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Research Paper

Antibacterial Potential of *Lippia Gracilis* Schauer Essential Oil Against a *Salmonella Sp.* Strain Isolated in Tilápia Fillet-Fish (*Oreochromis Niloticus*)

Microbiology

KEYWORDS : Salmonella, resistance, Lippia,carvacrol

LEANDRO ÍCARO SANTOS DANTAS	Food Microbiologyand Molecular Biology Laboratory - Campus CurraisNovos - Federal Institute of Education, Science and Technology of Rio Grande do Norte – CurraisNovos – Rio Grande do Norte – Brazil
GABRIELA MEDEIROS ARAÚJO	Immunological, AntimicrobialandCytotoxicityAssaysLaboratory-DepartmentofMicrobiologyandParasitology - Federal Universityof Rio Grande do Norte – Natal – Rio Grande do Norte – Brazil
NAYARA MARTINS RIBEIRO	Immunological, AntimicrobialandCytotoxicityAssaysLaboratory-DepartmentofMicrobiologyandParasitology - Federal Universityof Rio Grande do Norte – Natal – Rio Grande do Norte – Brazil
VÂNIA SOUSA ANDRADE	Immunological, AntimicrobialandCytotoxicityAssaysLaboratory-DepartmentofMicrobiologyandParasitology - Federal Universityof Rio Grande do Norte – Natal – Rio Grande do Norte – Brazil
FRANCISCO FÁBIO MESQUITA OLIVEIRA	PlantTissueCultureLaboratory- DepartmentofBiologicalSciences - StateUniversityof Rio Grande do Norte – Mossoró – Rio Grande do Norte – Brazil
CYNTHIA CAVALCANTI DE ALBUQUERQUE	PlantTissueCultureLaboratory- DepartmentofBiologicalSciences - StateUniversityof Rio Grande do Norte – Mossoró – Rio Grande do Norte – Brazil
FRANCISCO ÂNGELO GURGEL DA ROCHA	FoodMicrobiologyand Molecular BiologyLaboratory - Campus Currais Novos - Federal InstituteofEducation, Science and Technology of Rio Grande do Norte – Currais Novos – Rio Grande do Norte – Brazil

ABSTRACT

Salmonella spp. causer outbreaks and contaminated food intoxications throughout the world. In this perspective, the elucidation of bioactive compounds extracted from species of the Caatinga stands out as a promising source in the development of new antimicrobials. The present study aimed to elucidate the antibacterial potential of essential oil of L. gracilis Schauer on Salmonella spp. from the isolation and preliminary characterization in tilapia fillet-fish – (Oreochromisniloticus).The essential oil was evaluated in HPLC/MS, elucidating the major compounds Carvacrol (44.45%), Thymol (29.53%), and ρ-Cymene (19.89%). The minimum inhibitory concentration was determined by the agar disc diffusion method, using serial dilutions of the oil solution of 1% Tween 80, with the inhibitory concentration at 30 µL mL-1. Bactericidal activity was evident in the concentration of 100 µL mL-1. This result is related to the high composition of monoterpenes and sesquiterpenes characterized, which are compounds with reported ample biological activity.

INTRODUCTION

Pathogenic bacteria belonging to the family Enterobacteriaceae, specifically the genus *Salmonella spp*., Gram-negative bacilli are widely distributed in the environment. Their main natural reservoir is the gastrointestinal tract of humans and warm-blooded animals (FRANCO & LANDGRAFF, 2008). *Salmonella spp*. intoxication outbreaks are mainly associated with the consumption of meat, dairy, eggs, and derived products contaminated with this microorganism (MURMANN et al., 2008; KOTTWITZ et al., 2010).

Cross-contamination between industrial and fish viscera can lead to outbreaks of contamination by fecal coliform and *Salmonella spp*. in fillet-fish samples,such as the one that occurred in the state of São Paulo (LORENZON, 2009; GATTI -JR, 2011).

Intoxications may be limited to a single individual, a small group of related individuals, or even major outbreaks involving thousands of people (FRANCO & LANDGRAFF, 2008; KOTTWITZ et al., 2010).

Bacterial resistance to the usual antibiotics has been a chronic problem in global health. It is estimated that in the U.S., approximately 70% of bacteria associated with hospital infections are resistant to commonly used antibiotics (GOODMAN & GILMAN, 2010). MARTINS et al (1998, cited by OLIVEIRA et al, 2008) reported that in Brazil this pattern is repeated. According to the Ministry of Health, approximately 70% of bacteria that cause hospital infections are resistant to at least one antimicrobial agent commonly used in treatment.

Because of the accelerated bacterial mutagenicity to these antibiotics, research for new bioactive chemicals has been intensifying, especially in Brazil, focusing on plants (COSTA et al. 2008; SILVA et al., 2009; BOSE et al., 2011; KOTTWITZ et al., 2012).

In order to elucidate bioactive compounds, the traditional knowledge of medicinal plants is joined with the prospect of compounds with pharmacological activity. Among the Brazilian biomes, the Caatinga is currently gaining prominence in the chemistry of natural products, given that the regional flora have adapted to shifting water regimes, which promotes chemical adaptations that trigger the development of highly concentrated endogenous chemical compounds (SANTOS et al. 2009; FELIX-Silva et al., 2012; SANTOS & ALVES, 2012).

Among the most studied plant compounds are the essential oils from the aerial parts of several native medicinal species. These have proven effective antimicrobial activity against pathogens of medical-sanitary (COSTA et al., 2008; MURAKAMI, 2009; SILVA

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et al., 2009; AQUINO *et al.* 2010).

Of all the savanna's typical plants,*L. gracilis*Schauer, a member of the Verbenaceae family, stands out as a branched shrub up to 2 meters tall, with brittle stems, simple and small leaves (about 1 cm long), aromatic and spicy, popularly known in Brazil as alecrim-da-chapada (the plateau rosemary) and alecrim-de-tabuleiro (rosemary board)(MELO et al. 2010). When assessed, the essential oil of this species expressed a significant amount of biologically active organic compounds such as carvacrol, thymol, ρ-cymene, α-pinene, β-caryophyllene, γ-terpinenetrujeno α-, β-myrcene and other monoterpenes, and sesquiterpenes (NETO *et al.* 2010; BITU et al. 2012).

The prospect for development of new bioactive phytochemicals is promising, in view of the high incidence of contamination involving foodstuff. These studies enable the discovery of alternatives to combat food poisoning. In this scenario the present study evaluated the antibacterial activity of the essential oil of *L. gracilis*Schauer againsta strain of *Salmonella spp*. isolated from fillet fish (*Oreochromisniloticus*) sold in the public market of the city of Currais Novos/RN.

MATERIALS AND METHODS

Essential oil of *Lippia gracilis* extraction.

We collected fresh leaves of *L. gracilis*Schauer, from the Laboratory of Plant Tissue Culture of the State University of Rio Grande do Norte (UERN) cultivation. The essential oil extraction was performed by steam distillation using the Clevenger system in the Laboratory of Plant Tissue Culture - UERN. The material was stored in an airtight jar, away from light, placed on ice, and then transported to the Laboratory of the Institut Federal Education, Science and Technology of Rio Grande do Norte (IFRN) Currais-Novos Campus, where it remained under refrigeration at - 10 °C until the time of use.

Preliminary Phytochemical Analysis

The quantification of major compounds in the sample was determined by HPLC using a Shimadzu Class-VP chromatograph, consisting of three bombs U-10ATvp, detector scan by ultraviolet photodiode array SPD-M10Avp, oven CTO-10ASvp, sample injection systemSIL10AF, fraction collector FRC10A, and column oven for automatic degasser CTO10AS. For separation of the compounds of the oil, column chromatography was used on reversed phase analytical Hyperclone ODSIC18 3 microns sized 4.6 x 150 mm, and a gradient system solution with 50% acetonitrile and another solution with 90% acetonitrile for 20 min, with continuous flow of 0.8 mL min^{-1} and injection volume 1μL min^{-1}.

Preparation of dilutions of the essential oil

To obtain varied concentrations and a better distribution in the culture medium, the oil was diluted in a saline solution with 1% Tween 80. The concentrations tested were 100, 80, 40, 35, 30, 25, 20, 10, and 5 mL^{-1}. After adding the components, the tubes containing the dilutions were subjected to stirrer vortex for two minutes, repeating it immediately before use.

Microorganism test

The antibacterial activity of the essential oil was evaluated against the Salmonella strain. The respective pure culture was obtained from IFRN Currais Novos Campus' bacteria database, whose isolation and characterization were described by Dantas et al, 2012.

Standardization of inoculum

The salmonella strain was inoculated into BHI broth and incubated at 37°C for 2-6 hours. Then the turbidity was compared to 0.5 McFarland standard (1.5 x 108 CFU / mL). When required, the turbidity of the culture was adjusted by using sterile saline solution. The bacteria were inoculated in 100mm petri plates containing Mueller-Hinton agar by the spread plate method, using a sterile swab. All assays were performed in triplicate (NCCLS, 2003).

Evaluation of antibacterial activity and reading results

The test was performed in triplicate, and for each repetition two streaked plates with 6 and 7 drilled wells wereused. The minimum distance between them was approximately 20mm, and100μL of each essential oil dilution wastransferred to each well. Positive control consisted of 100μL of chloramphenicol (4μg ml-1) and negative control of the 100 μL saline tween 80 1%. The plates were incubated at 37°C/18h under aerobic conditions. After the incubation period, the halo diameters were evaluated and compared with the positive control halo (Chloramphenicol 4μg mL^{-1}); the sensitivity of the oil halo was measured with the aid of a pachymeter (NCCLS, 2003).

Evaluation of Bactericidal or Bacteriostatic Activity

After checking the serial dilution test results, the studied strain was inoculated in nutrient broth at 37°C 24h. Next, the inoculum was adjusted according to 0.5 McFarland scale; 1mL of this inoculum was transferred to a sterile tube containing 1 mL from the 100μL mL^{-1}*L. gracilis* Schauer essential oil dilution and then incubated again at 37°C for 24h. Subsequent to the incubation time, Mueller-Hinton agar plates were seeded in triplicate to confirm growth of colonies resistant to oil.

RESULTS

Table 1 shows the concentrations of the main constituents of *L. gracilis*Schauer essential oil. Table 2presents the inhibition growth zone diameter meansof the studied strain tested against different concentrations, while Table 3 providesthe averages of a second test in which serial dilutions were tested, starting from the smallest inhibitory concentration achieved in the initial test.

Table 1.Concentration of the main constituents of the oil of *Lippia gracilis* Schauer.

ESSENTIAL OIL	CONCENTRATION (%)
Carvacrol	44.45
Thymol	29.53
ρ – cymene	19.89

In line with the results, it can be said that *L. gracilis* Schauer essential oil potentially inhibitsgrowth of the *Salmonella spp*. strain, since theinhibition zone values when compared to the positive control, Chloramphenicol 4μg $mL^{-1,}$ are representative since the undiluted oil inhibition zone diameter is larger than the control.

Table 2. Mean inhibition zone diameter (mm) of *Salmonella spp*. exposed to different concentrations of *L. gracilis* Schauer oil.

TREATMENT	DIAMETER (mm)*
OE undiluted	30.00 a
100 μl mL^{-1}	25.75 b
80 μl mL^{-1}	23.75 c
40 μl mL^{-1}	14.25 d
20 μl mL^{-1}	0.00 e
10 μl mL^{-1}	0.00 e
5 μl mL^{-1}	0.00 e
Tween 1%	0.00 e
Chloramphenicol 4μg mL^{-1}	28.00 f

* Means followed by the same lowercase letters in the column do not differ significantly by Tukey test at 5% probability.

The first test did not identify inhibition at concentrations below 20μL mL^{-1}; thus, another test was performed with fresh dilutions with concentrations ranging from 20μL mL^{-1} to 40μL mL^{-1} to determine the minimum inhibitory concentration of *L. gracilis*S-

chauer essential oil. In accordance with Table 3, the lowest concentration of inhibition was 30μL mL^{-1}.

Table 3. Mean inhibition zone diameter (mm) of *Salmonella spp.* exposed to different concentrations of *L. gracilis* SCHAUER oil.

TREATMENT	DIAMETER(mm)*
35 μl mL^{-1}	13.50 a
30 μl mL^{-1}	11.75 b
25 μl mL^{-1}	0.0 c

* Means followed by the same lowercase letters in the column do not differ significantly by Tukey test at 5% probability.

In view that the 1% Tween negative control and the dilutions below 25 μL mL^{-1} did not create inhibition halos, it was inferred that the surfactant used did not contribute to the tested oil antibacterial activity, nor did itgenerate false results.

Based on the achieved results, the spectrum action of the *Lippiagracilis*Schauer oil was assessed to determine its bactericidal activity, since there was no microbial growth after 24h incubation of the 100 μL mL-1dilution of the essential oil with the bacteria tested.

DISCUSSION

Chromatography revealed that the major constituents of the *L. gracilis*Schauer essential oil are carvacrol, thymol, and ρ-cymene (Table 1). In a similar work, Melo-Neto et al. (2010) determined the chemical composition of *L. gracilis* Schauer essential oil and found an extensive constituent concentration of carvacrol (50.13%) with significant antibacterial activity to *Staphylococcus aureus* strains isolated from affected infections in diabetic rats. In contrast,Bitu et al. (2012)characterized the phenolic compounds of the same species and found the highest percentage of thymol (44.42%), followed by carvacrol (22.21%), which were also effective in combating strains of *E. coli* (ATCC 10536), *E. coli* (27), *P. aeruginosa* (ATCC 15442), *S. aureus* (ATCC 12692), and *S. aureus* (358) microorganisms of relevant medical-sanitary position.

Although the literature presents differences in concentrations of the essential oil constituents evaluated, it can be inferred that such variations are related to changes in abiotic factors predisposing every growing region. However, despite some differences with respect to variation in composition of the oil, this study confirmed its effectiveness as an antimicrobial agent. This activity is widely attributed in the literature to thymol and carvacrol monoterpenes, and this project has confirmed the major presence of these compounds.

Strengthening *L. gracilis* Schauer oil efficiency, Rocha et al. (2010) also demonstrated the inhibitory activity of these volatile compoundsagainst pathogens of medical and sanitary importance such as *Listeria monocytogenes*,*Salmonella sp.* serotype typhi, *Salmonella sp.* serotype typhimurium, *Staphylococcus* aureus ATCC 25923, and *Staphylococcus* epidermis. Also, according to the authors, the bacterial growth inhibition by the compounds present in the oil in many of the strains overcame the antibiotic used as control, especially for *Salmonella spp.* strains isolated from food.

Other studies have attempted to elucidate the antibacterial activity of essential oils against clinical and sanitary bacteria strains of interest. For instance,in a study by Santurio et al. (2007), whichevaluated the antibacterial activity of thyme, cinnamon, and oregano essential oils against enteric Salmonella serovars isolated from poultry, emphasized oregano essential oil's bactericidal characteristics. In a later study, Lima-Silva et al. (2010) evaluated five samples of oregano essential oil against Salmonella enteritidis ATCC 13076 and determined thatCarvacol was the main constituent of the brands from different regions, the same phenolic constituent evidenced in *L. gracilis* Schauer essential oil. Oregano essential oils have inhibited S. enteritidis ATCC 13076 growth.In 2010, Aquino et al. evaluated the antibacterial activity of lemongrass and basil essential oils (widely used cooking condiments), determining its minimum inhibitory concentrations against *E. coli*, *Salmonella spp.*, and*S.aures*obtainedfrom contaminated beef samples andverifying *Lippiaalba* (lemongrass) essential oil bactericide power against *Salmonella spp.*, thereby elucidating antibacterial activity data of the tested oil.

Assuming that the strain tested against *L. gracilis* Schauer essential oil was isolated and characterized from tilapia (*Oreochromis niloticus*) by Dantas et al. (2012). The bacterial growth control is crucial for sanitary purposes, which can inspire studies regarding new methods to maintain the quality of fish and meat products, which are the most important *Salmonella spp.* Carriersand are highly related to food poisoning, thus triggering outbreaks (FAI et al., 2011; MARCHI et al., 2011)

This test also shows the bactericidal efficacy of*L. gracilis* Schauer essential oil, contrary to chloramphenicol bacteriostatic activity, as the antibiotic choice for acute salmonellosis and typhoid (GOODMAN & GILMAN, 2010).

CONCLUSION

Finally, this study has shown thatthe *Lippiagracilis*Schauer essential oil,when tested against tilapia (*Oreochromisniloticus*) bacteria strains, has a satisfactory activity and, when in a 100μL mL-1 dilution, has a bactericidal activity, whichcouldbe useful ina possible food preservation method or for future new antimicrobial development.

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