Immunomodulatory Effects of *Lippia sidoides* Extract: Induction of IL-10 Through cAMP and p38 MAPK-Dependent Mechanisms

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ABSTRACT *Lippia sidoides* is an aromatic shrub that grows wild in the northeastern region of Brazil. In local traditional medicine, the aerial portions of this species are used as anti-infectives, antiseptics, spasmolytics, sedatives, hypotensives, and anti-inflammatory agents. In this research, we evaluate the potential immunological properties of *Lippia* extract through *in vitro* analysis of its ability to modulate intracellular cyclic adenosine monophosphate (cAMP) levels and interleukin-10 (IL-10) production. These results show that *Lippia* extract increases intracellular cAMP through the inhibition of phosphodiesterase activity. They also demonstrate that *Lippia* extract increases IL-10 production in THP-1 monocytes through both an increase in intracellular cAMP and the activation of p38 MAPK. These results suggest that the *Lippia*-mediated inhibition of phosphodiesterase activity and the subsequent increase in intracellular cAMP may explain some of the biological activities associated with *L. sidoides*. In addition, the anti-inflammatory activity of *L. sidoides* may also be due, in part, to its ability to induce IL-10 production through the inhibition of cyclic nucleotide-dependent phosphodiesterase activity and by its activation of the p38 MAPK pathway.

KEY WORDS: • *anti-inflammatory* • *cytokines* • *medicinal plant*

INTRODUCTION

*L*IPPIA SIDOIDES, POPULARLY KNOWN AS alecrim pimento, is an aromatic shrub that grows wild in the northeastern region of Brazil.[1] In traditional medicine, the aerial portions of this species are used as anti-infectives, antiseptics, spasmolytics, sedatives, hypotensives, and anti-inflammatory agents.[2,3] Essential oils extracted from its leaves have been shown to have antimicrobial properties for oral care, reducing gingivitis, and minimizing bone loss and inflammation associated with periodontal disease.[1,4–7] Its action as an antileishmanial shows that *L. sidoides* may also be an effective alternative treatment for protozoal infections,[8,9] and the essential oil and hydrolysates have been shown to be effective pesticides against mollusks,[10] mosquito larvae,[11] and spider mites.[12]

Elevation of intracellular cyclic adenosine monophosphate (cAMP) activates protein kinase A (PKA)-mediated phosphorylation and activation of cAMP response element-binding protein (CREB).[13] Once phosphorylated, CREB is involved in a variety of cellular processes, including prolif-

eration, survival, differentiation, and glucose homeostasis.[14–16] CREB also plays an important role in immune responses, stimulating interleukin-10 (IL-10) production to mediate a feedback loop that limits inflammation, and thereby provides an anti-inflammatory mechanism of action.[17]

During routine screening of botanical extracts, we found that *L. sidoides* (*Lippia*) extract repeatedly activated a cAMP response element (CRE)-linked luciferase reporter. These results suggest that some of *Lippia*'s medicinal activities may be due, in part, to elevation of intracellular cAMP. In the present study, we sought to determine if the CRE activation is through stimulation of adenylyl cyclase or inhibition of phosphodiesterase. In addition, due to the proposed role of increased cAMP and CRE activation in the stimulation of IL-10,[17,18] we investigated the potential anti-inflammatory action of *L. sidoides* by analyzing the effect of *Lippia* extract on IL-10 production in human monocytic cells.

MATERIALS AND METHODS

Plant material and preparation of the extract

Aerial portions of *L. sidoides* were collected from a crop planted at the Nutrilite farm in Ubajara, Brazil, in the summer of 2008. The identity of the crop (Voucher specimen EAC 54235) was verified by Dr. Ines Cordeiro,

Nucleus Research Curator of the Herbarium of São Paulo, Institute of Botany, Sao Paulo, Brazil. Aerial portions were air-dried, finely ground with mortar and pestle, and extracted with 85% ethanol by vortexing for 5 min. Typically, 50 mg of plant material is extracted with 1 mL solvent. The extract was centrifuged (250 g) to remove debris and the supernatant transferred to a clean container and evaporated at room temperature using a vacuum concentrator. The dried sample was weighed and reconstituted to 100 mg/mL in dimethyl sulfoxide.

Cell cultures and generation of stable cell line

Human embryonic kidney HEK293T cells (CRL-3216) and human acute monocytic leukemia THP-1 cells (TIB-202) were purchased from ATCC (Manassas, VA, USA). HEK293T cells were cultured in Dulbecco's modified Eagle's medium and THP-1 cells in RPMI 1640. All cell culture media were supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B. Cell cultures were grown in a humidified 5% CO_2 atmosphere at 37°C. Tissue culture reagents were from Mediatech (Manassas, VA, USA), except FBS, which was from HyClone (Logan, UT, USA).

A stable cell line was developed by transfecting a firefly luciferase reporter vector, pGL4.29 [luc2P/CRE/Hygro], from Promega (Madison, WI, USA) into HEK293T cells using Fugene 6 from Roche Applied Science (Indianapolis, IN, USA). The resulting clones were selected and maintained in 200 μg/mL hygromycin supplemented growth media. The vector construct consists of three tandem CRE sequences upstream of the promoter sequence to initiate transduction of the luciferase reporter gene.

CRE-luciferase reporter assay

Stably transfected HEK293T cells expressing the CRE-luciferase reporter were plated (6×10^4 cells/well) in white-walled, clear-bottom 96-well plates and incubated overnight. Cells were treated with select samples/compounds at specified concentrations for 4 h in a humidified 5% CO_2 incubator at 37°C. The assay was terminated with a lysis buffer (20 μL/well), D-luciferin (100 μL/well; Biotium, Hayward, CA, USA) was added, and the resulting luminescence was quantified using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Data analysis was performed using GraphPad Prism Software (La Jolla, CA, USA).

PDE-Glo™ phosphodiesterase assay

Phosphodiesterase (PDE) activity was quantitated using the PDE-Glo™ PDE assay kit as outlined by the manufacturer (Promega). The PDE activity was measured in 96-well plates using bovine brain PDE (1 mU/well; Sigma-Aldrich, St. Louis, MO, USA), 1 μM cAMP or 10 μM cyclic guanosine monophosphate (cGMP) per reaction, and the indicated concentrations of test compounds. Sample luminescence was quantified using a SpectraMax M5 microplate reader and data analysis performed using GraphPad Prism Software.

Enzyme-linked immunosorbent assay

THP-1 cells were differentiated with calcitrol (5 μM; Cayman Chemical, Ann Arbor, MI, USA) for 3 days. The differentiated cells were washed and treated overnight with *Lippia* extract and other compounds (forskolin [Fsk], polymyxin B [PMB], isoproterenol, 8-bromoadenosine-3′,5′-cyclic monophosphate [8-Br-cAMP], SB203580 [Sigma-Aldrich], and *Escherichia coli* K12 lipopolysaccharide [LPS; Invivogen, San Diego, CA, USA]). The cultures were incubated for 18 h, at which time the culture supernatants were collected. IL-10 levels in the culture supernatants of THP-1 cells were quantitated by an enzyme-linked immunosorbent assay according to the manufacturer's instructions (MabTech, Cincinnati, OH, USA). All test compounds were titrated before use in IL-10 induction assays to determine concentrations that were nontoxic using the water-soluble tetrazolium viability indicator WST-1 (Roche Applied Science).

Western blot

THP-1 cells were differentiated with calcitrol as stated above. The differentiated cells were washed and incubated in RPMI-1% FBS for 3 h, at which time *Lippia* extract and Fsk were added at 200 μg/mL and 10 μM, respectively. Pam$_3$Csk$_4$ (Invivogen) was used a positive control (1 μg/mL). Following treatment, cell lysates were made by incubating the cells on ice for 30 min in a 1 × cell lysis buffer with an added phosphatase inhibitor cocktail containing sodium fluoride, sodium pyrophosphate, β-glycerophosphate, and sodium orthovanadate (Cell Signaling Technology, Beverly, MA, USA). The lysates were centrifuged for 3 min at 4000 g to remove cellular debris. Protein content of the lysates was determined using the BCA protein assay (Pierce, Rockford, IL, USA). Samples (50 μg protein) were electrophoresed on 4–15% Miniprotean TGX polyacrylamide gels (Bio-Rad, Hercules, CA, USA) and were transferred to an Immobilon-P PVDF membrane (EMD Millipore, Billerica, MA, USA). The blot was blocked with 5% nonfat dry milk in Tris-buffered saline–Tween 20 (TBST; Cell Signaling Technology). Rabbit anti-p38 and anti-P-p38 antibodies (Cell Signaling Technology) were incubated for 18 h each at 4°C and p38 was probed as a loading control. A donkey anti-rabbit IgG-horseradish peroxidase (HRP) conjugate was used as the secondary antibody (Abcam, Cambridge, MA, USA). Between antibodies, blots were stripped with the Restore Plus Western Blot stripping buffer (Pierce). The blots were developed with ImmunStar HRP substrate (Bio-Rad) on a BioRad ChemiDoc XRS Imaging system.

Statistical analysis

The results of the experiments described in this study are expressed as mean ± standard deviation values and are representative of three independent experiments. Statistical analysis was carried out by Student's *t*-test using PRISM version 6.01 statistical analysis software (GraphPad Software, Inc., San Diego, CA, USA). Levels of statistical

significance between data sets were significant if the P-value $< .05$ (*), highly significant if $P < .01$ (**), and a very high level of significance if $P < .001$ (***).

RESULTS

Effect of Lippia *extract, IBMX, and forskolin on CRE-luciferase reporter assay*

During a routine screen of plant extracts using a CRE-luciferase reporter assay, *Lippia* extract was found to repeatedly activate the luciferase reporter. The response was shown to be concentration dependent with a maximum activity of approximately fourfold over control and an EC_{50} of 20 μg/mL (Fig. 1). As activation of CREB is dependent on an increase in intracellular levels of cAMP, we wanted to test if the CREB-activating effect of *Lippia* extract was due to stimulation of adenyl cyclase or inhibition of phosphodiesterase. Therefore, we compared the *Lippia* extract-induced reporter activity to reporter responses elicited by IBMX and Fsk. IBMX, a nonspecific competitive cAMP- and cGMP-dependent phosphodiesterase inhibitor,[19] increases intracellular cAMP by inhibiting its degradation by phosphodiesterases. Conversely Fsk, a botanical diterpene from *Coleus forskohlii*, increases intracellular cAMP through direct activation of adenylyl cyclase.[20] The maximum *Lippia* extract-induced reporter response was similar in potency to reporter responses to 100 μM IBMX and 100 nM of Fsk (Fig. 2).

Effect of IMBX alone and in combination with Fsk or Lippia *extract on CRE-luciferase reporter assay*

When combined, Fsk and IBMX increase intracellular cAMP levels by simultaneously increasing its production and
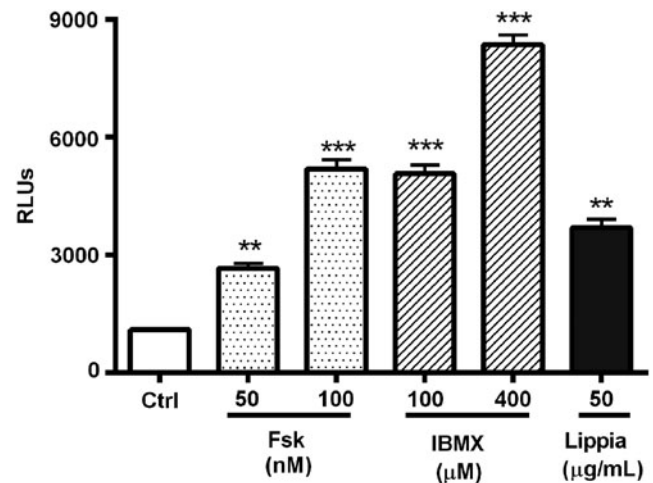

FIG. 2. Effects of forskolin (Fsk), IBMX, and *Lippia* extract on CRE reporter activity. HEK293T cells expressing a CRE-responsive luciferase reporter were treated with various concentrations of forskolin (50–100 nM), IBMX (100–400 nM), or *Lippia* extract (50 μg/mL) for 4 h. Reporter responses are plotted as RLUs. Results are representative of three separate experiments and data reported as mean ± 1 SD. ** Represents $P < .01$ and *** represents $P < .001$.

inhibiting its destruction. The maximal response of the CRE reporter cells to the combination of IBMX and *Lippia* extract was similar to the maximal response elicited by IBMX alone (Fig. 3). In addition, the maximal response elicited by the combination of *Lippia* extract with Fsk was similar to the maximal response elicited by IBMX with Fsk. The two sets of curves in Figure 3, therefore, suggest that *Lippia* extract acts through the same mechanism as IBMX and increases cAMP levels through the inhibition of PDE activity.
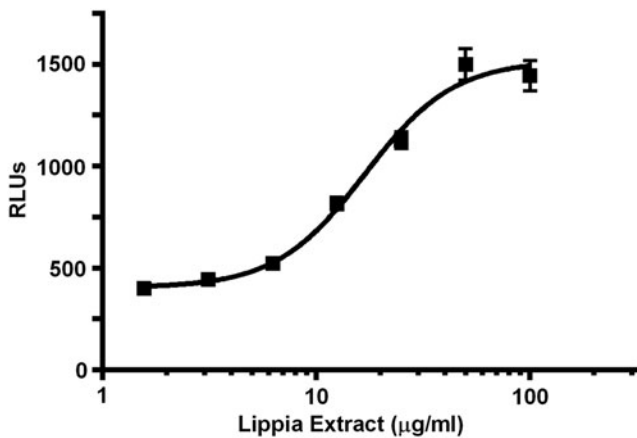

FIG. 1. Effects of *Lippia* extract on activation of cAMP response element (CRE)-responsive luciferase reporter. HEK293T cells expressing a CRE-responsive luciferase reporter were treated with increasing concentrations (1.6–100 μg/mL) of *Lippia* extract for 4 h. Results are plotted as relative luminescence units (RLUs) against increasing concentrations of *Lippia* extract ($EC_{50} = 20$ μg/mL). Results are representative of three separate experiments and data reported as mean ± one standard deviation (SD).
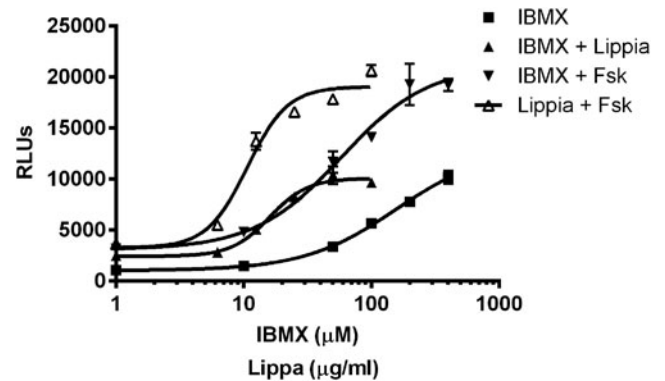

FIG. 3. Effects of IBMX in combination with forskolin and *Lippia* extract on CRE-luciferase reporter activity. HEK293T cells expressing a CRE-responsive luciferase reporter were treated with IBMX alone (25–400 μM) and in the presence of *Lippia* extract (100 μg/mL); *Lippia* extract (6.25–100 μg/mL) with Fsk (100 nM), and IBMX (25–400 μM) with Fsk (100 nM) for 4 h. The x axis depicts concentrations of IBMX in μM or *Lippia* extract in μg/mL. Reporter responses are plotted as RLUs. Results are representative of three separate experiments and data reported as mean ± 1 SD.

Effect of Lippia *extract on phosphodiesterase activity*

To confirm that *Lippia* extract inhibits PDE, we tested its effect in the PDE-Glo assay. We demonstrate that *Lippia* extract inhibits cAMP- and cGMP-dependent phosphodiesterases in a concentration-dependent manner with IC_{50} of 3 and 25 μg/mL, respectively (Fig. 4). *Lippia* extract did not inhibit the activity of the assay controls, luciferase, and PKA (data not shown).

Effect of Lippia *extract on IL-10 production in THP-1 cells*

An increase in intracellular cAMP and subsequent activation of CREB has been shown to play a role in IL-10 expression.[17,18] Since *Lippia* extract increases intracellular cAMP, it may also induce IL-10 production in immune cells. To test this hypothesis, we investigated the effect of *Lippia* extract on IL-10 production in calcitrol-differentiated THP-1 cells. Calcitrol-treated THP-1 cells differentiate along a monocytic maturation pathway such that they express CD11b and CD14.[21] Treatment of differentiated THP-1 cells with *Lippia* extract (4 and 20 μg/mL) slightly increased IL-10 levels compared with untreated controls (Fig. 5). *Lippia* extract was also tested at 100 μg/mL, but this concentration was toxic (data not shown). Isoproterenol, a β-adrenergic receptor agonist shown to induce IL-10 production in a murine macrophage cell line,[22] was used as a positive control. Undifferentiated THP-1 cells did not produce IL-10 when treated with *Lippia* extract or isoproterenol (data not shown).

Effect of Lippia *extract on IL-10 production in Fsk and 8-Br-cAMP-treated THP-1 cells*

To further investigate the potential role of cAMP in the production of IL-10, we treated differentiated THP-1 cells
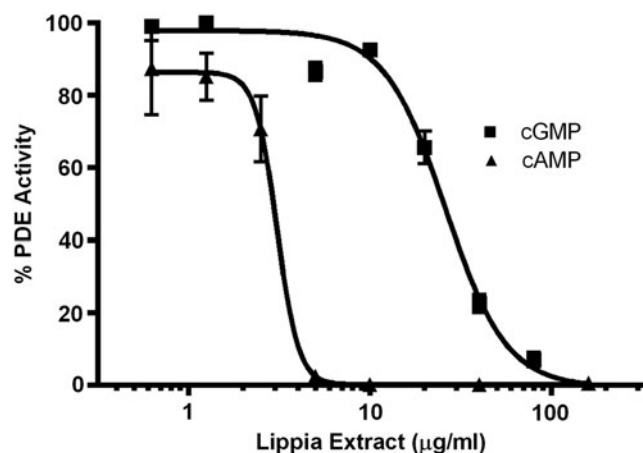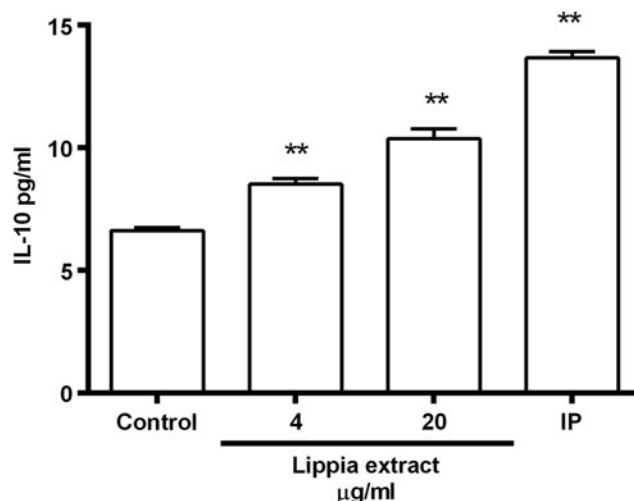

FIG. 5. Effect of *Lippia* extract on interleukin-10 (IL-10) production in differentiated THP-1 cells. Calcitrol-differentiated THP-1 cells in triplicate were treated with *Lippia* extract (4, 20 μg/mL) for 18 h, after which the cell culture supernatants assayed for IL-10 by enzyme-linked immunosorbent assay (ELISA). Isoproterenol (IP, 100 nM) was used as a positive control. Results are representative of three separate experiments and data reported as mean $\pm$ 1 SD (**$P < .01$).

FIG. 4. Effect of *Lippia* extract on cyclic adenosine monophosphate (cAMP)- and cyclic guanosine monophosphate (cGMP)-dependent phosphodiesterases (PDEs). PDE-Glo PDE assay was used to assess the inhibition of *Lippia* extract (1.6–160 μg/mL) on bovine brain PDE activity. IC_{50} values were 3 and 25 μg/mL for cAMP- and cGMP-dependent PDEs, respectively. Results are representative of three separate experiments and data reported as mean $\pm$ 1 SD.

with the adenylyl cyclase activator, Fsk, and the cell permeable cAMP analog, 8-Br-cAMP, alone and in the presence of *Lippia* extract. Our hypothesis was that by elevating cAMP levels while preventing its degradation through the PDE inhibitory activity of *Lippia* extract, we might maximize intracellular cAMP levels to a level that is able to maximize IL-10 production. Treatment with Fsk or 8-Br-cAMP alone produced an increase in IL-10 production between 25% and 30% (Fig. 6). However, when the cells were treated with *Lippia* extract (25 μg/mL) in combination with either Fsk (1 μM) or 8-Br-cAMP (100 μM), net IL-10 production above that of untreated control cells was greater than what might be expected by simply adding the IL-10 responses to each of these reagents singly (Fig. 6). For Fsk and *Lippia* extract, the net expected additive response would be $\sim$ 6.9 pg/mL IL-10, while the actual observed net IL-10 response to the combination of the two was 12.9 pg/mL. Likewise for 8-Br-cAMP, the net expected additive response would be $\sim$ 8 pg/mL, while the actual observed net IL-10 response was 13.1 pg/mL.

Comparison of Lippia *extract and IBMX for effect on IL-10 production in Fsk-treated THP-1 cells*

To further explore the role of the PDE inhibitory activity of *Lippia* extract in stimulation of IL-10 production, we compared levels of IL-10 production in differentiated THP-1 cells treated with *Lippia* extract or IBMX, alone and in combination with Fsk. The results demonstrate that the synergistic IL-10 response seen with *Lippia* extract in combination with Fsk was not mimicked by the treatment of THP-1 cells with a combination of IBMX and Fsk (Fig. 7). Similarly, THP-1 cells treated with rolipram, a cAMP-specific
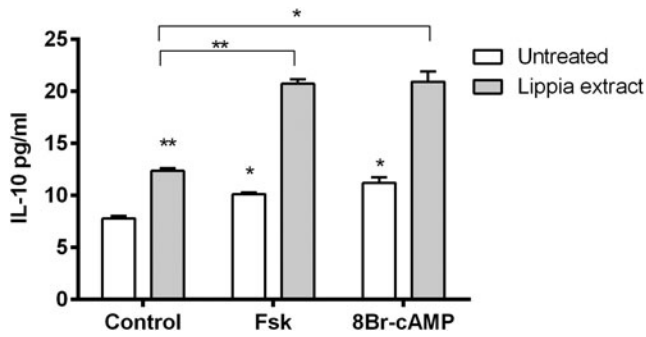
FIG. 6. Effect of *Lippia* extract with forskolin and 8Br-cAMP on IL-10 production by differentiated THP-1 cells. Calcitrol-differentiated THP-1 cells in triplicate were treated for 18 h with *Lippia* extract (25 μg/mL), Fsk (1 μM), and 8Br-cAMP (100 μM). The following day, cell culture supernatants were assayed for the presence of IL-10 by ELISA. Results are representative of three separate experiments and data reported as mean $\pm$ 1 SD (*$P < .05$, **$P < .01$). Significance levels for results of cells treated with a single sample and compared with the response of untreated cells are shown above the respective *bars*. Significance levels for results of cells treated with *Lippia* extract and Fsk or 8-bromoadenosine-3′,5′-cyclic monophosphate (8-Br-cAMP) are shown above the connector lines.

PDE inhibitor, in combination with Fsk did not mimic the synergistic IL-10 response seen with *Lippia* extract in combination with Fsk (data not shown).

Role of p38 MAPK in Lippia *extract-stimulated IL-10 production in THP-1 cells*

As neither Fsk and IBMX nor the combination of the two stimulated significant production of IL-10, our results suggest that the elevation of intracellular cAMP in response to *Lippia* extract is not sufficient to account for the *Lippia* extract-mediated increase in IL-10 production in THP-1 cells. As PKA and p38 MAPK have been shown to play roles in IL-10 expression in response to LPS in a murine
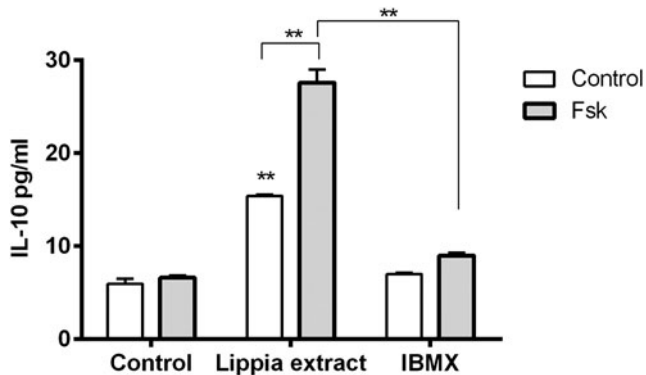

FIG. 7. Effect of IBMX and forskolin on IL-10 production by differentiated THP-1 cells. Calcitrol-differentiated THP-1 cells in triplicate were treated for 18 h with *Lippia* extract (25 μg/mL), Fsk (1 μM), and IBMX (100 μM). The following day, cell culture supernatants were assayed for the presence of IL-10 by ELISA. Results are representative of three separate experiments and data reported as mean $\pm$ 1 SD (*$P < .5$, **$P < .01$).

macrophage cell line,[22] we tested the effect of specific kinase inhibitors of these pathways on *Lippia* extract-stimulated IL-10 production (Fig. 8). The PKA inhibitor H89 only partially inhibited the IL-10 response similar to its effect on LPS-stimulated production of IL-10 in RAW 264.7 macrophages.[22] The p38 MAPK inhibitor SB203580, however, abolished IL-10 produced by cells treated with *Lippia* extract and Fsk. In addition, western blot analysis confirmed that *Lippia* extract and Fsk stimulated phosphorylation of p38 that peaked at 60 min.

Effect of Lippia *extract on IL-10 production in LPS-treated THP-1 cells*

Finally, as cAMP elevation has been shown to synergize with LPS to induce IL-10 in murine macrophages,[22] we also tested whether *Lippia* extract and/or Fsk would synergize with the TLR4 agonist LPS in our model. Treatment of differentiated THP-1 cells with LPS alone induced a modest, but significant, increase in IL-10 production. In addition, treatment of cells with LPS combined with *Lippia* extract, Fsk, or *Lippia* extract and Fsk resulted in significant additive increases in IL-10 produced in response to each of these treatment groups (Fig. 9).

Based on these results, however, it was possible that the ability of *Lippia* extract to induce IL-10 production was due
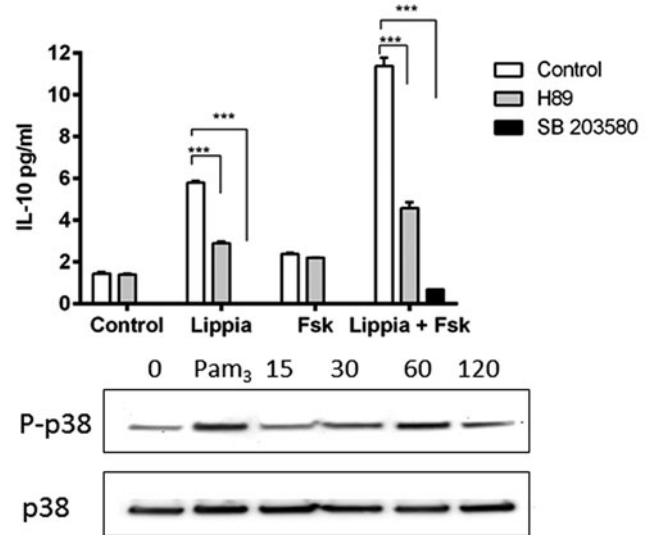

FIG. 8. Effect of protein kinase A (PKA) and p38 MAPK inhibitors on *Lippia* extract stimulation of IL-10 production in differentiated THP-1 cells. Calcitrol-differentiated THP-1 cells in triplicate were treated for 18 h with *Lippia* extract (25 μg/mL) and Fsk (1 μM) in the presence of either the PKA inhibitor H89 (5 μM) or the p38 MAPK inhibitor SB203580 (20 μM). The following day, culture supernatants were assayed for the presence of IL-10 by ELISA. Results are representative of three separate experiments and data reported as mean $\pm$ 1 SD (***$P < .001$). In addition, phosphorylation of p38 was assessed by western blot, in which differentiated THP-1 cells were incubated with *Lippia* extract (200 μg/mL) and Fsk (10 μM) for the times shown (min). In addition, Pam$_3$Csk$_4$ (1 μg/mL for 30 min) was used as a positive control for p38 phosphorylation. Total p38 was used as a loading control.
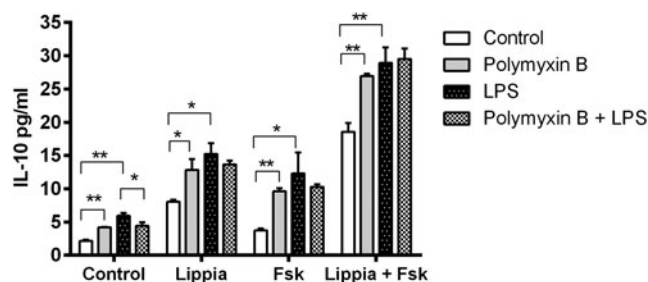
FIG. 9. Effect of TLR agonists and Polymxyin B on *Lippia* extract-induced IL-10 production by differentiated THP-1 cells. Calcitrol-differentiated THP-1 cells in triplicate were treated for 18 h with *Lippia* extract (25 μg/mL), Fsk (1 μM), polymyxin B (PMB; 10 μg/mL), and lipopolysaccharide (LPS; 100 ng/mL). The following day, cell culture supernatants were assayed for the presence of IL-10 by ELISA. Results are representative of three separate experiments and data reported as mean ± 1 SD (*$P<.05$, **$P<.01$). Significance levels are shown above the connector lines.

to the presence of LPS contamination as the *Lippia* extract was not prepared under pyrogen-free conditions. To exclude this possibility, *Lippia* extract was treated with PMB, an antibiotic peptide that binds to and neutralizes LPS.[23,24] Surprisingly, the addition of PMB to cells exposed to *Lippia* extract, Fsk, or *Lippia* extract and Fsk had a stimulatory effect on IL-10 production (Fig. 9). As expected, however, PMB inhibited IL-10 produced in response to LPS (100 ng/mL) alone or LPS combined with *Lippia* extract or Fsk. The addition of PMB had no effect on IL-10 production by cells treated with *Lippia* extract, Fsk, and LPS, suggesting that the capacity to produce IL-10 by these cells had reached a plateau. To eliminate any complexity that PMB might be adding to our model, we did not include it in any of the other experiments described in this study.

DISCUSSION

In traditional Brazilian medicine, *L. sidoides* has been used as an anti-infective, antiseptic, spasmolytic, sedative, hypotensive, and anti-inflammatory agent.[2] Changes in intracellular cAMP levels are known to affect many of these biological activities. Compounds that inhibit the PDE activity to increase cAMP relax smooth muscles and act as spasmolytics and hypotensives.[25] An increase in intracellular cAMP reduces inflammatory responses, and compounds that inhibit PDE4 are potential targets to minimize inflammatory diseases.[26,27] Compounds that increase cAMP have been shown to control pest populations and researchers suggest that plants synthesize PDE inhibitors as part of their defense mechanisms against invasive pests.[28,29]

Despite these medicinal uses, no cAMP-regulating activity has yet to be identified for *L. sidoides*. We propose that many of the medicinal and biological effects of *L. sidoides* are a result of its inhibition of cyclic nucleotide-dependent PDE activity. Our results demonstrate that *Lippia* extract stimulates cAMP levels as measured by a CRE-luciferase reporter assay (Fig. 1) and that this increase is due to its inhibition of cAMP- and cGMP-dependent PDE activity (Fig. 4).

Since *Lippia* extract increases intracellular cAMP as evidenced by activation of the CRE luciferase reporter, we expected that it may also induce IL-10 production as CREB activation is known to be involved in IL-10 stimulation pathways.[17,18] However, while *Lippia* extract with and without Fsk does stimulate the production of IL-10 by differentiated THP-1 cells (Fig. 5), the inability of the combination of Fsk and the PDE inhibitor, IBMX, to mimic the effect of the combination of *Lippia* extract and Fsk (Fig. 8) suggests that the extract may increase IL-10 production through an additional pathway besides simply the inhibition of PDE.

As the p38 MAPK pathway and, to a lesser extent, the PKA pathway have been shown to play a role in the LPS-stimulated production of IL-10,[18,22] we also tested the effect of inhibitors of these pathways on *Lippia* extract-induced IL-10 production. H89 partially inhibited the production of IL-10 in response to *Lippia* extract alone and in the presence of Fsk. These results support our hypothesis that elevation of cAMP by *Lippia* extract is not sufficient to induce IL-10 production. In contrast, the p38 inhibitor SB203580 completely inhibited the IL-10 response elicited by *Lippia* extract and Fsk, suggesting that p38 MAPK is necessary for *Lippia* extract-induced IL-10 production.

Other signaling pathways have been shown to play a role in the expression of IL-10. In particular, phosphotidylinositol 3-kinase (PI3K) has been shown to be upregulated by calcitrol[21,30] and to play a role in IL-10 production.[31,32] It is also interesting to note that curcumin, a component of the spice turmeric, has been shown to enhance IL-10 production in the gut mucosa of patients with inflammatory bowel disease by inhibition of p38.[33]

As mentioned above, bacterial LPS has also been shown to induce IL-10 production through a p38 and MSK-1-dependent mechanism in RAW 264.7 macrophages.[22] The effect of *Lippia* extract on IL-10 production could, therefore, be explained by the possibility of LPS contamination of the *Lippia* extract. To rule out this possibility, we tested whether the LPS neutralizing antibiotic, PMB,[23,24] would eliminate the IL-10-inducing activity of *Lippia* extract. While PMB did abolish any effects of added LPS in our model, the data in Figure 9 rule out the possibility of LPS contamination as the addition of PMB to *Lippia* extract and Fsk surprisingly augmented IL-10 production. While it is not in the scope of this work to define the mechanism by which PMB augmented IL-10 production, one possible explanation might be similar to Sugiura *et al.* who recently demonstrated induction hepatocyte growth factor by PMB in human dermal fibroblasts through MAPK activation.[34]

An additional reason to test whether LPS has an impact on *Lippia* extract-induced IL-10 production is due to the fact that if *Lippia* extract were consumed as a medicinal food and was able to encounter myeloid cells in the lamina propria, it is plausible that such cells might also encounter LPS derived from the gut microbiota. LPS added to our model did induce significant increases in IL-10 by itself and in combination with *Lippia* extract and Fsk. It is tempting, therefore, to speculate that the amount of IL-10 produced in proximity to the intestine may be augmented by *Lippia* extract in the

presence of LPS. A possible health benefit, therefore, of enhanced IL-10 production under such conditions might be in controlling intestinal inflammation as $IL-10^{-/-}$ mice and people who are low IL-10 producers are prone to development of inflammatory bowel disease.[35,36]

Finally, it may be feasible to utilize *L. sidoides* to facilitate healthy aging. We believe that an efficacious dose is achievable for *Lippia* extract as the EC_{50} for the CREB reporter assay was 20 μg/mL. While we do not yet know the active ingredient(s) in *Lippia* extract, for an assumption of 10% absorption multiplied by an average blood volume of 5 L of a human adult, the calculated dosage would be ∼1 g of extract per day. As the average mass of a typical nutritional supplement tablet is 500 mg, a 1 g dose is feasible.

Aging is characterized by chronic low-grade inflammation, a phenomenon known as inflammaging, which is thought to contribute to long-term tissue damage and is associated with mortality risk from all causes in older persons.[37] In addition, inflammation is associated with most conditions that impact health as a person ages. Interestingly, high IL-10 production due to genetic polymorphism is associated with healthy aging, increased longevity, and decreased incidence of Alzheimer's disease.[38] Therefore, it seems likely that modulating the diet with plants, such as *L. sidoides*, may increase systemic or local expression of IL-10 to facilitate healthy aging by limiting inflammation and the long-term damage it causes.

In addition, activation of the CREB pathway indirectly inhibits activation of NFκB as CREB competes for the coactivator proteins, CBP and p300, that are also involved in NFκB activation.[17] We have tested *Lippia* extract in an IL-1β-stimulated NFκB-luciferase reporter assay and have found that *Lippia* extract inhibits the response (D. Fast, unpublished observation). Therefore, *Lippia* may also inhibit production of inflammatory mediators under the control of NFκB, such as IL-6, IL-8, PGE_2, and nitric oxide.

In conclusion, these results demonstrate that *L. sidoides* extract increases intracellular cAMP through inhibition of phosphodiesterase activity. We propose that this activity may explain some of the traditional medicinal activities associated with *L. sidoides*. In addition, the anti-inflammatory activity of *Lippia* may also be due, in part, to its ability to induce IL-10 production through the inhibition of cyclic nucleotide-dependent PDE activity and through activation of the p38 MAPK.

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AUTHOR DISCLOSURE STATEMENT

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