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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; leishmanial 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 duckei 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

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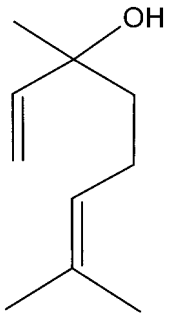

FIG. 1. Chemical structure of linalool.

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 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 (10^6 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). The MIC was considered the lowest concentration of each substance used that prevented the growth of *L. amazonensis* in vitro.

Mouse peritoneal macrophages. Thioglycolate-elicited peritoneal macrophages from female Swiss mice (age, 6 to 8 weeks) were collected in 0.85% saline and allowed to adhere onto coverslips placed in 24-well culture plates for 30 min at 37°C in a 4% CO_2 atmosphere. Nonadherent cells were then removed, and the adhered macrophages were washed twice with 0.9% saline and cultured for 24 h in culture medium (RPMI; Gibco-BRL, Gaithersburg, Md.) supplemented with 10% FBS.

Purification of amastigotes. Intact living *L. amazonensis* promastigotes in the stationary growth phase were added to plate wells containing cultured peritoneal macrophages. Free promastigotes were removed after 2 h of incubation, and the infected macrophages were kept for 48 h under the conditions described above. Free amastigotes were then collected from the supernatants of the culture plate wells. The amastigotes were washed twice in 0.1 M phosphate buffer (pH 7.2) and then resuspended in RPMI culture medium for the antileishmanial activity assays.

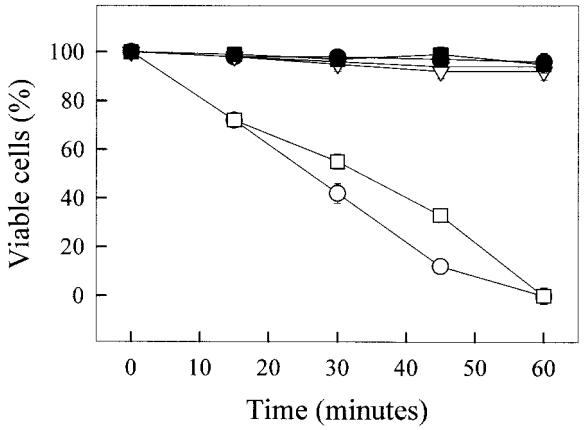

FIG. 2. Time courses of the viabilities of *L. amazonensis* promastigotes and mouse peritoneal macrophages in the absence or presence of linalool-rich essential oil (15.0 ng/ml) extracted from *C. cajucara*. The viabilities of both parasites and macrophages were calculated as described in the text. ●, *L. amazonensis* promastigotes (control); ○, *L. amazonensis* promastigotes and essential oil; ■, *L. amazonensis* amastigotes (control); □, *L. amazonensis* amastigotes and essential oil; ▼, mouse peritoneal macrophages (control); ▽, mouse peritoneal macrophages and esssential oil

Antileishmanial activity. *L. amazonensis* promastigotes or amastigotes (10^6 parasites/ml) were incubated in RPMI culture medium in the absence or presence of several concentrations (1 pg/ml to 1 mg/ml) of essential oil or purified linalool at 37°C in order to evaluate parasite survival and cell morphology by optical microscopy at 10-min intervals. Parasite viability was assessed before and after the incubations by evaluation of the motility and by the trypan blue method (5) with a hemocytometer. Cell viability was determined by using the following formula: $[100 - (L2/L1)] \times 100$, where L1 is the percentage of viable control cells and L2 is the percentage of viable treated cells, as described previously (11). The 50% lethal dose (LD_{50}) was determined by logarithmic regression analysis of the data obtained by the formula described above, 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% $CaCl_2$) and fixed in a solution containing 2.5% glutaraldehyde, 4% formaldehyde, and 3.7% sucrose in 0.1 M phosphate buffer (pH 7.2) for 1 h 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% OsO_4, 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 (900; 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

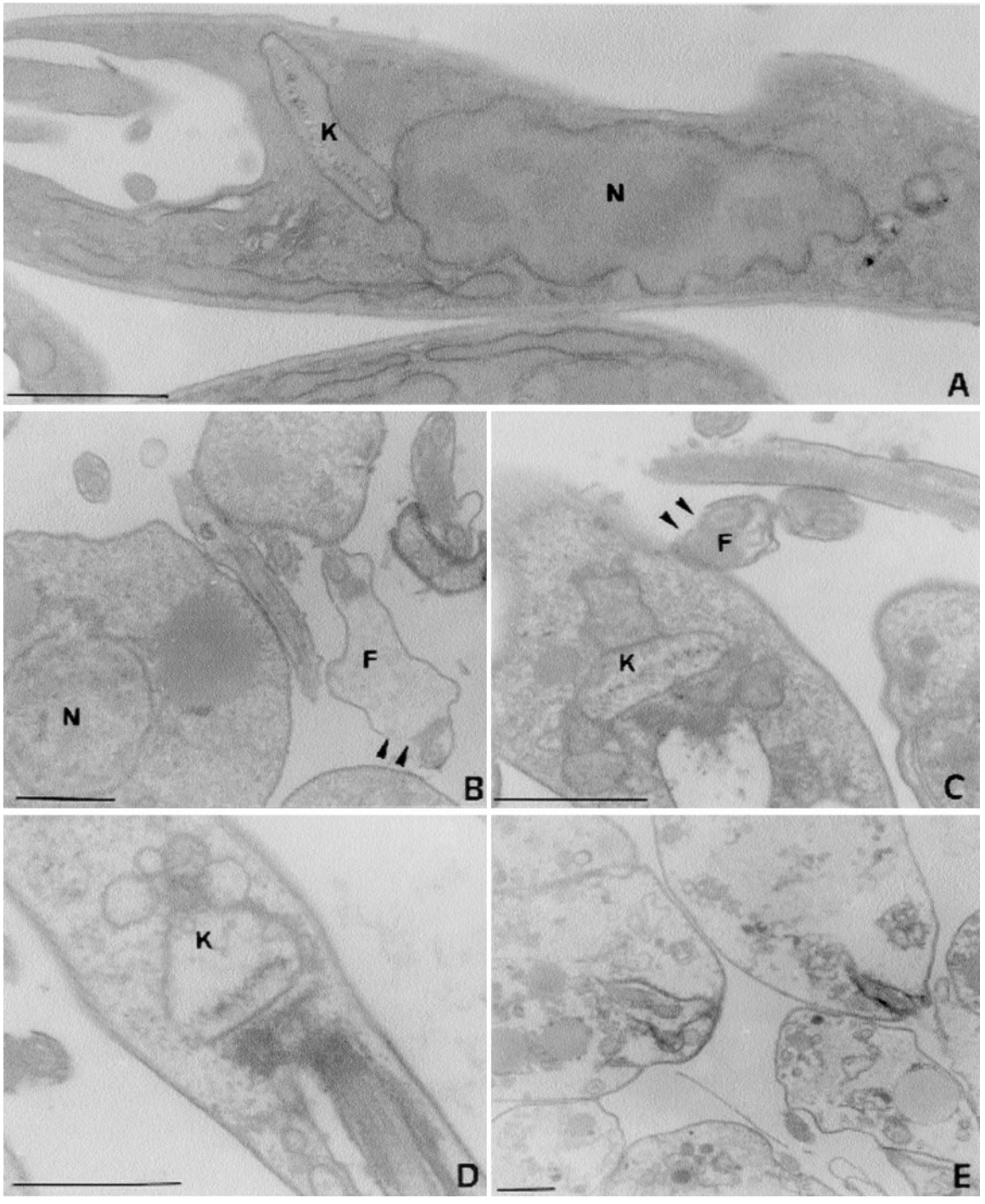

FIG. 3. Effects of linalool-rich essential oil (15.0 ng/ml) extracted from *C. cajucara* on promastigote forms of *L. amazonensis* observed by transmission electron microscopy. (A) Control parasites; (B to E) parasites treated for 5 (B), 10 (C), 15 (D), and 30 (E) min, showing promastigotes with different 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.

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

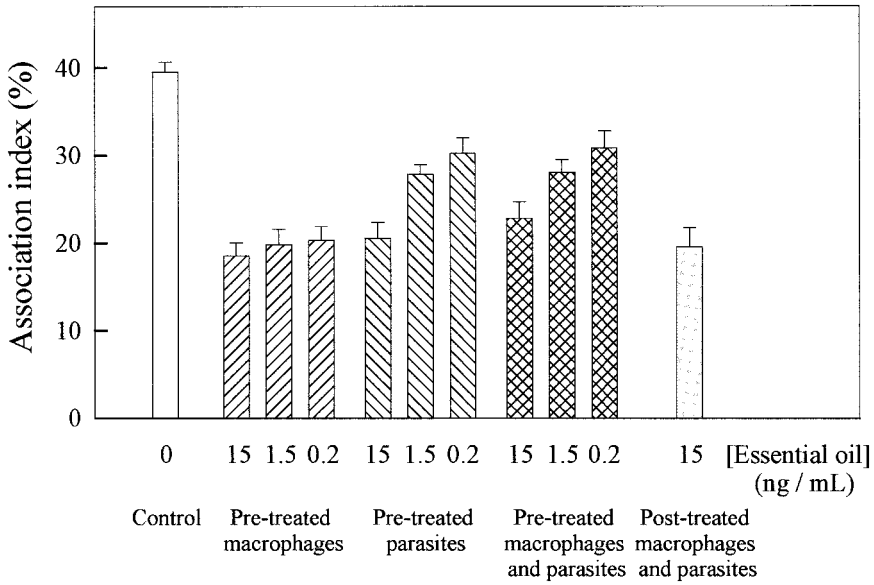

FIG. 4. Effects of linalool-rich essential oil extracted from *C. cajucara* on the *L. amazonensis*-macrophage interaction. The parasites and/or the mouse peritoneal macrophages were either not treated or treated with 15, 1.5, and 0.2 ng of essential oil per ml 20 min prior to the macrophage-parasite interactions. 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 then added to the macrophage culture plate wells. Association indices were determined by light microscopy, with 600 cells in triplicate coverslips counted after 90 min of interaction. Each bar represents the mean ± standard error of at least three independent experiments, which were performed in triplicate. The association indices from assays performed with macrophages and/or parasites pretreated with essential oil are significantly different from the association indices for control (nontreated) macrophages.

infected the macrophages. The supernatants from control and *L. amazonensis*-infected macrophages were analyzed for their nitrite contents by the Griess reaction, as described previously (19). The absorbance at 550 nm was measured, and the concentration of nitrite was calculated by using a linear regression of a standard curve, as described previously (25). The mice were first killed to obtain mouse peritoneal macrophages for both infection with *Leishmania* and nitric oxide measurements, and all federal guidelines and institutional policies for the treatment of mice were adhered to.

Cytotoxicity assay. The cytotoxicity of the drug for mammalian cells was measured by the neutral red uptake assay (26) with mouse peritoneal macrophages and a transformed cell line (Vero cells) (29). The cells were cultivated in 96-well microtiter plates (150 μl containing 10^5 cells/ml in Eagle minimal essential medium [Eagle MEM]/well) at 37°C in a humidified 5% CO_2 atmosphere. The medium was completed by the addition of L-glutamine (0.10 g/liter), HEPES (2.38 g/liter), penicillin G (10^5 IU/liter), streptomycin sulfate (0.10 g/liter), and 4% FBS. After a 24-h incubation, 50 μl of ethanolic or aqueous crude extracts, essential oil, or linalool was added to the cell cultures at the respective MICs and LD_{50}s for *L. amazonensis*. A total of 50 μl of Eagle MEM was added to the control cells. After further incubation for 48 h, control and treated cells were washed three times with phosphate-buffered saline (PBS) solution (pH 7.2). A total of 100 μl of neutral red solution (0.3% in Eagle MEM) was added to each well. After a 3-h incubation at 37°C in this solution, the cells were then washed three times with PBS. A total of 100 μl of a solution containing acetic acid (1%; vol/vol) and ethanol (50%; vol/vol) was added to the wells, and the optical densities of the supernatants were measured at 540 nm.

Statistical analysis. All experiments were performed in triplicate. The mean and standard deviation of at least three experiments were determined. Statistical analysis of the differences between mean values obtained for the experimental groups was done by Student's *t* test. *P* values of 0.05 or less were considered significant.

RESULTS

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. LD_{50}s for promastigotes were 8.3 ng/ml for essential oil and 4.3 ng/ml for purified linalool, and the LD_{50}s 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 chromatins 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 LD_{50}s 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).

Infection of macrophages. Figure 4 shows the effects of the essential oil on the *L. amazonensis*-macrophage interaction. The parasites and/or the mouse peritoneal macrophages were

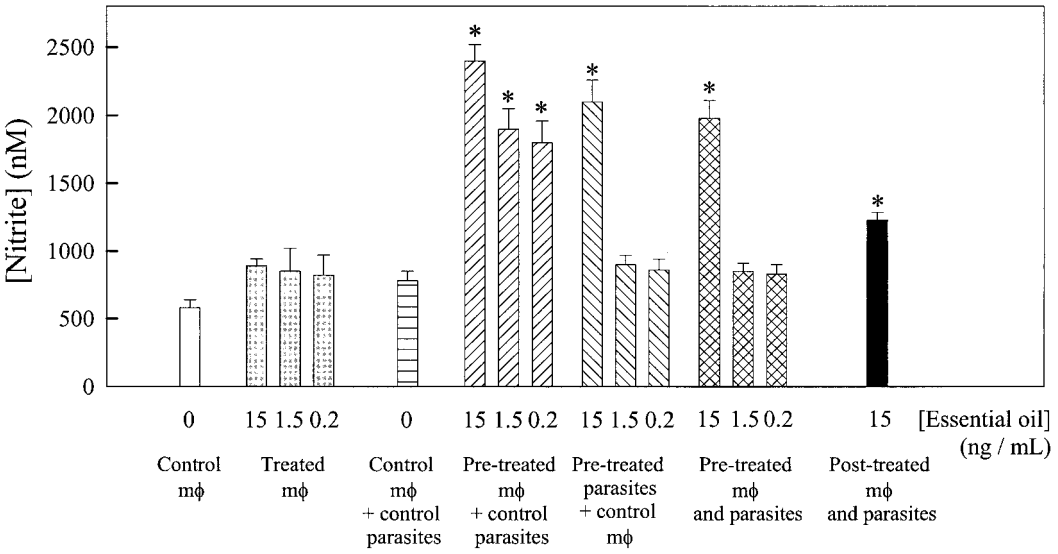

FIG. 5. Effects of linalool-rich essential oil extracted from *C. cajucara* on nitric oxide production by mouse peritoneal macrophages (mϕ). The parasites and/or the macrophages were either not treated or treated with 15, 1.5, and 0.2 ng of essential oil per ml 20 min prior to the macrophage-parasite interactions. 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 then added to the macrophage culture plate wells. The supernatants from control and *L. amazonensis*-infected macrophages were recovered, and the nitrite concentration of each system was determined by the Griess reaction, as described in the Materials and Methods section. Each bar represents the mean ± standard error of at least three independent experiments, which were performed in triplicate. The asterisks indicate that the result is significantly different from that for the infected macrophages when neither the macrophages nor the parasites were treated with essential oil.

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. Infected macrophages produced 30% more nitric oxide than noninfected macrophages. When infected macrophages were pretreated with 15, 1.5, and 0.2 ng of essential oil per ml, the levels of nitric oxide production were 220, 150, and 130% higher, respectively, than those for the control infected macrophages. When parasites were pretreated 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 parasites were pretreated with 1.5 or 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). Therefore, 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). Interestingly, recent 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 this restriction are that the spread of leishmaniases 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 LD_{50}s 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 (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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