Bioassay-guided isolation of antibacterial compounds from the leaves *of Tetradenia riparia* with potential bactericidal effects on food-borne pathogens

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Abstract

Ethnopharmacological relevance: *Tetradenia riparia* (commonly known as ginger bush) is frequently used in traditional African medicine to treat foodborne infections including diarrhoea, gastroenteritis, and stomach ache.

Aim of the study: The present study aims to identify in *Tetradenia riparia* the compounds active against foodborne pathogens.

Materials and Methods: Dried *Tetradenia riparia* leaf powder was consecutively extracted with hexane, ethyl acetate, methanol and water. The hexane extract was counter-extracted with methanol:water (9:1), and after evaporation of the methanol, this phase was extracted with dichloromethane. The water extract was counter-extracted with butanol. All these fractions were tested against a panel of foodborne bacterial pathogens. A bioassay-guided purification was performed to isolate antimicrobial compounds using *Staphylococcus aureus* as a target organism. Further, antibiofilm activity was evaluated on *S. aureus* USA 300.

Results: The dichloromethane fraction and ethyl acetate extract were the most potent, and therefore subjected to silica gel chromatography. From the dichloromethane fraction, one active compound was crystalized and identified using NMR as 8(14),15-sandaracopimaradiene-7alpha, 18-diol (compound 1). Two active compounds were isolated from the ethyl acetate extract: deacetylumuravumbolide (compound 2) and umuravumbolide (compound 3). Using a microdilution method, their antimicrobial activity was tested against eight foodborne bacterial pathogens: *Shigella sonnei*, *S. flexneri*, *Salmonella enterica* subsp. *enterica*, *Escherichia coli*, *Micrococcus luteus*, *S. aureus*, *Enterococcus faecalis*, and *Listeria innocua.* Compound 1 had the strongest activity (IC_{50} ranging from 11.2 – 212.5 µg/mL), and compounds 2 and 3 showed moderate activity (IC_{50} from 212.9 – 637.7 µg/mL and from 176.1- 521.4 µg/mL, respectively).

Interestingly, 8(14),15-sandaracopimaradiene-7alpha, 18-diol is bactericidal, and also showed good antibiofilm activity with BIC_{50} (8.8±1.5 µg/mL) slightly lower than for planktonic cells (11.4±2.8 µg/mL).

Conclusions: These results support the traditional use of this plant to conserve foodstuffs and to treat gastrointestinal ailments, and open perspectives for its use in the prevention and treatment of foodborne diseases.

Keywords: *Tetradenia riparia,* antimicrobial activity, bacterial foodborne pathogens, biofilm, 8(14),15-sandaracopimaradiene-7alpha 18-diol, deacetylumuravumbolide, umuravumbolide

1. Introduction

Foodborne pathogens are mainly bacteria, but also viruses or even parasites, that can be present in food, causing a range of diseases with major effects on human health and the economy (Green-Johnson 2006; Bintsis 2017). According to the U.S. Food and Drug Administration, foodborne illness is often caused by consuming food contaminated by bacteria and/or their toxins, parasites, viruses, chemicals, or other agents (FDA 2020). Over 200 diseases are caused by foodborne pathogens. Each year worldwide, unsafe food causes 600 million cases of foodborne disease, and 420,000 deaths. Over 30% of foodborne deaths occur in children under 5 years of age, Foodborne pathogens can cause severe diarrhoea or debilitating infections, including meningitis (WHO 2020). In the European Union (EU) for the year 2018, 26 member states reported 5,146 foodborne and waterborne outbreaks, 48,365 cases of illness, 4,588 outbreak-related hospitalizations and 40 deaths, *Salmonella* was the most commonly detected agent, with *S. enterica* causing one in five outbreaks. In the United States, foodborne infections trigger an estimated 76 million illnesses,

with 5,000 deaths each year (Mead et al., 1999). The top five foodborne pathogens are Norovirus, *Salmonella, Clostridium perfringens, Campylobacter* and *Staphylococcus aureus*. Moreover, some other foodborne germs do not cause illness frequently, but more likely lead to hospitalization, including *Clostridium botulinum, Listeria, E. coli*, and *Vibrio* (CDC 2020).

Antibiotics used for human treatment are increasingly prohibited for other applications such as food, agriculture or veterinary use, in part to decrease the development of resistance. Therefore, research on natural products could yield sustainable alternatives for chemically synthesized antimicrobials (Panda et al., 2019). Resistant foodborne pathogens represent one of the most important public health problems related to the emergence of antibacterial resistance in the food supply chain. Indeed, several foodborne pathogens developed a tolerance or resistance to different antibiotics (Olsen et al., 2004; Hummel, Holzapfel, et Franz 2007; Alfredson et Korolik 2007; Werner et al., 2013). This can result in treatment failure, increased mortality as well as treatment costs, reduced infection control efficiency, and spread of resistant pathogens from hospitals to the community (Hashempour-Baltork et al., 2019). Therefore, many research projects try to find new alternative approaches to control and prevent this problem. Plant extracts have long been considered as a natural source of antimicrobial agents, that may be nutritionally safe and easily degradable. Many potential antibacterial agents against foodborne pathogens have been purified from plants (Ma et al., 2018; Pereira et al., 2008; Bajpai, et al., 2017a; Bajpai, et al 2017b). Natural preservatives such as herbal extracts and essential oils, as well as their components, are used increasingly as alternatives for inhibiting pathogenic and spoilage microorganisms (Schirone et al., 2019).

Tetradenia (*T.*) *riparia* (Hochst.) Codd (Lamiaceae), is an African medicinal herb, widely distributed throughout Eastern and tropical Africa (Gairola et al., 2009). This plant is well known

for its medicinal properties against a number of infectious diseases (malaria, yaws, gastroenteritis, gonorrhoea, dental abscesses), chest pain (angina), several kinds of fevers and aches, and for treating stomach-related ailments (Van Puyvelde et al., 2018; Van Puyvelde et al., 1987; Van Wyk and Wink, 2004). Interestingly, the leaves are used as a spice in foods, for the conservation of food products in traditional silos, as well as for dry storage of crops, mostly to repel insects (Van Puyvelde, et al 1975; Xaba 2009).

Therefore, we used bioassay-guided purification to identify compounds from this plant which could be used against foodborne pathogens and help to reduce the emergence of drug resistance.

2. Material and Methods

2.1. Plant material:

"T. riparia plant was harvested in Mukoni, Huye, Rwanda and identified by an expert botanist (Vedaste Minani). A voucher specimen (No. 86) was deposited in the National Herbarium at the National Industrial Research and Development Agency (NIRDA), Huye, Rwanda" (Van Puyvelde et al., 2018). The leaves were air-dried, ground to a powder using a mechanical grinder, and stored in a cold room (4 °C) till use.

2.2. Extraction of plant and isolation of active compounds:

Briefly, powdered air-dried leaves (10 g) were successively extracted (for 5 x 30 min each) with n-hexane, ethyl acetate, methanol and water (each solvent: 5 x 150 mL) with the help of sonication, and the extracts were filtered (Whatman filter paper). The ethyl acetate and methanol extracts were dried by rotary evaporation, yielding 460 and 600 mg dry residue, respectively. The filtered hexane extract was counter-extracted (5 x) (liquid-liquid) with methanol-water (9:1). The hexane phase was dried, yielding 390 mg residue. The methanol was removed from the aqueous methanolic

phase by rotary evaporation. The remaining aqueous phase was extracted (5 x) (liquid-liquid) with dichloromethane, which gave after evaporation a brown-yellow residue (490 mg). The water extract was extracted (3 x) with butanol, and after evaporation of the solvents yielded 100 and 900 mg, respectively for the butanol and aqueous phase (for a schematic representation of the extraction procedure, see supplementary material S1). The activity of the six dried residues was tested against a panel of bacteria. Only the dichloromethane fraction and the ethyl acetate extract showed significant antimicrobial activity.

The active dichloromethane extract was adsorbed on silica gel (20 g) and fractionated by column chromatography (h: 50 cm- Ø 5 cm) on silica gel (300 g- 230-400 mesh) by elution with a hexane-ethyl acetate-methanol, step-gradient (20 mL/min; 100 mL per solvent step; fractions of 20 mL). Fractions were combined after TLC analysis (using a hexane-ethyl acetate mobile phase) into 10 pools of similar composition. Fractions eluted with hexane-ethyl acetate (10:90), and hexane-ethyl acetate (30:70), yielded one active compound after crystallization in hexane (compound 1).

The active ethyl acetate fraction was also fractionated on silica gel as described above. Fractions with similar band profiles were combined after TLC analysis (using a hexane-ethyl acetate mobile phase) into 9 pools. Two pools from fractions eluted with hexane-ethyl acetate (70:30) gave after crystallization in hexane one active compound each (compounds 2 and 3).

2.3. Bioassay

2.3.1 Bacterial strains

The bacterial strains used in this study were: *Shigella sonnei* (LMG 10473), *Shigella flexneri* LMG 10472, *Salmonella enterica* subsp. *enterica* (ATCC13076), *Escherichia coli* (ATCC47076) (all Gram-negative), as well as *Micrococcus luteus* (DPMB3), *Staphylococcus aureus* (ATCC 65385),

Enterococcus faecalis (HC-1909-5), *Listeria innocua* (LMG 11387) (all Gram-positive). For the biofilm assay, *S. aureus* USA 300 was used.

2.3.2 Antibacterial activity against planktonic cells

Antibacterial activity was assessed as described previously (Panda et al., 2017) using a microdilution broth protocol. The purified compounds were dissolved in DMSO (Chem-Lab, Cell Biology grade) to a final stock concentration of 10 mg/mL. Ten µL of the test sample was transferred into the wells of a multiwall-96 test plate, as well as the positive control (ciprofloxacin, stock 200 µg/mL) and blank (solvent) controls (5% final concentration DMSO). The wells of the microdilution plate were then inoculated with 190 µL of a diluted standardized inoculum in Luria-Bertani broth for all test bacteria (OD = 0.003 at 620 nm), except *E. faecalis*, *L. innocua* and *S. enterica* (OD = 0.01 at 620 nm, Tryptic Soy Broth, TSB). Control wells were prepared with 190 µL sterile broth plus 10 µL extract, to correct for any absorption due to extract components. The microdilution plates were placed in a shaker-incubator at 37°C for 24 h and then read on a Multiskan FC microplate photometer. The OD was measured at a wavelength of 620 nm, and wells with a plant extract were corrected for the absorption contributed by the extract. Throughout the experiments, 5% DMSO was used as the solvent control, and none of the test strain showed any notable inhibition when tested at this DMSO concentration. All experiments were repeated in duplicate. The relative inhibition (%) of the test sample was calculated as 100% - $\{[(OD_{\text{test saample}}-OD_{\text{extract control}})x100\%]/OD_{\text{solvent control}}\}$ Data from dose-response experiments were represented as the percentage of inhibition and analysed with Prism™ (GraphPad Prism 5.0 Software Inc., San Diego, CA). The IC_{50} for each growth condition was calculated by fitting the data to a non-linear least-squares sigmoid regression curve, keeping the minimum and maximum fixed at 0 and 100%, respectively (Jouneghani et al., 2020).

2.3.3 Antibiofilm test on *S. aureus* USA 300

Antibiofilm activity was assessed with a broth microdilution method as described earlier (Panda et al., 2020). An overnight *S. aureus* culture in TSB was used as inoculum after adjusting to OD= 0.1 (10^6 cells), and 100 µL of cell suspension was aliquoted into 96-well flat-bottom polystyrene plates (Costar, USA). Plates were incubated for 90 min at 37 °C in a stationary incubator to permit cell adhesion. The medium was then aspirated carefully without disturbing the cells at the bottom of the well, and wells were gently rinsed with phosphate-buffered saline (PBS). Fresh TSB (190 µL) was added to the wells, and gently mixed with 10 µL of each test compound prepared from a stock solution as a dilution series ranging from 10 mg/mL to 0.01 mg/mL. The plates were then incubated at 37 °C in a stationary incubator for 24 hours, followed by carefully removal of the TSB broth, and subsequent washing twice with PBS. Each well was then stained with 100 µL resazurin dye (0.4% v/v, Acros Organics, Belgium), followed by a 1-hour incubation in the dark at 37 °C. Fluorescence was measured with a Flexstation II spectrofluorometer; with λex at 535 nm and λem at 590 nm. The percentage of surviving biofilm cells was calculated relative to the growth controls. Control wells were filled with 10 µL of DMSO (solvent controls), or antibiotic ciprofloxacin (200 µg/mL) in DMSO (positive control).(Kipanga et al., 2020). All experiments were repeated in duplicate.

2.3.4 Determination of minimum inhibitory concentration-50 (IC_{50}), biofilm inhibitory concentration-50 (BIC_{50}), and minimum bactericidal concentration (MBC)

Two-fold serial dilutions of test compounds (ranging from 10 mg/mL to 0.01 mg/mL) were prepared in DMSO and tested against biofilms and planktonic cells. The BIC_{50} (minimum concentration required to inhibit 50% of growing biofilms), and IC_{50} (minimum inhibitory concentration required to inhibit 50% of growing planktonic cells) were then estimated by non-

linear regression using Prism. (GraphPad Prism 6.0 Software Inc., San Diego, CA, USA). The MBC was determined for the treated cells by plating aliquots (~5 µL) from all test concentration wells on TSA using a replica plater for 96-well plates (Sigma-Aldrich). The TSA plates were then incubated overnight at 37 °C, and growth was then determined and compared with controls, where a 10-fold serial dilution was carried out (supplementary material S2).

2.4. NMR analysis:

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance II+ 600 spectrometer (Bruker, Fallanden, Switzerland) (working at 150 MHz). Deuterated chloroform was used as internal standard (CDCl3 77.16 ppm, triplet), and the chemical shifts are expressed in δ scale (ppm) (Van Puyvelde et al., 2018)

3. Results

3.1. Antimicrobial activity against foodborne bacteria:

Six different dried residues were tested against bacterial foodborne pathogens (Table 1). The dichloromethane fraction and ethyl acetate extract had the broadest activity, inhibiting both Gram-positive and Gram-negative bacteria when tested at a final concentration of 250 µg/mL (stock 5 mg/mL in DMSO).

Table 1: Antibacterial activity of different crude extracts (growth inhibition in % compared to the solvent, OD at 620 nm).

Dried residues	Foodborne bacteria					
	S. aureus	*M. luteus*	*E. faecalis*	*E. coli*	*S. enterica*	*S. sonnei*
Hexane fraction	54	31	29	4	23	36
Dichloromethane fraction	52	44	26	15	51	26
Ethyl acetate extract	88	89	23	29	52	41
Methanol extract	11	38	43	9	34	23

Butanol fraction	53	37	47	1	40	1
Water fraction	–	–	–	–	–	–
Ciprofloxacin (50 µg/mL)	98	97	–	92	99	100

Percentage growth inhibition of various bacteria by *Tetradenia* extracts in different solvents. "–" No activity, activity above 50% inhibition is underlined.

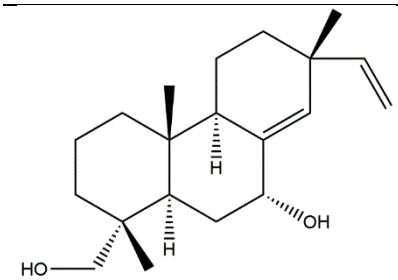
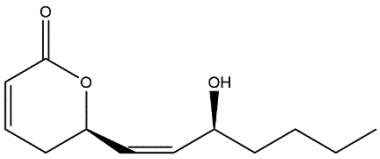
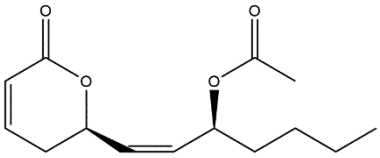
Consequently, these were further fractionated on silica gel. Those fractions were analysed by TLC, similar fractions were pooled, and tested for antibacterial activity. From each of three active pools, a single compound was crystallised.

3.2. NMR identification:

The three purified active compounds obtained after fractionation and crystallization were analysed by NMR for identification (Table 2).

Table 2: ^{1}H and ^{13}C NMR spectral data

Compound identification	^{1}H NMR (600 MHz, $CDCl_3$): δ	^{13}C NMR (101 MHz, $CDCl_3$) δ	References
Compound 1 : 8(14),15-sandaracopimaradiene-7alpha, 18-diol (diterpenediol)	5.77 (dd, 1H) 5.47 (s, 1H), 4.92 (m, 2H), 4.16 (s, 1H), 3.54 (brd, 1H), 3.49 (d, 1H), 2.87 (d, 1H), 2.38 (brd, 1H), 2.14 (m, 1H), 1.84 (dd,1H), 1.66 (m, 5H), 1.48 (m, 5H), 1.37 (m, 1H), 1.25 (m, 3H), 1.19 (d, 1H), 1.07 (dt, 1H),	148.55, 139.48, 134.13, 110.72, 73.39, 70.66, 46.37, 39.40, 38.75, 38.35, 37.76, 37.58, 35.11, 34.34, 31.74, 28.54, 25.84, 22.80, 18.45, 18.42, 18.29, 14.94, 14.26	(De Kimpe et al., 1982; Van Puyvelde et al., 2018)

	1.03 (s, 3H), 0.87 (t, 2H), 0.79 (s, 3H), 0.72 (s, 3H)			
Compound 2: deacethylumuravumbolide (alpha-pyrone) 	6.89 (m, 1H), 6.05 (m, 1H), 5.66 (m, 2H), 5.33 (m, 1H), 4.42 (m, 1H), 2.43 (m, 1H), 2.36 (m, 1H), 1.84 (d, 1H), 1.61 (m, 1H), 1.45 (m, 1H), 1.34 (m, 3H), 1.26 (1H), 0.90 (t, 3H)	163.84, 144.80, 138.03, 127.74, 121.73, 73.81, 68.01, 36.92, 30.05, 27.61, 22.75, 14.14		(Shekhar et al., 2011)
Compound 3: umuravumbolide (alpha-pyrone) 	6.86 (m, 1H), 6.05 (m, 1H), 5.72 (m, 1H), 5.53 (m, 1H), 5.41 (m, 2H), 2.45 (m, 1H), 2.28 (m, 1H), 2.02 (s, 3H), 1.67 (m, 1H), 1.51 (m, 1H), 1.28 (m, 4H), 0.89 (t, 3H)	170.35, 163.65, 144.36, 131.85, 130.24, 121.85, 74.21, 69.59, 34.43, 30.19, 27.37, 22.61, 21.29, 14.06		(Shekhar et al., 2011)

The three compounds (1: 8(14),15-sandaracopimaradiene-7alpha, 18-diol (diterpenediol), 2: deacetylumuravumbolide (alpha-pyrone), and 3: umuravumbolide (alpha-pyrone) (Table 2) were identified by comparison with NMR spectra reported earlier (De Kimpe et al., 1982; Shekhar et al., 2011; Van Puyvelde et al., 2018). For details on chemical shifts of the NMR spectra, see Table 2.

3.3. Antimicrobial activity against foodborne pathogens

The activity of the three purified compounds was tested at a concentration of 500 µg/mL against a panel of foodborne pathogens. Compound 1 has a broad spectrum, inhibiting both Gram-positive and Gram-negative pathogens, while compound 2 and 3 are active only against Gram-positives (Table 3).

Table 3:| Antibacterial activity of three purified compounds against foodborne pathogens (growth inhibition in % compared to the solvent, OD at 620 nm).

Strains	Compound 1	Compound 2	Compound3	Ciprofloxacin
Gram-negative				
Escherichia coli	77	51	14	90
Salmonella enterica	55	64	25	99
Shigella flexneri	58	56	46	100
Shigella sonnei	94	63	17	100
Gram-positive				
Enterococcus faecalis	100	40	66	−
Listeria innocua	100	40	58	100
Micrococcus luteus	100	72	92	100
Staphylococcus aureus	100	57	87	100

Compound 1: 8(14),15-sandaracopimaradiene-7alpha, 18-diol.

Compound 2: deacetylumuravumbolide.

Compound 3: umuravumbolide.

(−) No activity.

Percentage growth inhibition of various foodborne pathogens. Inhibition by >50% is underlined.

Compounds that showed more than 50% inhibition were further tested by 2-fold serial dilution to evaluate their IC_{50} (Table 4).

Table 4: IC_{50} (µg/mL) against foodborne bacterial pathogens of three purified compounds

Strains	Compound 1	Compound 2	Compound 3
Gram-negative			
Escherichia coli	209	530	ND
Shigella flexneri	212	465	253
Shigella sonnei	210	ND	ND
Gram-positive			
Enterococcus faecalis	16	ND	252
Listeria innocua	21	ND	521
Micrococcus luteus	16	213	176
Staphylococcus aureus	11	638	252

ND- Not determined

The lowest IC_{50} value range (11.4-21 µg/mL) was observed for compound 1 against Gram-positives such as *M. luteus*, *S. aureus*, *E. faecalis* and *L. innocua*; while moderate activity was observed (IC_{50} value range 208.8-212.5 µg/mL) against Gram-negatives such as *S. flexneri, S. sonnei* and *E. coli*. Compound 2 has lower activity compared to compound 1 (Table 3), e.g. against Gram-positive bacterium *M. luteus* (MIC_{50} = 212.9 µg/mL), while weak activity was noted against *S. aureus* (MIC_{50} = 637.7 µg/mL). Compound 3 has potency intermediate between compounds 1 and 2, e.g. against Gram-positives such as *M. luteus* and *S. aureus,* with IC_{50} values of 176.1 and 251.5 µg/mL, respectively (Table 4). Interestingly, compound 1 can inhibit biofilm formation with BIC_{50} = 8.8 µg/mL (Table 4). i.e., lower than its IC_{50} for planktonic growth (Figure 1 a and b). Moreover, compound 1 is bactericidal with a minimum bactericidal concentration (MBC) of 31.25 µg/mL (Table 5).

Table 5: BIC_{50} (µg/mL) and MBC of three purified compounds

	Compound 1	Compound 2	Compound 3

BIC_{50} (µg/mL)	8.8±1.5	>500	>500
Survival % w.r.t. C.F.U., MBC	0.0001%, 31.25	100%, >500	100%, >500

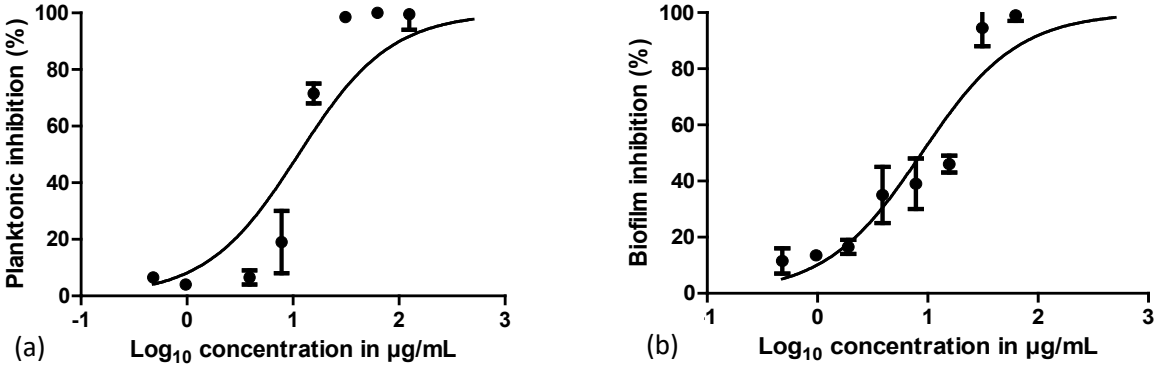


Figure 1. Dose-response experiments are represented as the percent of growth inhibition, and analysed with GraphPad Prism 6 software (San Diego, CA, USA). A non-linear regression of log(inhibitor) vs. response, with bottom constrained to 0 and top constrained to 100, and Hill slope set equal to 1, was used to estimate the IC_{50}. (a) Planktonic growth inhibition for *S. aureus* Rosenbach, (b) Biofilm growth inhibition for *S aureus* USA 300.

4. Discussion

T. riparia is well known for its medicinal properties (Demarchi et al., 2015; Cardoso et al., 2015; Campbell et al., 1997; Melo et al., 2015). It has good anti-parasitic as well as antispasmodic and antimicrobial activity (Van Puyvelde et al., 1987), strong acaricidal activity against *Rhipicephalus* (*Boophilus*) *microplus* (Gazim et al., 2011), anthelmintic activity (Van Puyvelde et al., 2018), antidermatophytic activity (of leaf extract; Endo et al., 2015), anti-mycobacterium activity (Baldin et al., 2018), as well as anti-inflammatory and wound-healing properties (Ghuman et al., 2019). Also, its essential oil has antifungal effects against postharvest plant pathogenic fungi (Hannweg

et al., 2016). It is moreover used for flavouring food and drinks, as well as for use in perfumes and cosmetics (Khuzwayo, 2011).

Phytochemical studies of *T. riparia* reported the isolation of the diterpenoid ibozol (7a-hydroxyroyleanone) and sitosterol (Zelnik et al., 1978), 8 (14), 15- sandaracopimaradiene-7α,18-diol (Van Puyvelde et al., 1987), deacetylumuravumbolide, deacetylboronolide and umuravumbolide (5,6-dihydro-6-(3-acetoxy-1-heptenyl)-2-pyrone) (Van Puyvelde et al., 1979; Davies-Coleman et Rivett 1995; Van Puyvelde et Kimpe 1998), 1',2'-dideacetylboronolide (Van Puyvelde et al., 1981), etc.

Most studies on antimicrobial properties used the essential oil, whose composition differs greatly from that of solvent extracts. Gazim et al., (2010), reported the antimicrobial activity against the yeast *Candida albicans* and a panel of pathogenic bacteria such as *S. aureus*, *Bacillus subtilis*, and several Gram-negatives viz. *E. coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. According to these authors, the essential oil is more effective against Gram-positive bacteria compared to the tested Gram-negatives. These authors could not establish the active constituents responsible for the antimicrobial effect, but proposed that the activity is due to monoterpenes, diterpenes and sesquiterpenes. Later, Melo et al., (2015), established the effect of the essential oil against oral pathogens. Their conclusions are similar to the observation of Gazim et al., (2010), and they presume that the antimicrobial activity is due to presence of the monoterpene fenchone, the diterpene calyculone, and several sesquiterpenes (14-hydroxy-9-*epi*-caryophyllene, *cis*-muurolol-5-en-4-α-ol, and α-cadinol), as well as the presence of a new compound: (*E,E*)-farnesol, as one of the major constituents (Melo et al., 2015). The essential oil is particularly rich in terpenes; the main components are: α-terpineol (22.6%), fenchone (13.6%), β-fenchyl alcohol (10.7%), β-caryophyllene (7.9%) and perillyl alcohol (6.0%) (Campbell et al., 1997); other researchers found

that 14-hydroxy-9-epi-(E)-caryophyllene is the main component (16.48%) (Araújo et al., 2018). However, the major constituents vary between publications, and depend on seasonal variability, geographic location (Gazim et al.,, 2010; Melo et al.,, 2015; Baldin et al.,, 2018) and shading (Araújo et al., 2018). Although there are several reports on the antimicrobial activity of *T. riparia*, most deal with essential oils, while the leaves are most extensively used in traditional practice.

In a previous study, 8(14),15-sandaracopimaradiene-7e,18-diol, a compound purified from a *T. riparia* leaf extract, showed fairly strong activity against several Gram-positive bacteria such as *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis, Streptococcus pyogenes* (MIC 6.25-12.5 µg/mL), as well as *Mycobacterium smegmatis*, (6.25 µg/mL) (Van Puyvelde et al., 1986). Later, Van Puyvelde et al., (1994) demonstrated that *T. riparia* extracts show activity against a wide range of mycobacteria, and that this was due to the presence of 8(14),15-sandaracopimaradiene,7α,18-diol (MIC ranging from 25 to 100 µg/mL). Recently, Baldin et al. (2018) studied the *in vitro* anti-*M. tuberculosis* and cytotoxic activity of essential oil, prepared from *T. riparia* leaves, as well as one of its most abundant (12.5%) components (6,7-dehydroroyleanone). Interestingly, the MIC values obtained with the diterpene 6,7-dehydroroyleanone (MIC 31.25 µg/ml) were similar to those of 8(14),15-sandaracopimaradiene,7α,18-diol. Baldin et al., (2018), also studied the activity profile of 6,7-dehydroroyleanone against other pathogenic bacteria, and noted strong activity against Gram-positive bacteria, but low or no activity against Gram-negative bacteria. Njau et al. (2014) studied the antimicrobial activity of *T. riparia* leaves from Tanzania and found significant inhibitory effects against *E. coli*, *E. faecalis* and *S. aureus*. They conclude that "comprehensive research may lead into isolation and purification of the active ingredients that will be useful for the development of pharmaceutical as a therapy against diseases caused by the three investigated bacteria".

Recently, Endo et al., (2018) demonstrated the anti-methicillin-resistant *Staphylococcus aureus* (MRSA) potential of *T. riparia* in both planktonic and biofilm forms (Endo et al., 2018). Fernandez et al., (2017) studied both antimicrobial and antioxidant activities of *T. ripari*a leaves from Brazil. Fraction I (dichloromethane:hexane, 9:1) was the most active on several test pathogens, including *S. aureus,* and the active compound was identified as abieta-7,9 (11)-dien-13-b-ol, an abietane-type diterpene. From the somewhat less active fraction II (dichloromethane:ethyl acetate, 1:1) they isolated ibozol, a diterpenoid. Fraction III (ethyl acetate) also has antimicrobial properties and contains a mixture of two isomers: 8 (14), 15-sandaracopimaradiene-2a, 18-diol and 8 (14), 15-sandaracopimaradiene-7a, 18-diol. Fraction IV (ethyl acetate-methanol: 8:2) presented the highest antioxidant activity in terms of total phenol content, and led to the identification of the flavonoids astragalin and luteolin, as well as the α-pyrone boronolide (Fernandez et al., 2017).

Using bioassay-guided purification, the present study led to the isolation of three antimicrobial compounds: 8(14),15-sandaracopimaradiene-7alpha,18-diol, deacetylumuravumbolide and umuravumbolide, whose IC_{50} values against a panel of foodborne pathogens range from 11.2 to 212.5 µg/mL, 212.9 to 637.7 µg/mL and 176.1 to 521.4 µg/mL, respectively. Although these IC_{50} values are low for compound 1 and modest for compounds 2 and 3, these three compounds appear to be the major contributors to the antibacterial effect of our *T. riparia* extract. Moreover, they are in the same range as for commercially used antimicrobial food preservatives (Hintz et al., 2015). It is not clear why Fernandez et al. (2017) identified largely different antibacterial compounds from their *T. riparia* extract. They used another extraction method and solvent ("dynamic maceration process with solvent renovation using 70% (v/v) ethyl alcohol"). Also, the solvents used on the silica gel column show some differences (they used dichloromethane in addition to the solvents that we used, and collected different fractions than we did). Perhaps more importantly,

their plant was grown in Brazil, and since the *T. riparia* essential oil composition can vary considerably depending on geographic location (Gazim et al., 2010), the other phytocomponents likely do so as well. It is difficult to compare our antimicrobial activity with that of Fernandez et al., since we used IC_{50} values and they used MIC values. Moreover, their antimicrobial tests were often carried out with mixtures rather than pure compounds. Finally, the bacteria they used differ considerably from ours. In any case, we report for the first time the activity of *T. riparia* extracts against a wide range of foodborne pathogens and identify the main compounds responsible for this activity. However, we cannot exclude the presence of other antibacterial compounds of low potency and/or abundance in our extracts, which could even exhibit synergistic effects.

Diterpenes are well known for their antimicrobial activity, which is thought to depend on the ability of functional groups (carboxyl, hydroxyl, aldehydes or ketones, among other groups) to donate or receive hydrogen as a target in the microorganism (Gigante et al., 2002; Fernandez et al., 2017). Alpha pyrones are a common pharmacophore in many naturally occurring and bioactive synthetic compounds. They are a promising class of bio-renewable platform chemicals with diverse biological activities, including antimicrobial ones (Bhat et al., 2017).

The potent antimicrobial activity of 8(14),15-sandaracopimaradiene-7e,18-diol against foodborne pathogens may explain the use of *T. riparia* by the native Rwandese to conserve foodstuffs in their traditional silos (Van Puyvelde et al., 1975), as well as its use in treating stomach-related ailments in South African traditional medicine (Van Wyk and Wink, 2004). The plant is used there for the treatment of stomach ache, mouth ulcers and toothaches (Khuzwayo, 2011). This compound also showed antibiofilm activity, with an BIC_{50} that is even slightly lower than its IC_{50} for planktonic cells. This is quite unusual, since biofilms are typically much less sensitive to antimicrobials than planktonic cells. It is also cidal, which is preferable for food pathogens since bacteriostatic

compounds may permit microbial growth to continue slowly, or even resume, upon prolonged storage Therefore, our findings scientifically validate the use of this plant for food preservation and foodborne illness. Extracts of the plant may prove useful for incorporation into food packaging (Friedrich et al., 2020).

5. Conclusion

In conclusion, crude extracts of *T. riparia* leaves from Rwanda showed antimicrobial activity against a wide range of foodborne pathogens, due essentially to the presence of 8(14),15-sandaracopimaradiene-7e,18-diol. This supports the traditional use of this plant to conserve foodstuffs and to treat stomach-related ailments and opens perspectives for its use in combating foodborne illnesses.

6. Abbreviations

ATCC, American Type Culture Collection, Manassas, Virginia, USA; BIC, Biofilm inhibitory concentration; DMSO, Dimethyl sulfoxide; IC, Inhibitory concentration; LB, Luria-Bertani medium, MBC, Minimum bactericidal concentration; NMR, Nuclear magnetic resonance; OD, Optical density, TLC, Thin layer chromatography

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10. Author contribution

Study conception and design: LVP, SKP, WL

Acquisition of data: LVP, AA, WDB

Analysis and interpretation of data: LVP, AA, MJM, WDB, WL

Drafting of manuscript: LVP, AA, SKP

Critical revision: SKP, WL

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