphate buffer (pH 7.2) at room temperature. The parasites were then washed in 0.1 M cacodylate buffer (pH 7.2), and the parasites were gently scraped off with a rubber policeman and postfixed in a solution containing 1% OsO₄, 0.8% potassium ferricyanide, and 5 mM calcium chloride in 0.1 M cacodylate buffer (pH 7.2) for 1 h at room temperature in the dark. The parasites were then rinsed in cacodylate buffer, dehydrated in acetone, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and were examined in a transmission electron microscope (90; Carl Zeiss, Oberkochen, Germany) operated at 80 kV.

**Infection of macrophages and nitric oxide production.** Mouse peritoneal macrophages were obtained as described above. The parasites and/or the macrophages were either not treated or treated with 15, 1.5, or 0.2 ng of essential oil per ml 20 min prior to the macrophage-parasite interactions. The adherent cultured macrophages and the free parasites were washed once and resuspended in fresh culture medium. Dead parasites were removed from the medium by centrifugation (1,000 × g, 5 min), and intact living *L. amazonensis* promastigotes were counted using a hemocytometer.
in the stationary growth phase were then added to the macrophage culture plate wells. The parasite-macrophage interaction studies were performed at 37°C for 90 min by using parasites and/or macrophages pretreated with the essential oil or macrophages that had already been infected with the parasites for 24 h and then treated with the essential oil. In the last system, all promastigotes had already differentiated into amastigotes before the treatment with the essential oil. A ratio of 10 promastigotes to 1 macrophage was used for both infection assays. After the interaction assays were done, the coverslips were fixed and Giemsa stained, and the percentage of infected macrophages was determined by counting 600 cells in triplicate coverslips. The association indices were determined by multiplying the percentage of infected macrophages by the mean number of parasites per infected cell. Association indices were the number of parasites that actually

FIG. 3. Effects of linalool-rich essential oil (15.0 ng/ml) extracted from *C. cajucara* on promastigote forms of *L. amazonensis* observed by transmission electron microscopy. (A) Control parasites; (B to E) parasites treated for 5 (B), 10 (C), 15 (D), and 30 (E) min, showing promastigotes with different degrees of damage. Note the disruption of flagellar membranes (arrowheads in panels B and C), the mitochondrial swelling (C and D), and the gross alterations in the organization of the nuclear and kinetoplast chromatins (C and D). After 30 min in the presence of essential oil the parasites were completely destroyed (E). N, nucleus; K, kinetoplast; F, flagellum. Bars, 1 μm.
Inhibition of parasite growth. The MICs of *C. cajucara* essential oil and purified linalool for the growth of *L. amazonensis* promastigotes were 85.0 and 22.0 pg/ml, respectively.

Antileishmanial activity. The effects of *C. cajucara* essential oil and purified linalool on the viability of *L. amazonensis* were tested. LD₅₀ for promastigotes were 8.3 ng/ml for essential oil and 4.3 ng/ml for purified linalool, and the LD₅₀ for amastigotes were 22.0 ng/ml for essential oil and 15.5 ng/ml for purified linalool. Figure 2 shows the time course of the viabilities of *L. amazonensis* promastigotes, amastigotes, and mouse peritoneal macrophages in the absence or presence of linalool-rich essential oil. Essential oil at 15.0 ng/ml was able to kill 100% of the parasites in 60 min. On the other hand, mouse macrophages were unaffected by essential oil at 15.0 ng/ml (Fig. 2).

Transmission electron microscopy. Untreated and treated (15.0 ng of essential oil per ml) promastigotes were observed by transmission electron microscopy, and photomicrographs of the promastigotes are shown in Fig. 3A to E, which show promastigotes with different degrees of damage. Disruption of flagellar membranes, mitochondrial swelling, and gross alterations in the organization of the nuclear and kinetoplast chromatin were detected. After 30 min in the presence of essential oil the parasites were completely destroyed.

Cytotoxicity for mammalian cells. The effects of the essential oil and linalool (at the MICs and LD₅₀ for *L. amazonensis*) on mouse peritoneal macrophages and a transformed cell line (Vero cells) were tested as described previously (12). No cytotoxic effects on mammalian cells were observed at the concentrations used (data not shown).
either not treated or treated with different concentrations (15.0, 1.5, and 0.2 ng/ml) of essential oil 20 min prior to the macrophage-parasite interactions. When macrophages were pretreated with essential oil, the association indices were 50% lower than those in the control system (in which control macrophages and control parasites were used), regardless of the concentration of essential oil used in those assays. When parasites were pretreated with 15, 1.5, and 0.2 ng of essential oil per ml, the association indices were 50, 30, and 25% lower, respectively, than those in the control system; these results were approximately the same as those obtained for pretreated macrophages and parasites. When the macrophages were preinfected with *L. amazonensis* for 24 h and then treated with 15 ng of essential oil per ml, the association index was 50% lower than that for the control system.

**Nitric oxide production.** Mouse peritoneal macrophages were either noninfected or infected with *L. amazonensis*, and then the culture supernatants were evaluated for nitrite contents. The parasites and/or the macrophages were either not treated or treated with 15, 1.5, and 0.2 ng of essential oil per ml 20 min prior to the macrophage-parasite interactions. Figure 5 shows that noninfected macrophages that were treated with essential oil produced 40 to 50% more nitric oxide than the control (noninfected and nontreated) macrophages. When infected macrophages were preinfected with *L. amazonensis* for 24 h and then treated with 15 ng of essential oil per ml, the level of nitric oxide production was 170% higher than that for the control infected macrophages, although no significant difference was obtained when the macrophages were pretreated with 15, 1.5, and 0.2 ng of essential oil per ml. When both macrophages and parasites were pretreated with 15 ng of essential oil per ml, the level of nitric oxide production was 150% higher than that for the control infected macrophages, although no significant difference was obtained when the parasites were pretreated with 1.5 and 0.2 ng of essential oil per ml. When the macrophages were preinfected with *L. amazonensis* for 24 h and then treated with 15 ng of essential oil per ml, the level of nitric oxide production was 60% higher than that for the control infected macrophages.

**DISCUSSION**

The ability to survive and multiply within macrophages is a feature of several infectious agents including *Trypanosoma cruzi* and *Leishmania*. In order to sustain a chronic infection, parasites must subvert macrophage-accessory cell activities and ablate the development of protective immunity (2). Nevertheless, the most important mechanism for the killing of *Leishmania* and the control of leishmaniasis is the production of nitric oxide by macrophages of draining lymph nodes (11). New drug therapy regimens have taken advantage of the knowledge obtained from studies on *Leishmania*-macrophage interactions (2). Concerning mouse peritoneal macrophage infection with *L. amazonensis*, when the macrophages were pre-
treated with *C. cajucara* essential oil, as well as when the macrophages were preinfected with the parasites and then treated with the essential oil, the association indices were 50% lower than those for the control system (in which control macrophages and control parasites were used), regardless of the concentration of essential oil used in those assays (Fig. 4). Interestingly, the finding that macrophages pretreated with *C. cajucara* essential oil produced twice the amount of nitric oxide as the nontreated macrophages is hardly surprising (Fig. 5). Recently, several studies have demonstrated that sand fly saliva suppresses macrophage leishmanicidal activity, inhibiting nitric oxide production (10, 22). This activity has been attributed to the sand fly peptide maxadilan, which diminishes the ability of macrophages to produce nitric oxide and kill *Leishmania* in vitro (11). Cooperation between *Leishmania* species and their vectors is presumably a result of coevolution of the vector and the parasite. The epidemiological consequences of these restrictions are that the spread of leishmaniasis is restricted by the distribution of suitable vectors (27, 28).

Linalool-rich essential oils extracted from other plants have antimicrobial properties (35), so we decided to test the effects of *C. cajucara* extracts on the growth and viability of *L. amazonensis*. The LD₅₀ of the essential oil and purified linalool from *C. cajucara* for both *L. amazonensis* promastigotes and amastigotes were very low. It is remarkable that 15.0 ng of essential oil per ml was able to kill 100% of both promastigotes and amastigotes in 60 min (Fig. 2). On the other hand, mouse macrophages were unaffected by 15.0 ng of essential oil per ml was able to kill 100% of both promastigotes and amastigotes in 60 min (Fig. 2). Also, the MICs of essential oil purified from *Helichrysum italicum* for fungal growth (4) are much higher than the MIC of essential oil from *C. cajucara* for *L. amazonensis* presented here (85.0 pg/ml).

Mitochondrial swelling and important alterations in the organization of the nuclear and kinetoplast chromatins were observed by electron microscopy when *L. amazonensis* parasites were treated for 20 to 30 min with 15.0 ng of essential oil from *C. cajucara* per ml (Fig. 3). Although linalool was extremely potent when used directly on *L. amazonensis* parasites, it had little effect when used in the assays for measurement of the association between macrophages and parasites, as well as assays for measurement of the levels of nitric oxide production by the infected macrophages (data not shown). These data could be explained by synergistic effects of the different compounds of the plant extracts used in this work, which is a phenomenon widely known for several other systems (41). On the other hand, the present work shows that the decrease in the association between macrophages and parasites, concomitant with the increase in the level of nitric oxide production by the infected macrophages when both cell types were pretreated with essential oil, was less than that when macrophages alone were pretreated. In this case one could infer that linalool and another substance of the essential oil could present distinct and yet opposite effects on the macrophages.

The results presented in this paper further support the antimicrobial activity of linalool-rich essential oil. The extreme toxicity of *C. cajucara* leaf extracts for *L. amazonensis*, with no effect upon mammalian cells, enables linalool-rich essential oil to be a source of a new lead compound for novel antileishmanial drugs.

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