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Antimicrobial activity of *Tetradenia riparia* leaf essential oil

[Actividad antimicrobiana del aceite esencial de hoja de *Tetradenia riparia*]

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Abstract: Food spoilage is a widely neglected problem and the constant use of synthetic fungicides could develop resistant fungi. The objective of this study was to evaluate the chemical composition and antimicrobial activity of *Tetradenia riparia* leaf essential oil against foodborne disease microorganisms. Leaf essential oil was obtained by hydrodistillation and identified by gas chromatography coupled to mass spectrometry. The antimicrobial activity was studied by broth microdilution. The major compounds identified were oxygenated sesquiterpenes (43.6%): 14-hydroxy-9-epi-(E)-cariophylene (20.8%) and τ-cadinol (18.4%); followed by oxygenated diterpenes (24.6%): 6,7-dehydroroyleanone (12.6%) and 9β, 13β-epoxy-7-abiethene (10.6%); sesquiterpenic hydrocarbons (17.1%) and oxygenated monoterpenes (7.4%): fenchone (5.6%). The essential oil had broad antibacterial and antifungal activity, mainly against *A. versicolor* and *P. ochrochloron* with fungistatic and fungicidal activities and *B. cereus*, *L. monocytogenes*, and *S. aureus* with bacteriostatic and bactericidal activities. *T. riparia* leaf essential oil is a potential alternative to control microorganisms.

Keywords: Antimicrobials; *Bacillus*; Bioproducts; *Listeria*; *Staphylococcus*.

Resumen: El deterioro de los alimentos es un problema ampliamente desatendido y el uso constante de fungicidas sintéticos podría desarrollar hongos resistentes. El objetivo de este estudio fue evaluar la composición química y la actividad antimicrobiana del aceite esencial de hoja de *Tetradenia riparia* contra microorganismos patógenos transmitidos por los alimentos. El aceite esencial de hoja se obtuvo por hidrodestilación y se identificó mediante cromatografía de gases acoplada a espectrometría de masas. La actividad antimicrobiana estudiada fue por microdilución en caldo. Los compuestos principales del aceite esencial se identificaron como sesquiterpenos oxigenados (43,6%): 14-hidroxi-9-epi-(E)-cariofileno (20,8%) y τ-cadinol (18,4%); seguido de diterpenos oxigenados (24,6%): 6-7-deshidroroileanona (12,6%) y 9β, 13β-epoxi-7-abieteno (10,6%); hidrocarburos sesquiterpénicos (17,1%) y monoterpenos oxigenados (7,4%): fenchona (5,6%). Tenía amplia actividad antibacteriana y antifúngica, principalmente contra *A. versicolor* y *P. ochrochloron* con actividades fungistáticas y fungicidas, y principalmente contra *B. cereus*, *L. monocytogenes* y *S. aureus* con actividades bacteriostáticas y bactericidas. El aceite esencial de hoja de *T. riparia* es una alternativa potencial para controlar microorganismos.

Palabras clave: Antimicrobianos; *Bacillus*; Bioproductos; *Listeria*; *Staphylococcus.*

INTRODUCTION

Food spoilage is a serious widely neglected problem, mainly due to harvesting practices such as inappropriate drying, handling, packaging, storage, and transport conditions (Bhat *et al.*, 2010). Several microorganisms could cause food spoilage such as *Aspergillus ochraceus* and/or *Penicillium verrucosum* responsible for the production of some toxins like ochratoxin-A, a mycotoxin with pathogenic effects in animals and possible human carcinogen (Bui -Klimke & Wu, 2015; Malir *et al.*, 2016) and citrinin, a nephrotoxic, hepatotoxic, and cytotoxic mycotoxin (Larsen *et al.*, 2001). Moreover, food bacterial contamination has resulted in billions of dollars in losses annually in developing countries (WHO, 2015; Bintsis, 2017) such as *Pseudomonas aeruginosa*, a multidrug-resistant bacterium, (Pang *et al.*, 2019) *Listeria monocytogenes*, which develops multidrug resistance, (Olaimat *et al.*, 2018), and *Bacillus cereus*, which causes food poisoning (El-Arabi & Griffiths, 2013) among others such as *Salmonella enterica* and *Staphylococcus aureus* (Khare *et al.*, 2018).

The use of synthetic fungicides – in the postharvest treatments of vegetables – could develop resistant fungal strains (Koul *et al.*, 2008) and other synthetic chemicals against bacteria and fungi such as nitrates, benzoates, sulfites, and sorbates that are used to preserve foods have been associated to adverse effects on human health such as allergic or carcinogenic effects (Sultana *et al.*, 2014; Gyawali & Ibrahim, 2014; Pisoschi *et al.*, 2018). It was suggested the use of bio-based essential oils from plants as fungicides are an alternative to control microorganisms (Anyanwu & Okoye, 2017) and to reduced microbial resistance. In addition, Sivakumar & Bautista-Baños (2014) recommended the use of essential oils to preserve food and Burt (2004) reported that most essential oils to preserve and flavor food are considered Generally Recognized as Safe (GRAS) at concentrations from 0.1 to 6%. Thus, the identification of alternative compounds for controlling microbial foodborne diseases is relevant for food preservation, especially with the increase in microbial resistance to conventional chemicals.

Tetradenia riparia (Hochst.) Codd, belonging to Lamiaceae family, native to South Africa, is a shrub ranging from 1.20 to 1.60 m height, and popularly known as myrrh, umuravumba, incense, lemon verbena, and fake-myrrh. Its leaves are large, heart-shaped, coarsely toothed, thick, and sticky; they are easily identified when touched because of the strong smell due to its great amount of essential oil (Lorenzi & Souza, 1999; Gairola *et al.,* 2009). The essential oil from *T. riparia* leaves has been reported to present antibacterial activity against *Bacillus subtilis*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Lactobacillus casei*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Staphylococcus aureus*, *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus sanguinis*, and *Streptococcus sobrinus*, but the antifungal activity was only reported against *Candida albicans* (Gazim *et al.*, 2010; Melo *et al.*, 2015; Baldin *et al.*, 2018). Moreover, the hydroalcoholic extract of *T. riparia* leaves (Fernandez *et al.,* 2017) and *T. riparia* leaf isolate compound 8(14), 15-sandaracopimaradiene-7α, 18-diol were also reported to have antibacterial activity (Van Puyvelde *et al.,* 1986). Thus, despite the reports on the antimicrobial activity, mostly antibacterial, for the essential oil from *T. riparia* leaves, no reports have been found on its antimicrobial activity against a broad-spectrum of fungi and bacteria that cause foodborne diseases as a potential alternative to synthetic chemical compounds for food preservation.

MATERIALS AND METHODS
Plant material
Tetradenia riparia was cultivated in the medicinal garden of *Paranaense* University, Brazil, at coordinates S23º46.225' and WO 53º16.730' and altitude of 391 m. A voucher specimen was authenticated and deposited at the Educational Herbarium of *Paranaense* University under the number 2502. Plant propagation was carried out in a 6.0×1.2 m garden bed, where four plants were planted 1 m apart from each other. The plants reached adult stage six months after planting when the leaves were collected and the essential oil extracted. The research study was registered in the National System of Genetic Heritage Management and Associated Traditional Knowledge (SisGen, acronym in Portuguese) under the number AA6C8A8. The leaves of *T. riparia* were collected when the plant reached the adult stage with 2.1 m of height and 16 cm of stem diameter. The leaves were manually collected early in the morning, from 7 to 9 h, just after early dew evaporation in the period from 03/01/2014 to

04/01/2015.

Essential oil extraction

The essential oil was obtained from fresh leaves by hydrodistillation using a Clevenger type apparatus for 3 h (Gazim *et al.*, 2010). The distilled essential oils were collected and dried with anhydrous sodium sulfate and stored at -20°C in a closed glass vial wrapped in aluminum foil. The essential oil yield was calculated dividing the essential oil mass by fresh leaf mass (wet basis) and multiplied by 100.

Chemical identification of essentials oils by GC-MS

The essential oil chemical identification was carried out by a gas chromatograph (Agilent 5973 MSD) coupled to a mass spectrometer (Agilent 5973 MSD) (GC-MS), and a fused silica capillary column (30 m × 250 × 0.25 μm; Agilent 19091S-433 HP-5MS) with initial oven temperature from 60°C to 285°C at a rate of 4.3°C min^{-1}. Helium was used as the carrier gas; the inlet pressure was 56 kPa and linear speed was 1 mL min^{-1} at 300°C. The injector temperature was 280°C and the injection mode was spitless. MS scan conditions were source temperature at 200°C, interface temperature at 250°C, E energy of 70 eV, mass scan range of 40-350 *amu*. The transfer line, ion source and quadrupole were 280, 230, and 150°C, respectively. The identification of the compounds was based on the comparison of their retention indices (RI), obtained using various n-alkanes (C7-C30). In addition, their electron ionization (EI) mass spectra were compared with the NIST 11.0 library spectra and with the data available in the Adams (2017).

Antibacterial activity assay

The following Gram-positive bacteria were used: *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC 6538), *Bacillus cereus* Frankland and Frankland (clinical isolate), and *Listeria monocytogenes* (Murray *et al.*) Pirie (NCTC 7973), and the following Gram-negative ones: *Pseudomonas aeruginosa* (Schroeter) Migula (ATCC 27853), *Escherichia coli* (Migula) Castellani and Chalmers (ATCC 35218), *Enterobacter cloacae* (Jordan) Hormaeche and Edwards (clinical isolate), and *Salmonella enterica* subsp. *enterica* (ex-Kauffmann and Edwards) Le Minor and Popoff serovar Typhimurium (ATCC 13311). The microorganisms were from the culture collection of the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research 'Siniša Stanković', Belgrade, Serbia. The antibacterial assay was carried out by a microdilution method (CLSI, 2009) in order to determine the antibacterial activity of the compounds tested against bacteria. The bacterial suspensions were adjusted with sterile saline to a concentration of 1.0×10^5 CFU mL^{-1}. The inocula were prepared daily and stored at +4°C. Dilutions of the inocula were cultured on solid medium to verify the absence of contamination and to check the validity of the inoculum. All experiments were performed in duplicate and repeated three times.

Minimal inhibitory and bactericidal concentrations

The bacterial suspensions were adjusted with sterile saline solution to a concentration of 1.0×10^5 CFU mL^{-1}. Essential oils were dissolved in a 5 mL 100 mL^{-1} dimethyl sulfoxide (DMSO) solution, containing 0.1 mL 100 mL^{-1} polysorbate-80, and added in tryptic soy broth (TSB) medium (100 μL) with bacterial inoculum (1.0×10^4 CFU per well). The lowest concentrations without visible growth (optical microscope) were defined as concentrations that completely inhibited bacterial growth (MIC). The MIC, obtained from the susceptibility testing of various bacteria to tested extracts, was determined by a colorimetric microbial viability assay based on the reduction of an INT (p-iodonitrotetrazolium violet) [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride; Sigma]) color and compared with positive control for each bacterial strains. The minimum bactericidal concentration (MBC) was determined by serial sub-cultivation of 2 μL into microtiter plates, containing 100 μL of broth per well, and further incubation for 24 h. The lowest concentration with no visible growth was defined as the MBC, indicating 99.5% killing of the original inoculum. The optical density of each well was measured at 655 nm by Microplate manager 4.0 (Bio-Rad Laboratories) and compared with a blank (broth medium plus diluted extracts) and the positive control. Streptomycin (Sigma P 7794) and ampicillin (Panfarma, Belgrade, Serbia) were used as positive controls (1 mg mL^{-1} in sterile physiological saline), and 5 mL 100 mL^{-1} DMSO was used as a negative control. All experiments were performed in duplicate and repeated three times.

Antifungal activity assay

For the antifungal bioassays, eight fungi were used: *Aspergillus fumigatus* Fresenius (ATCC 1022), *Aspergillus niger* van Tieghem (ATCC 6275), *Aspergillus ochraceus* Batista et Maia (ATCC 12066), *Aspergillus versicolor* (Vuillemin) Tiraboschi (ATCC 11730), *Penicillium funiculosum* Thom (ATCC 8725), *Penicillium ochrochloron* Biourge (ATCC 90288), *Penicillium verrucosum* var. *cyclopium* (Westling) Samson, Stolk & Hadlok (food isolate), and *Trichoderma viride* Pers. (IAM 5061). The microorganisms were obtained from the culture collection of the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research 'Siniša Stanković', Belgrade, Serbia. The micromycetes were maintained on malt agar, and the cultures stored at +4°C and sub-cultured once a month. In order to investigate the antifungal activity of the compounds, a modified microdilution technique was used (Espinel-Ingroff, 2001). The fungal spores were washed from the surface of agar plates with 0.85 g 100 mL^{-1} sterile saline solution, containing 0.1 mL 100 mL^{-1} polysorbate-80, to check the validity of the inoculum. The spore suspension was adjusted with sterile saline solution to a concentration of approximately 1.0×10^5 CFU mL^{-1} in a final volume of 100 μL per well. The inocula were stored at +4°C for further use. Dilutions of the inocula were cultured on solid malt agar to verify the absence of contaminants.

Minimal inhibitory and fungicidal concentrations

MIC determinations were performed by a serial dilution technique using 96-well microtiter plates. The essential oil was dissolved in 5 mL 100 mL^{-1} DMSO solution (Merck KGaA, Germany), containing 0.1 mL 100 mL^{-1} polysorbate-80, and added to a malt extract cultivation medium with inoculum. The microplates were incubated in a rotary shaker (160 rpm) for 72 h at 28°C. The lowest concentrations without visible growth (optical microscope) were defined as MIC. The minimum fungicidal concentration (MFC) was determined by serial subcultivation of 2 μL of tested compounds, dissolved in medium, and inoculated for 72 h into microtiter plates containing 100 μL broth per well, and further incubated for 72 h, at 28°C. The lowest concentration with no visible growth was defined as MFC, indicating 99.5% killing of the original inoculum. A solution of 0.1 mL 100 mL^{-1} polysorbate-80 was used as negative control, and the commercial fungicides bifonazole (Srbolek, Belgrade, Serbia) and ketoconazole (Zorkapharma, Šabac, Serbia) were used as positive controls (1-3500 μg mL^{-1}). All experiments were performed in duplicate and repeated three times.

Statistical analysis

For each species, three samples were used, and all the assays were carried out in triplicate. The results were expressed as arithmetic mean with standard errors and analyzed using one-way analysis of variance (ANOVA), followed by Tukey's HSD (honestly significant difference) test with α = 0.05. This analysis was carried out using SPSS v. 18.0 software.

RESULTS

The yield of the essential oil from *T. riparia* leaves was 0.25%. GC-MS identified 36 compounds representing 95.2% of the volatile constituents (Table No. 1). The major compounds were oxygenated sesquiterpenes (43.6%), mainly 14-hydroxy-9-*epi*-(*E*)-caryophyllene (20.8%) and *τ*-cadinol (18.4%) (Figure No. 1); followed by oxygenated diterpenes (24.6%), mainly 6,7-dehydroroyleanone (12.6%) and 9*β*,13*β*-epoxy-7-abietene (10.6%). (Figure No. 1); sesquiterpene hydrocarbons (17.1%) and oxygenated monoterpenes (7.4%).

Table No. 1
Chemical composition of *Tetradenia riparia* leaf essential oil

Peak	[a]Compound	[b]RI	Composition (%)	Methods of Identification
	Monoterpenes Hydrocarbons			
1	*α*-pinene	913	0.4	a,b,c
2	Camphene	931	0.3	a,b,c
3	Sabinene	963	0.5	a,b,c
4	*β*-pinene	967	0.3	a,b,c

| 5 | *β*-phellandrene | 1023 | 0.4 | a,b,c |

Monoterpenes Oxygenated

6	Fenchone	1078	5.6	a,b,c
7	*endo*-fenchol	1105	0.3	a,b,c
8	Camphor	1134	1.1	a,b,c
9	Borneol	1157	0.3	a,b,c
10	*α*-terpineol	1185	0.1	a,b,c

Sesquiterpene Hydrocarbons

11	*δ*-elemene	1339	0.1	a,b,c
12	*α*-copaene	1372	0.3	a,b,c
13	*β*-elemene	1389	0.5	a,b,c
14	*α*-gurjunene	1405	1.0	a,b,c
15	*β*-caryophyllene	1416	5.3	a,b,c
16	*α*-(*E*)-bergamotene	1432	0.4	a,b,c
17	*α*-humulene	1452	0.3	a,b,c
18	*allo*-aromadendrene	1459	0.2	a,b,c
19	*γ*-muurolene	1476	0.1	a,b,c
20	Germacrene D	1480	0.1	a,b,c
21	*β*-selinene	1485	0.3	a,b,c
22	Bicyclogermacrene	1495	4.1	a,b,c
23	*α*-muurolene	1499	0.2	a,b,c
24	*γ*-cadinene	1513	1.1	a,b,c
25	n.i.	1513	0.3	a,b,c
26	*δ*-cadinene	1520	3.1	a,b,c
27	n.i.	1570	0.9	a,b,c
28	n.i.	1573	0.1	a,b,c

Oxygenated Sesquiterpene

29	Carotol	1580	0.8	a,b,c
30	n.i.	1601	0.3	a,b,c
31	*τ*-cadinol	1642	18.4	a,b,c
32	*epi*-*α*-Muurolol	1650	1.4	a,b,c
33	Cubenol	1655	1.3	a,b,c
34	14-hydroxy-9-*epi*-(*E*)-caryophyllene	1669	20.8	a,b,c
35	Eudesm-7(11)-en-4-ol	1698	0.6	a,b,c
36	14-hydroxy-*α*-humulene	1715	0.3	a,b,c
37	n.i.	1935	0.1	a,b,c

Diterpenes (Hydrocarbons and Oxygenated)

38	9*β*,13*β*-epoxy-7-abietene	1988	10.6	a,b,d
39	Manool oxide	1996	0.4	a,b,c
40	Abietatriene	2062	0.2	a,b,c
41	Abietadiene	2075	0.4	a,b,c
42	n.i.	2120	0.1	a,b,c
43	n.i.	2128	0.1	a,b,c
44	n.i.	2135	0.1	a,b,c
45	Abienol	2161	0.2	a,b,c
46	Calyculone	2176	0.8	a,b,c
47	6,7-Dehydroroyleanone	2206	12.6	a,b,d

48	n.i.	2356	0.2	a,b,c
		Total identified	**95.2**	

Grouped components

Monoterpenes hydrocarbons	1.9
Oxygenated monoterpenes	7.4
Sesquiterpenes hydrocarbons	17.1
Oxygenated sesquiterpenes	43.6
Diterpene hydrocarbons	0.6
Oxygenated diterpenes	24.6
Not identified	2.2

ᵃCompounds listed in order of elution from a HP-5MS column. ᵇRI calculated = identification based on retention index; c = identification based on comparison of mass spectra in NIST 11.0 libraries; n.i. = not identified. d = formula and molecular mass similar to Gazim *et al.* (2014) that used nuclear magnetic resonance spectroscopy to identify the compounds in the same plant extract type
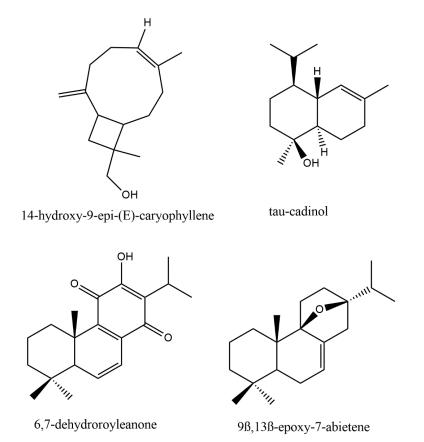

14-hydroxy-9-epi-(E)-caryophyllene   tau-cadinol

6,7-dehydroroyleanone   9ß,13ß-epoxy-7-abietene

Figure No. 1
Chemical structures of the major compounds of *Tetradenia riparia* leaf essential oil

Antibacterial activity

The essential oil from *T. riparia* leaves presented antibacterial activity against all tested bacteria, with MIC ranging from 0.05 to 0.60 mg mL^{-1} and MBC from 0.075 to 1.25 mg mL^{-1} (Table No. 2). MIC for streptomycin varied from 0.05 to 0.125 mg mL^{-1} and MBC from 0.10 to 0.50 mg mL^{-1}, and for ampicillin MIC ranged from 0.10 to 0.30 mg mL^{-1} whereas MBC was from 0.15 to 0.50 mg mL^{-1} (Table No. 2). The Gram-positive bacteria were the most sensitive to the essential oil: *B. cereus* (0.05 mg mL^{-1}), *L.*

monocytogenes (0.05 mg mL^{-1}), and *S. aureus* (0.05 mg mL^{-1}), while the greatest resistance was against *P. aeruginosa* (MIC of 0.60 mg mL^{-1} and MBC of 1.25 mg mL^{-1}) compared to the controls streptomycin and ampicillin (Table No. 2). Thus, the essential oil presented bacteriostatic activity with MIC equal to or lower than the controls against *B. cereus*, *L. monocytogenes*, and *S. aureus*. Similarly, the bactericidal activity of the essential oil occurred with MBC lower than or equal to the controls against *B. cereus*, *L. monocytogenes*, and *S. aureus*.

Table No. 2
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Tetradenia riparia* leaf essential oil and positive controls streptomycin and ampicillin

Bacterium	Essential oil (mg mL^{-1})	Streptomicyn (mg mL^{-1})	Ampicillin (mg mL^{-1})
	MIC MBC	MIC MBC	MIC MBC
Bacillus cereus	0.05 ± 0.003^a 0.075 ± 0.003^A	0.05 ± 0.03^a 0.10 ± 0.03^{AB}	0.10 ± 0.01^b 0.15 ± 0.03^B
Enterobacter cloacae	0.30 ± 0.06^b 0.60 ± 0.003^B	$0.05 \pm <0.001^a$ 0.10 ± 0.06^A	0.30 ± 0.02^b 0.50 ± 0.09^B
Escherichia coli	0.30 ± 0.03^b 1.00 ± 0.43^B	0.15 ± 0.03^a 0.30 ± 0.03^A	$0.15 \pm <0.01^a$ 0.30 ± 0.03^A
Listeria monocytogenes	$0.05 \pm <0.001^a$ 0.20 ± 0.03^A	0.25 ± 0.06^b 0.50 ± 0.03^B	0.10 ± 0.03^a 0.15 ± 0.01^A
Pseudomonas aeruginosa	0.60 ± 0.006^b 1.25 ± 0.03^C	0.05 ± 0.01^a 0.10 ± 0.03^A	0.10 ± 0.06^a 0.20 ± 0.01^B
Salmonella enterica	0.075 ± 0.03^a 0.50 ± 0.06^C	0.05 ± 0.03^a $0.10 \pm <0.01^A$	0.15 ± 0.01^b $0.20 \pm <0.01^B$
Staphylococcus aureus	0.05 ± 0.001^a 0.075 ± 0.006^A	0.125 ± 0.03^b 0.25 ± 0.06^B	0.10 ± 0.03^{ab} 0.15 ± 0.01^{AB}

Different lowercase and uppercase letters on the same line differ among themselves by Tukey HSD test ($p \leq 0.05$)

Antifungal activity

The essential from *T. riparia* leaves inhibited the growth of fungal strains with MIC ranging from 0.06 to 10.00 mg mL^{-1} and MFC from 0.30 to 20.0 mg mL^{-1} (Table No. 3). The control bifonazole had fungistatic effect with MIC from 0.10 to 0.20 mg mL^{-1} and fungicidal effect with MFC from 0.20 to 0.25 mg mL^{-1} whereas the control ketoconazole presented MIC from 0.15 to 2.50 mg mL^{-1} and MFC from 0.20 to 3.50 mg mL^{-1} (Table No. 3). The most sensitive fungus to the essential oil was *A. versicolor*, followed by *P. ochrochloron*, while the others were more resistant (Table No. 3). The MIC value (0.06 mg

mL^{-1}) of the essential oil against *A. versicolor* was lower than the controls, and the MFC value (0.30 mg mL^{-1}) was lower than the control ketoconazole (0.50 mg mL^{-1}) and higher than the control bifonazole (0.20 mg mL^{-1}) (Table No. 3). The most resistant fungus to the essential oil was *A. ochraceus* with MIC value 67-fold higher and MFC value 100-fold higher than the controls. For *P. ochrochloron*, the essential oil had MIC 2.5-fold higher for the control bifonazole, and 5-fold lower for the control ketoconazole, whereas MFC was 2.4-fold higher for the control bifonazole, and 5.8-fold lower for the control ketoconazole. The fungistatic and fungicide activity

of the essential oil were similar, resulting in a more efficient control of *A. versicolor*, followed by *P. ochrochloron*, and the antifungal activity was more effective only at greater concentrations of the essential oil for the other fungi such as *A. ochraceus*.

Table No. 3
Minimum inhibitory (MIC) and fungicidal (MFC) concentrations of *Tetradenia riparia* leaf essential oil and positive controls bifonazole and ketoconazole

Fungus	Essential oil (mg mL^{-1})	Bifonazole (mg mL^{-1})	Ketoconazole (mg mL^{-1})
	MIC MFC	MIC MFC	MIC MFC
Aspergillus fumigatus	5.00 ± 0.43^b 10.00 ± 2.58^B	0.15 ± 0.03^a 0.20 ± 0.01^A	0.20 ± 0.03^a $0.50 \pm < 0.01^A$
Aspergillus niger	2.50 ± 0.41^b 6.25 ± 0.43^B	0.10 ± 0.03^a 0.20 ± 0.06^A	0.20 ± 0.03^a $0.30 \pm < 0.01^A$
Aspergillus ochraceus	10.00 ± 1.58^b 20.00 ± 1.58^B	0.15 ± 0.01^a $0.20 \pm < 0.01^A$	0.15 ± 0.01^a 0.20 ± 0.03^A
Aspergillus versicolor	0.06 ± 0.003^a 0.30 ± 0.003^B	0.10 ± 0.03^a $0.20 \pm < 0.01^A$	0.20 ± 0.03^b 0.50 ± 0.01^C
Penicillium funiculosum	5.00 ± 0.43^b 10.00 ± 1.58^B	0.20 ± 0.03^a 0.25 ± 0.02^A	$0.20 \pm < 0.01^a$ 0.50 ± 0.01^A
Penicillium ochrochloron	$0.50 \pm < 0.01^a$ $0.60 \pm < 0.01^B$	$0.20 \pm < 0.01^a$ 0.25 ± 0.03^A	2.50 ± 0.33^b 3.50 ± 0.06^B
Penicillium verrucosum	1.25 ± 0.41^b 5.00 ± 4.53^A	0.15 ± 0.06^a $0.20 \pm < 0.01^A$	0.20 ± 0.06^a 0.50 ± 0.06^A
Trichoderma viride	$0.30 \pm < 0.01^b$ 1.25 ± 0.43^B	$0.15 \pm < 0.01^a$ 0.20 ± 0.01^A	1.00 ± 0.01^c 1.00 ± 0.02^B

Different lowercase and uppercase letters on the same line differ among themselves by Tukey HSD test ($p \leq 0.05$)

DISCUSSION

Tetradenia riparia is a shrub easily spread and well adapted to Brazilian soil and climate. There is no data in the literature on the development of this plant but from our personal experience it takes six months to reach the adult stage (producing flowers), which makes its cultivation viable for the production of essential oil. The essential oil is resinous with a low volatility rate and with low variation in concentration in applications. The use of *T. riparia* leaves to preserve food in silos is a traditional practice in Rwanda to control the attack of fungi and insects (Van Puyvelde *et al.*, 1986).

The essential oil yield (0.25%) of *T. riparia* leaves was close to the ones reported by Gazim *et al.* (2010), from 0.17% to 0.26%. For Gazim *et al.* (2010), the greatest yield was obtained in the winter, making the soil and climate effect evident on the production of *T. riparia* essential oil. According to the European Pharmacopoeia, the minimum yield for essential oil extraction is 2 mL kg^{-1} for application

development (Nemeth & Bernath, 2008). The essential oil yield in our study was 2.5 mL kg^{-1} (dry basis). This value is within the parameters established by the European Pharmacopoeia for the development of applications in products (European Pharmacopoeia, 2013).

Campbell *et al.* (1997) reported that the main compounds of the essential oil from *T. riparia* fresh leaves and stems cultivated in South Africa were α-terpineol (22.6%), fenchone (13.6%), fenchyl alcohol (10.7%), β-caryophyllene (7.9%), and perillyl alcohol (6.0%). Godoy *et al.* (1999) also evaluated the essential oil from *T. riparia* fresh leaves cultivated in Manaus, Amazonas, Brazil, and found the following major compounds: fenchone (19.9%), 14-hydroxy-9-*epi*-(*E*)-caryophyllene (12.3%), α-cadinol (5.2%), isocaryophyllene (3.9%), camphor (3.4%), and δ-cadinene (3.1%). Gazim *et al.* (2010) verified great chemical variability of the essential oil from *T. riparia* fresh leaves harvested in different seasons of the year. The samples were collected and divided into

three groups: winter, fall, and spring-summer. The samples harvested in the winter had greater percentages of calyculone (24.7%), abietadiene (13.5%), and viridiflorol (4.2%). In the fall, the main constituents were ledol (8.7%) and *cis*-muurolol-5-en-4-α-ol (13.8%). The samples collected in the spring-summer presented greater amounts of fenchone (12.7%), 14-hydroxy-9-*epi*-(*E*)-caryophyllene (24.4%), and α-cadinol (8.3%). Oxygenated sesquiterpenes were predominant in all analyzed samples. According to our results, the major compounds of *T. riparia* leaf essential oil were oxygenated sesquiterpenes 14-hydroxy-9-*epi*-(*E*)-caryophyllene (20.8%) and *τ*-cadinol (18.4%), followed by oxygenated diterpenes 6,7-dehydroroyleanone (12.6%) and 9*β*,13*β*-epoxy-7-abietene (10.6%); the oxygenated monoterpenes fenchone (5.6%) and sesquiterpene hydrocarbon *β*-caryophyllene (5.3%) were also present. The oxygenated diterpenes 6,7-dehydroroyleanone and 9*β*,13*β*-epoxy-7-abietene were not listed in the NIST 11.0 libraries, however, Gazim *et al.* (2014) reported the same type of extract from the same plant and identified molecular mass identical to those found in our study, by chromatography, for compounds 38 and 47 (Table No. 1). In addition, Gazim *et al.* (2014) isolated both compounds and identified by NMR (^{1}H, ^{13}C, DEPT, HSQC, HMBC, and NOESY) and high-resolution ESI-MS techniques and therefore those compounds were considered in our study as 6,7-dehydroroyleanone and 9*β*,13*β*-epoxy-7-abietene. Comparing the results obtained in our study to the ones reported in the literature, a similarity was found regarding the predominant classes such as oxygenated sesquiterpenes and diterpenes, but we observed differences regarding the major compounds. The variation of the main chemical compounds of the essential oil of this plant could be related to factors such as environmental aspects, genetic variability, growth conditions, and soil type (Glamočlija *et al.*, 2011; Linde *et al.,* 2016; Raimundo *et al.*, 2017; Cazella *et al.*, 2019; Tedesco *et al.*, 2020).

Antibacterial activity
The most sensitive bacteria to the essential oil were the Gram-positive bacteria *B. cereus* (0.05 mg mL^{-1}), *L. monocytogenes* (0.05 mg mL^{-1}), and *S. aureus* (0.05 mg mL^{-1}), whereas the most resistant ones were the Gram-negative bacterium *P. aeruginosa* (MIC of

0.60 mg mL^{-1} and MBC of 1.25 mg mL^{-1}) compared to the controls streptomycin and ampicillin. For streptomycin, there is no cut-off data for MIC values, but there is a correlation for resistance for MIC values ≥ 1000 µg mL^{-1} for broth microdilution (CLSI, 2005). For ampicillin, the cut-off points for MIC are 16 µg mL^{-1} intermediate and ≥ 32 µg mL^{-1} resistant (CLSI, 2005). Melo *et al.* (2015) verified that *T. riparia* essential oil presented promising antimicrobial activity against some cariogenic bacteria, including *Streptococcus mutans* with MIC and MBC of 0.062 mg mL^{-1}. Baldin *et al.* (2018) reported the antimicrobial activity of *T. riparia* essential oil (MIC of 0.06 mg mL^{-1}) and the isolate compound 6,7-dehydroroyleanone (MIC of 0.03 mg mL^{-1}) against *Mycobacterium tuberculosis* H$_{37}$Rv alcohol-acid resistant bacilli. Our results made evident that *T. riparia* leaf essential oil presented MIC of 0.05 mg mL^{-1} and the control ampicillin of 0.10 mg mL^{-1} against *S. aureus*, showing that the essential oil, a mixture of compounds, had bacteriostatic activity 2-fold higher than the ampicillin control.

Essential oils are considered to have high antimicrobial activity when MIC ranges from 0.05 to 0.50 mg mL^{-1}, moderate activity with MIC ranges from 0.6 to 1.50 mg mL^{-1}, low activity when MIC is over 1.50 mg mL^{-1} (Aligiannis *et al.*, 2001). In addition, essential oils whose MIC is lower than 0.1 mg mL^{-1} are considered very promising for the research of new antimicrobial agents (Gibbons, 2004; Rios & Recio, 2005). Thus, our results show that *T. riparia* leaf essential oil presents high antibacterial activity for most of the evaluated bacteria, except for the moderate activity for *P. aeruginosa* (MIC of 0.60 mg mL^{-1}), and with potential to be a natural source of antibacterial agents.

In our study, we identified that 95.2% of the essential oil constituents were terpenoids, and that 43.6% of them are oxygenated sesquiterpenes. The high antibacterial activity presented by *T. riparia* leaf essential oil can be associated to these compounds as most of the antimicrobial action mechanisms are related to hydrophobicity of monoterpenes and sesquiterpenes (Shaaban, 2020). Terpenoid hydrophobicity reduces the cell membrane permeability and causes collapse to the proton bomb and ATP depletion, resulting in membrane permeability and cellular death (Bakkali *et al.,* 2008).

Antifungal activity

In our study, *A. versicolor* was the most sensitive fungus to the essential oil, followed by *P. ochrochloron*, while the others were more resistant. The cut-off values for ketoconazole MIC range from 0.03 to 16 µg mL^{-1} (CLSI, 2002). In our study, 14-hydroxy-9-*epi*-(*E*)-caryophyllene was the major compound of *T. riparia* leaf essential oil. *Trans-β*-caryophyllene is a bicyclic hydrocarbon sesquiterpene classified as safe by the Research Institute for Fragrance Materials (RIFM) and by Food and Drug Administration (FDA) in the USA to use as an aromatic agent in cosmetics products and in food additives (Api *et al.,* 2018). Furthermore, *β*-caryophyllene from *Murraya paniculata* has antibacterial activity against *S. aureus*, *Salmonella typhimurium*, *E. coli*, and *Enterococcus faecalis* (MBC ranging from 2.0 to 4.0 mg mL^{-1}) and antifungal activity against *A. niger*, *A. fumigatus*, *Aspergillus parasiticum*, and *Fusarium solani* with MFC from 0.5 to 4.0 mg mL^{-1} (Selestino Neta *et al.,* 2017). Thus, the caryophyllene found in the essential oil from *T. riparia* leaves of our study is probably related to the antimicrobial activity.

Another major sesquiterpene in our study was τ-cadinol. Essien *et al.* (2018) reported in *Senna hirsuta* fruit essential oil, τ-cadinol (18.9%) as the major compound with antimicrobial activity (MIC of 0.625 mg mL^{-1}) against *S. aureus*, *E. coli*, *P. aeruginosa*, and *C. albicans.*

Oxygenated diterpenes were the second major class of the essential oil, mainly two diterpene-type abietanes: 6,7-dehydroroyleanone (12.6%) and 9*β*,13*β*-epoxy-7-abietene (10.6%). Diterpene-type abietanes are known to possess a broad range of biological activities, including antimicrobial, antiulcer, antioxidant, anti-inflammatory, cardiovascular, and cytotoxic activities, which have attracted a considerable interest of pharmaceutical and medical communities (Neto *et al.,* 2015). Antimicrobial activity of diterpenes can be associated the presence and the position of the functional groups (carboxyl, hydroxyl, aldehydes or ketones, among other groups) within the hydrocarbon chain and the ability of these functional groups to donate or receive hydrogen with the microbial target (Gigante *et al.*, 2002; Neto *et al.*, 2015).

The antimicrobial activity of diterpene-type abietanes such as 6,7-dehydroroyleanone, the main diterpene found in our study, was reported against *M. tuberculosis* (Baldin *et al.,* 2018) besides their antileishmanial (Demachi *et al.*, 2015), antioxidant and antitumoral (Gazim *et al.* 2014) activities. Melkani *et al.* (2016), when evaluating the essential oil from *Anisomeles indica* aerial parts, rich in abietadiene (20.5%), observed antimicrobial activity against *K. pneumoniae*, *Agrobacterium tumefaciens*, *S. aureus*, *Pasteurella multocida*, and *Aspergillus flavus*.

The oxygenated monoterpene fenchone (5.3%), one of the major compounds of *T. riparia* leaf essential oil, presents antimicrobial activity reported in the literature against Gram-positive bacteria such as *S. aureus, B. cereus, Bacillus megaterium, Bacillus subtilis, Sarcina lutea*, and *Streptococcus* sp., and Gram-negative ones such as *Salmonella typhi, Shigella dysenteriae, Shigella shiga, Shigella sonnei, Shigella boydii, E. coli, Klebsiella* sp., *P. aeruginosa*, and *Proteus* sp. (Kazemi *et al*., 2012).

Sivakumar & Bautista-Baños (2014) and Burt (2004), recommended that the use of essential oil content to preserve food must be from 0.1 to 6% (0.1 to 6 g 100 mL^{-1}). The antimicrobial concentrations of *T. riparia* leaf essential oil in our study ranged from 0.075 to 10 mg mL^{-1} (equivalent to 0.0075 to 1%, respectively) making this essential oil a promising alternative for microbial control in foods.

In conclusion, the essential oil of *T. riparia* leaves presents as major compounds oxygenated sesquiterpenes (43.6%): 14-hydroxy-9-*epi*-(*E*)-caryophyllene (20.8%) and τ-cadinol (18.4%), followed by oxygenated diterpenes (24.6%): 6,7-dehydroroyleanone (12.6%) and 9*β*,13*β*-epoxy-7-abietene (10.6%), hydrocarbon sesquiterpenes (17.1%), oxygenated monoterpenes (7.4%), and fenchone (5.6%). The essential oil had broad antibacterial and antifungal activity, mainly against *A. versicolor* and *P. ochrochloron* with fungistatic and fungicidal activities and *B. cereus*, *L. monocytogenes*, and *S. aureus* with bacteriostatic and bactericidal activities. *T. riparia* leaf essential oil is a potential alternative to control microorganisms.

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