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Light-mediated antibacterial activity of *Lippia origanoides* H.B.K. *in vitro*

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An ethanol extract and different partition fractions obtained from *Lippia origanoides* H.B.K. were assayed for light-mediated activity against strains of *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*). Identical assays were conducted with and without exposure to UV-A (400–315 nm) light to test for light enhanced activity. The ethanol extract and dichloromethane fraction showed light-mediated activity against the *S. aureus* strain, but not against the *E. coli* strain. The dichloromethane fraction was more active than the ethanol extract. Naringenin did not display light-mediated activity against the tested bacteria, indicating that the light-mediated antimicrobial activity of the dichloromethane fraction is not due to its major component. The results represent the first report of light-mediated antimicrobial activity of *Lippia origanoides* and show that its phytochemicals could be used as light-mediated antimicrobial agents.

Introduction

The high prevalence of infectious diseases worldwide has motivated the search for new strategies to control the dissemination of multi-drug resistant bacteria, and the search for new antibacterial agents has contributed to the increase in the availability of antimicrobial agents.[1,2]

In this perspective, a large amount of attention has been given to the use of biologically active compounds isolated from medicinal plants in the prophylaxis and treatment of infectious diseases.[3–6] Despite the great amount of published data regarding the antimicrobial properties of medicinal plants, little attention has been given to the light-mediated antimicrobial activities of these natural resources.[7,8]

Lippia origanoides H.B.K. (Verbenaceae) is a medicinal plant commonly used in culinary seasoning and in traditional medicine as a remedy for gastrointestinal disorders and respiratory diseases.[9] *Lippia origanoides* essential oil (LOEO) exhibits an antigenotoxic effect in bacterial cells, demonstrating that this plant can be an important source of compounds with applications in cancer chemoprevention.[10] LOEO has also shown antioxidant activity and low toxicity,[11,12] as well as, antimicrobial activity against several pathogens.[13–15]

However, the biological activities of extracts obtained from this species remain rarely studied. In this work, the ethanol extract and different partition fractions obtained from *Lippia origanoides* were tested as a source of photoactive secondary metabolites against *Staphylococcus aureus* and *Escherichia coli*.

Materials and methods

Bacterial strains used were: *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. All strains were maintained on heart infusion agar slants (HIA, Difco Laboratories Ltd) and prior to assay, the cells were grown overnight at 37 °C in brain heart infusion (BHI, Difco Laboratories Ltd).

Leaves of *Lippia origanoides* were collected in the county of José de Freitas (latitude 04°45′23″ south and longitude 42°34′32″ west), Piauí, Brazil. The plant material was identified and a voucher specimen was deposited with the number TEPB09205 at the Herbarium "Graziela Barroso" of Universidade Federal do Piauí – UFPI. The leaves were dried at room temperature and made into a powder. The powdered material was extracted by maceration using ethanol as the solvent in a ratio of 1 : 3 ($m:v$) and the homogenate was allowed to stand for 72 h at room temperature. This procedure was carried out in triplicate. The supernatants were then filtered, gathered and

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concentrated under vacuum in a rotary evaporator (model Q-344B – Quimis, Brazil) using an ultrathermal bath (model Q-214M2 – Quimis, Brazil). This yielded 309.5 g of the ethanol extract (LOEE). A part of this extract was suspended in ethanol–water (1 : 1, *v* : *v*) and partitioned in solvents with increasing polarity (hexane, dichloromethane and ethyl acetate), obtaining hexane (LOHEX), dichloromethane (LODCM) and ethyl acetate (LOEA) fractions, respectively. The fractions were concentrated under vacuum in a rotary evaporator and lyophilized. After extraction with ethanol, the residue was macerated with ethanol–water (1 : 1, *v* : *v*) in a ratio of 1 : 3 (*m* : *v*), and the homogenate was allowed to stand for 72 h at room temperature. This procedure was repeated three consecutive times. The supernatants were then filtered, gathered and concentrated under vacuum in a rotary evaporator and lyophilized, thereby obtaining the hydro-alcoholic extract (LOHA).

The dichloromethane fraction constituents were converted to silylated derivatives according to Isidorov *et al.*[16] with modifications. Two milligrams of the LODCM fraction were mixed with 100 μL of bis(trismethylsilyl) trifluoroacetamide and trimethylchlorosilane 1%. The mixture was maintained at 85 °C under agitation for 1 hour. The silylated LODCM fraction was analyzed by injecting 1.0 μL into a gas chromatograph (Shimadzu GC-17A) with a flame ionization detector (FID) model ISQ and coupled to a mass spectrometer model GCMS-QP5050A equipped with a DB-5 HT (Agilent, Palo Alto, CA) 95% methylpolisiloxane and 5% phenyl capillary column (internal diameter = 0.25 mm, length = 30 m, film thickness = 0.1 μm). The operating conditions were as follows: injector temperature 260 °C, detector temperature 300 °C, helium carrier gas, and a flow rate of 1.0 mL min^{-1}. The oven temperature was initially 60 °C (0.5 min), then it was raised to 260 °C (5.0 min) at a rate of 6 °C min^{-1}, and then it was raised to 300 °C (10.0 min) at a rate of 12 °C min^{-1}. The mass spectrometry conditions were as follows: scan mode with an acquisition time of 52.21 min, ionization voltage of 70 eV, mass range of 40–650 Da, and an ion source temperature of 200 °C. The compounds were preliminarily identified by characteristic fragmentation and by comparing to the NIST 2.0 mass spectra libraries. Naringenin and 8-methoxypsoralen (8-MOP) were obtained from Sigma Chemical Co., St. Louis, MO, USA. Naringenin was dissolved in DMSO and 8-MOP was dissolved in a hydro-alcohol (40%, *v*/*v*) solution. Disks with norfloxacin (10 μg per disk) and tetracycline (30 μg per disk) were obtained from Laborclin, Brazil.

The assays were performed using an agar-diffusion method according to Lopez *et al.*[17] Ten microliters of each extract and naringenin dissolved in DMSO (10 000 μg mL^{-1}) were added to blank disks (100 μg per disk). The disks were placed on the surface of medium inoculated with bacteria by the spread plate method (suspended in saline at a density of *ca.* 10^8 CFU mL^{-1}). To monitor the light-activated antimicrobial activities, two replicate experiments were carried out. One replicate dish (without lid) was exposed from above to UV-A light (5.0 W m^{-2}, 320–400 nm from four Sylvania F20T12-BLB lamps, maximum emission at 350 nm) for 2 h, while the other dish was kept in the dark. The fluence rate was monitored with a Viber Lourmat VLX-3W radiometer (UVA photocell). The plates were incubated at 37 °C overnight and the inhibition zones were determined. As positive controls, a disk of norfloxacin (10 μg per disk) and a disk of tetracycline (30 μg per disk) were used as standard antibiotics for the bacteria. 8-Methoxypsoralen (8-MOP – 5 μg per disk) dissolved in a hydro-alcohol solution 40% (*v*/*v*) was utilized as a positive control requiring light for activation. Blank disks with DMSO (10 μL) added were used as a negative control.

Each experiment was performed three times and the results were normalized by calculating the arithmetic average values. Statistical analysis was performed using a data bank from the statistical software SPSS (Statistical Package for Social Sciences), version 21. The assays were considered independent samples and were evaluated with a *t* test of mean, standard deviation and standard errors of mean, using a significance level of $p \leq 0.05$.

Results and discussion

The light-mediated antibacterial activities of different extracts obtained from *Lippia origanoides* were evaluated using an agar-diffusion method. This method was chosen due to the evident practicality and because the properties of photoactivated compounds from natural products, *e.g.* from plants, can be verified by the observation of inhibition zones in the presence of UV light. UV-A light was used in these assays due to the fact that this kind of light presents enough energy to photoactivate the phytocompounds. This light does not have antimicrobial potential, and so is used for the phototoxic evaluation of natural products. Other luminous radiation sources such as infrared were not assayed due the heat emission, which could kill the microorganisms in the test.

The LOEE showed activity against *S. aureus* 25923 only when exposed to UV-A light, indicating the presence of photoactive phytochemicals in the *L. origanoides* leaves (Table 1). Tests performed with different fractions obtained from the LOEE showed that only the LODCM fraction was photoactive against *S. aureus* 25923. This fraction showed antimicrobial activity in the dark, but UV-A exposure caused an enhancement in the anti-staphylococcal activity of the LODCM fraction. On the other hand, the natural products did not show light mediated-antimicrobial activity against *E. coli* (Table 1). Light-mediated antimicrobial activity of plant products has already been verified in several species, such as *Eugenia jambolana* L., *E. uniflora* L., *Hyptis martiusii* Benth., *Momordica charantia* L., *Mentha arvensis* L., *Turnera ulmifolia* L., *Croton campestris* A., *Ocimum gratissimum* L. and *Cordia verbenacea* DC.[4,5,18] As far as we know, light-mediated antimicrobial activity for *Lippia origanoides* has not been reported before.

In the present study, GC-MS analysis of the LODCM fraction showed the presence of naringenin in the keto and enol forms as the majority constituents (Table 2). The presence of naringenin in Verbenaceae species has already been reported,[19]

Table 1 Mean values (± standard errors of mean) of the diameter of the inhibition zones (mm) caused by the light-mediated antimicrobial activity of extracts and fractions from *Lippia origanoides* H.B.K.[a]

	S. aureus ATCC 25923		*E. coli* ATCC 25922	
	Dark	UV-A	Dark	UV-A
LOEE	6.0 ± 0.000	7.0 ± 0.577	6.0 ± 0.000	6.0 ± 0.000
LOHEX	6.0 ± 0.000	6.0 ± 0.000	6.0 ± 0.000	6.0 ± 0.000
LODCM	7.0 ± 0.577	9.7 ± 0.333*	6.0 ± 0.000	6.0 ± 0.000
LOEA	6.0 ± 0.000	6.0 ± 0.000	6.0 ± 0.000	6.0 ± 0.000
LOHA	6.0 ± 0.000	6.0 ± 0.000	6.0 ± 0.000	6.0 ± 0.000
Naringenin	6.0 ± 0.000	6.0 ± 0.000	6.0 ± 0.000	6.0 ± 0.000
Norfloxacin	26.0 ± 0.000	27.0 ± 0.667	31.0 ± 0.577	31.0 ± 0.577
Tetracycline	30.3 ± 0.333	30.0 ± 2.000	27.5 ± 1.000	25.0 ± 0.577
8-MOP	6.0 ± 0.000	12.0 ± 0.000*	6.0 ± 0.000	8.0 ± 0.000*
DMSO	6.0 ± 0.000	6.0 ± 0.000	6.0 ± 0.000	6.0 ± 0.000

[a] LOEE: ethanol extract; LOHEX: hexane fraction; LODCM: dichloromethane fraction; LOEA: ethyl acetate fraction; LOHA: hydro-alcohol fraction; norfloxacin (10 μg per disk) and tetracycline (30 μg per disk): positive standard; 8-MOP (8-methoxypsoralen, 5 μg per disk): light-mediated antimicrobial activity positive control; DMSO (10 μL per disk): solvent control; (–): no inhibition zone. *Significant statistical difference considering $p \leq 0.05$ for the t test using independent samples.

Table 2 Relative amounts of the chemical constituents of *Lippia origanoides* H.B.K. found in the dichloromethane (LODCM) fraction

N°	Compound	Retention time	Relative amount (%)
1	Thymol	9.524	1.26
2	Carvacrol	9.864	1.77
3	2,3-Dimethylcyclo-pentenol	12.983	1.41
4	Theophylline	13.239	0.48
5	Cycloleucine	13.357	0.34
6	7-Methylxanthine	14.700	0.64
7	3′-Hydroxy-irilona	27.474	0.48
8	Dihydro-glycitein	32.615	4.77
9	Naringenin (enol form)	33.593	15.56
10	Naringenin (keto form)	33.637	10.4
11	Eriodictyol	33.830	4.49
12	Hesperetin	33.976	0.54
13	(+) Catechin	34.899	2.62
14	Acid 2,4,6-trihydroxybenzoic	36.439	0.79

including in some *Lippia* species, such as *L. salviaefolia*, *L. sidoides* and *L. graveolens*.[20–23] Recently, naringenin was also found in *Lippia origanoides*.[24]

Besides naringenin, other aglycone flavonoids were found in the LODCM fraction: the flavanones eriodictyol and hesperetin, the isoflavones dihydroglycitein and 3′-hydroxi-irilone, and the flavan-3-ol (+)-catechin. To our knowledge, this is the first report of (+)-catechin found in *L. origanoides*. Flavonoids play important roles in plants, including reproduction, protection against UV-B radiation and defence against phytopathogens.[25–27]

A large variety of plants and fungi synthesize substances which exhibit phototoxicity once excited by visible or UV light, serving as a natural defence against predation by insects and nematodes.[28–30] This phototoxic activity can be a consequence of reactive oxygen species generation and direct production of singlet oxygen, or it can be a consequence of direct photoreaction with DNA, *e.g.* intercalating agents such as furocoumarins.[31,32]

Although flavonoids are considered effective antioxidants, these compounds can also act as pro-oxidants, generating reactive oxygen species *via* Fenton-type reactions or by the formation of flavonoid phenoxyl radicals, which cause cell damage.[31] Antimicrobial and apoptotic activity on tumor cells of natural products have been attributed to the pro-oxidant activity of flavonoids present in their composition.[33–36] The bactericidal activity of catechin against several bacterial strains seems to be related to hydrogen peroxide generation.[35] Oxidative stress stimulated by naringenin, hesperetin, quercetin and morin causes cytotoxicity in human lymphocytes as a consequence of DNA strand breakage and lipid peroxidation of cell membranes by hydroxyl radicals, hydrogen peroxide and superoxide anion formation.[37] In this cell model, naringenin was able to bind to the antioxidant enzyme glutathione transferase, decreasing its activity and promoting oxidative stress.[37] UV-A radiation alone also induces the generation of reactive oxygen species.[38] On the basis of the information mentioned above, we can hypothesize that the components in the LODCM fraction, such as flavonoids, diffuse from the disk causing an increase in the generation of reactive oxygen species during UV-A exposure. Similar to compounds such as 8-MOP (which is a known photosensitizing agent), the components in the LODCM fraction would be able to diffuse from the disk, inducing intracellular oxidative stress, which would be higher in the presence of UV-A than in the dark, causing a larger inhibitory effect on *S. aureus* growth in the presence of UV-A.

Naringenin alone did not display any photoactivity against the strains tested. This result indicates that the light-mediated inhibitory effect of the LODCM fraction is not only due to its major component. Further studies are needed to elucidate if the light-mediated activity of the LODCM fraction is due to a phytochemical specific or whether it is due to a synergism among its components.

Flavonoids were not found in the LOHEX fraction or in the LOHA extract. However in the LOEA fraction, irilone *O*-heterosides, such as 3′-hydroxi-5-furan-dihydroirilone,

3′-hydroxi-5-inososе-irilone, 3′-hydroxi-5-pyran-hydroirilone, 3′-hydroxi-5-inososе-dihydroirilone and catechin, were found (data not shown). As previously shown, the addition of hydrophilic groups such as glycosides, make a compound less effective at inhibiting Gram-positive bacteria.[39,40] Interestingly, these fractions did not display light-mediated antimicrobial activities against *S. aureus*.

None of the extracts and fractions tested were able to inhibit *E. coli* 25922 growth, either in the absence or in the presence of UV-A light. Gram-negative bacteria such as *E. coli* have an outer membrane rich in lipopolysaccharide molecules, which confer a hydrophilic character to the cell wall. The lack of light-mediated activity against the *E. coli* strain could be related to this natural barrier, which makes the uptake of hydrophobic compounds such as flavonoids difficult.

The results indicate that the LODCM fraction is a source of natural photoactive compounds, which could be considered for use in photodynamic therapy of mucosa and cutaneous diseases, such as psoriasis, vitiligo, bacterial infections and periodontal disease. Phototoxic compounds isolated from the LODCM fraction could also be an alternative in the production of light-mediated disinfectants for eliminating pathogens from materials and surfaces. However, additional studies are needed to elucidate the photoactive phytochemicals related to this biological activity.

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