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Chemical characterization of an aqueous extract and the essential oil of *Tithonia diversifolia* and their biocontrol activity against seed-borne pathogens of rice

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5 Abstract

6 The high cost of chemical pesticides and their negative impact on the environment prompted
7 the search for natural pesticides from plants. The objective of our study was to control rice
8 seed pathogenic fungi and bacteria using aqueous extract and essential oil from *Tithonia*
9 *diversifolia* leaves. We obtained aqueous extract and essential oil, respectively, by maceration
10 and hydrodistillation; the antimicrobial activities were determined *in vitro* on a solid medium
11 by the food poisoning method. The secondary metabolites were determined by qualitative and
12 quantitative assays; the chemical composition of the essential oil obtained from *Tithonia*
13 *diversifolia* was studied using gas chromatography coupled with mass spectrometry. The
14 results showed that phenols, tannins, flavonoids, alkaloids, terpenoids, sugars and saponins
15 were present in the aqueous extract. The essential oil contained mainly hydrocarbonated,
16 oxygenated monoterpenes, terpenoids and sesquiterpenes. α -terpineol (20.3%), eucalyptol
17 (14.6%), camphor (14.3%) and α -pinene (13.5%) as the main compounds. Regarding the
18 antimicrobial activity, all tested bacteria were sensitive to aqueous extract and essential oil.
19 The activity of the aqueous extract on the tested fungi showed an inhibitory concentration 50
20 (IC₅₀) of 50 mg/mL against *Bipolaris oryzae* and *Fusarium moniliforme*. The activity of the
21 essential oil on bacteria and fungi showed MIC of 125 μ g/mL (*Xanthomonas oryzae* pv.
22 *oryzae* and *Pseudomonas fuscovaginae*) and MFC of 5,000 μ g/mL (*Bipolaris oryzae* and
23 *Fusarium moniliforme*). These results allow us to consider *Tithonia diversifolia* as a potential
24 source of natural biopesticides against rice seed-borne pathogens.

25 **Keywords:** *Tithonia diversifolia*, Seed-borne pathogens, Biopesticides, Secondary
26 metabolites.

27 Declarations

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33 Cameroon.

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35 **Authors' contributions.** Laboratory experiments were carried out by Dongmo Nanfack
36 Albert, Emilio Stefani, and Nguefack Julienne. The essential oil was characterized by and
37 Fouelefack Romain François and Nkengfack Augustin Ephrem. The manuscript was written
38 by Emilio Stefani, Azah Udom Rene, and Dongmo Lekagne Blaise Joseph.

39 Introduction

40 The rice demand in Cameroon has more than doubled over the last decade; milled rice
41 imports rose from 469,450 to 728,433 tons, while the paddy yield fell from 2.74 to 1.33
42 tons/ha from 2009 to 2017, respectively. (FAOSTAT 2019). Yield reductions are mainly due
43 to the increasing impact of pests and diseases and their limited control, especially in the case
44 of seed-borne pathogens (Oerke 2006).

45 Disease surveys of rice grown in Cameroon revealed the existence of brown spot (*Bipolaris*
46 *oryzae*) and bakanae disease (*Fusarium moniliforme*), which can respectively lead to a yield
47 reduction of about 67% and 20% (Barnwal et al. 2013; Nguefack et al. 2013). Bacterial leaf
48 blight (*Xanthomonas oryzae* pv. *oryzae*) is present in Cameroon, as in several rice-growing
49 areas worldwide, and can lead to a yield loss of 30-35% (Jones et al. 1993; Sere et al. 2005)
50 or even rise to 50% or more, depending on variety, growth stage, and climatic conditions
51 (Kala et al. 2015). *Pseudomonas fuscovaginae*, the causal agent of sheath brown rot, although
52 not yet officially reported in Cameroon is an emerging threat for rice cultivation (CABI
53 2019). Like *X. oryzae* pv. *oryzae*, *P. fuscovaginae* is a seed-borne and seed-transmitted
54 pathogen of rice, and contribute to the reduction of the photosynthetic capacity of plants
55 (Lamichhane et al. 2015; Słomnicka et al. 2018), thus causing severe yield losses, estimated
56 from 30% to 60%, depending on the species susceptibility (Olczak-Woltman et al. 2008).

57 In Cameroon, as in other developing countries, synthetic pesticides used in plant disease
58 management are frequently unavailable, expensive for poor farmers, and often have negative
59 effects on the ecosystems, including their action on untargeted organisms and the
60 development of pathogen resistance (Wasim 2009). Nowadays, the development and
61 implementation of innovative and sustainable pest management strategies, based on the use
62 of plant extracts as an alternative to synthetic agrochemicals, is becoming more and more
63 explored. Plant extracts are important sources of new agrochemicals with satisfying

antimicrobial properties for the control of plant diseases (Fouelefack et al. 2018; Mekam et al. 2019). Plant extracts are usually broad-spectrum antimicrobials, eco-friendly and with minor effects as environmental pollutants; sometimes they are beneficial to soil organisms (Sharma et al. 2015).

Tithonia diversifolia (Hemsl.) A. Gray (*T. diversifolia*) is a pan-tropical plant species belonging to the *Asteraceae* family; it is commonly known as Mexican sunflower and is traditionally used for medicinal purposes in tropical and sub-tropical regions. In traditional agricultural systems, *T. diversifolia* is used by farmers as biofertilizer for soil amendment (Kaho et al. 2009; Nguefack et al. 2020). Linthoingambi et al. (2013) reported an excellent antimicrobial activity of *T. diversifolia* extracts against several phytopathogenic fungi. This work aims to describe the biochemical characteristics and evaluate the activity of the aqueous extracts and the essential oil from *T. diversifolia* against the most challenging seed-borne fungi and bacteria (*B. oryzae*, *F. moniliforme*, *X. oryzae* pv. *oryzae* and *P. fuscovaginae*) that dramatically reduce rice production in Cameroon.

Materials and methods

Plant material and media

Plant material consisted of leaves of *T. diversifolia* (Hemsl.) A. Gray (*Asteraceae*) harvested in June 2018, in Cameroon, in the council of Yaoundé 3, and identified at the Cameroon National Herbarium by comparison to official samples of the botanical species from the herbarium collection number 57410 HNC. Plants were grown until the flowering stage, harvested and shade dried for two weeks. Dried leaves were then milled into a powder, which was stored in small bags at room temperature until use.

Culture media (Potato dextrose agar, Nutrient sucrose agar, and the reference antibiotic (gentamycin) for biocontrol activities were purchased from Sigma-Aldrich (Milan, Italy).

88 Deionized water was obtained from a Milli-Q System (Bedford, MA, USA). Reference
89 fungicide Banko plus[®] was purchase from ADER, Douala, Cameroon.

90 **Preparation of aqueous extract and essential oil from *T. diversifolia***

91 The aqueous plant extract was obtained by maceration in distilled water. One hundred grams
92 (100 g) of powder of *T. diversifolia* leaves were weighed and macerated into 600 mL (1:6,
93 w/v) of distilled water under a magnetic stirrer at 120 rpm for 24 h, at a temperature of 25 °C.
94 After filtration through a Whatman No. 1 paper, the filtrate was centrifuged at 5,000 rpm for
95 5 min and the supernatant was collected and dried in an oven at 48 ± 2 °C overnight. The
96 extraction yield was calculated by weighing the dried extract per total mass of powder used
97 and extracts were stored at 4 °C until use.

98 Besides, the collected fresh *T. diversifolia* leaves were subjected to steam distillation using a
99 Clevenger type apparatus; 2.5 kg of fresh leaves in 5 litres of water were boiled for 4 h. The
100 extracted essential oil was then dried over anhydrous sodium sulphate and stored in a dark
101 amber glass vial at 4 °C until its use. The yield was calculated.

102 **Phytochemical Screening**

103 The standard modified methods of qualitative analysis described by Harbone (1998) and
104 Edeoga et al. (2005) were used for the determination of phenols, tannins, saponins,
105 flavonoids, alkaloids, glycosides, triterpenes, steroids and anthocyanins in the aqueous
106 extract.

107 **Quantitative assay of phenols and flavonoids in aqueous extracts of *T. diversifolia***

108 The determination of phenols and flavonoids was chosen since the majority of the biological
109 properties of the plant are attributed to them (Boizot and Charpontier 2006). *T. diversifolia*
110 leaf powder was weighed and dissolved in the corresponding volume of distilled water to
111 obtain different concentrations (1%, 3%, 5%, and 10%). After 24 h, the mixture was decanted

112 and filtered. The filtrate was kept at 4 °C for the determination of the phenol and flavonoid
113 content.

114 The Folin-Ciocalteu's assay was used for the quantification of total soluble phenols, using the
115 method described by Siddhuraju and Becker (2007), and gallic acid as a standard. Briefly, 15
116 µL extract at 1%, 3%, 5%, and 10% concentrations were each mixed with 3 mL of distilled
117 water, 250 µL of Folin-Ciocalteu's reagent, 750 µL of 70% Na₂CO₃ and vortexed thoroughly.
118 The mixture was then incubated at room temperature (18-25 °C) for 10 min and allowed to
119 stand for 2 h at room temperature, after adding 950 µL of distilled water. The optical density
120 was measured at $\lambda = 765$ nm. The experiments were performed in triplicate and the total
121 phenol content was expressed as gallic acid equivalents (mg of GAE/g of dry powder)
122 through the calibration curve [OD = f (weight of gallic acid)].

123 The total flavonoid content was evaluated using the aluminium chloride protocol as described
124 by Enujiugha (2010). Briefly, 0.25 mL of aqueous extract (as prepared above) was mixed
125 with 1.25 mL distilled of water (1:5, v/v) and 50% NaNO₃ (75 µL) and allowed to stay for 6
126 min at room temperature. Then, 150 µL of a 10% aluminium chloride solution was added in
127 the mixture and incubated for 5 min at room temperature. The reaction solution was brought
128 to 5 mL with distilled water and 0.5 mL of 1M sodium hydroxide was added. After
129 homogenization, the absorbance was measured at $\lambda = 510$ nm using a spectrophotometer (UV
130 160, Shimadzu, Japan). The total flavonoid content of each treatment was expressed as
131 catechin equivalents (mg of CE/g of dry powder) using a calibration curve [OD = f (weight of
132 catechin)].

133 **Determination of the chemical composition of essential oil**

134 The composition of the essential oil from *T. diversifolia* leaves was determined by using an
135 analytical gas-chromatography (GC-FID) and gas-chromatography coupled with mass
136 spectrometry (GC/MS) techniques. The column used and experimental conditions were both

the same in GC and GC/MS. An Agilent 6890N Network GC system for gas chromatography was equipped with an HP-5MS column [30 m × 0.25 mm (5%-phenyl)-methylpolysiloxane capillary column, film thickness 0.25 µm], a splitless injector heated at 250° C and a flame ionization detector (FID) at 240° C. The oven temperature was programmed as follows: initial temperature 50° C for 1.50 min, increase 10° C/min up to 180° C, 2 min at 180° C, and then increase by 6° C/min up to 280° C, 10 min at 280° C. Helium (99.999%) was used as a carrier gas at a flow rate of 1.0 mL/min. The injection volume was 1.0 µL (split ratio 1:20). GC/MS analyses were performed using an Agilent 6890N Network GC system with an Agilent 5973 Network mass selective detector, mass spectrometer in EI mode at 70 eV in m/e range 10-550 amu. The essential oil components were identified by comparison of their mass spectra with NIST 2002 library data of the GC-MS system. The retention index was calculated according to the formula set by Kovàts (1958).

Antimicrobial activity *in vitro*

Bacterial and fungal strains

The antibacterial activity of the aqueous extract and the essential oil of *T. diversifolia* were evaluated against two rice seed bacteria: *Xanthomonas oryzae* pv. *oryzae* and *Pseudomonas fuscovaginae*, isolated from Cameroon and Italy, respectively. Furthermore, the extracts were tested on two pathogenic fungi of rice seed: *Bipolaris oryzae* (teleomorph: *Cochliobolus miyabeanus* (S. Ito & Kurib.), strain DLS 1586, isolated from rice in Italy and *Fusarium moniliforme* (teleomorph: *Gibberella fujikuroi*) belonging to the *F. fujikuroi* species complex provided by the Institute of Agricultural Research for Development (IRAD), PO. BOX. 2123 Messa-Yaoundé, Cameroon.

Antibacterial activity

To check the possible antibacterial activity of both aqueous extract and essential oil, assays were done using the modified disk diffusion method (CLSI 2007). This method is based on

the diffusion of extracts from filter paper discs in contact with the solid culture medium (NSA) into Petri dishes, previously inoculated with a bacterial inoculum (10^6 CFU/mL). Essential oil was diluted in 5% Tween 20 (v/v) to obtain a concentration of 100 mg/mL; the same concentration was used for the aqueous extract (100 mg/mL). Then, 10 μ L of essential oil and 30 μ L of extract were spotted on different sterilized paper disks, before plating them on the agar surface with the two phytopathogenic bacteria. Gentamicin (1 mg/mL) was used as a control (5 μ L were spotted on sterilized paper disks, before plating them on the agar surface). The inoculated Petri dishes were then incubated at 27 °C and the antibacterial inhibition was assessed after 48 h by measuring the inhibition haloes. The microbial sensitivity was classified according to the diameter of the zones of inhibition as follows: not sensitive for diameters less than 8 mm; sensitive for diameters between 9-14 mm; very sensitive for diameters between 15-19 mm and extremely sensitive for diameters \geq 20 mm (Moreira et al. 2005).

Antifungal activity

The antifungal activity of the aqueous extract and the essential oil from *T. diversifolia* were checked *in vitro* by measuring the inhibition of the mycelial growth on PDA, supplemented with increasing concentrations of the extract and essential oil. Three increasing concentrations of the aqueous extract were used: 10, 50, and 100 mg/mL; five increasing concentrations were considered for the essential oil: 625; 1,250; 2,500; 5,000; and 10,000 μ g/mL. The positive control used was Banko plus[®], the most common fungicide indicated for rice (Chlorothalonil 550 g/L - Carbendazm 100 g/L as active substances) at following concentrations: 62.5; 125; 250; 500; and 1,000 μ g/mL; the negative control was represented by PDA plates supplemented with sterile distilled water. Each agar plate was then inoculated with a 5 mm mycelium plug taken from the margin of a 6-days old culture of each fungus and kept in an incubator at 27 °C. Growth inhibition was assessed after seven days: this was done

187 by measuring the two perpendicular diameters of the fungal colony (Nyegue et al. 2014). The
188 mycelium growth inhibition relative to the controls was then calculated according to the
189 following equation:

$$190 \text{ Mycelium growth inhibition (\%)} = (D-d)/D \times 100$$

191 where, D = mycelium diameter in the control PDA plate, d = mycelium diameter in the
192 amended PDA plate. The tests were carried out in triplicates and the experiments were
193 independently repeated 3 times.

194 The concentration of plant extracts required to inhibit by 50% the fungal growth (IC₅₀) was
195 determined by plotting the growth inhibition percentage as a function of final plant extract
196 concentration (base-10 logarithm). IC₅₀ values were expressed as mg of extract/mL. The
197 antifungal activity of the aqueous extract and essential oil was evaluated as follow: strong
198 activity, when mycelial growth inhibition was > 50%; weak activity when mycelial growth
199 inhibition was <50% or not active when no inhibition was observed (Nyegue 2006).

200 **Determination of the Minimum Inhibitory Concentrations, Minimum Bactericidal** 201 **Concentration and Minimum Fungicidal Concentration**

202 The modified microdilution method described by CLSI (2007) was used for the determination
203 of the minimum inhibitory concentrations (MIC). The MIC was defined as the lowest
204 concentration of aqueous extract or essential oil visibly inhibiting bacterial growth after 48 h
205 of incubation at 27 °C. Into each well, 100 µL of broth enriched with 5% red phenol was
206 added. Then, 100 µL of aqueous extract or essential oil were added in every first well of the
207 microplate. Geometric dilutions ranging from 50 to 0.781 mg/mL were carried out and
208 subsequently, 100 µL of media containing 10⁶ CFU/mL of the target strain was added to all
209 wells to yield 25 to 0.0152 mg/mL of concentration. The plates were then incubated at 27 °C
210 for 48 h. For both extract and essential oil, the experiment was done in triplicate. A colour
211 change from red to yellow indicated a bacterial growth. To obtain the minimum bactericidal

212 concentration (MBC), 20 μ L of each well coloured in red was spotted on the agar surface and
213 incubated at 27 °C for 48 h. The MBC was defined as the lowest concentration of aqueous
214 extract and essential oil where less than 10 colonies growing in the plate were counted. The
215 ratio MBC/MIC was calculated.

216 The MICs of the fungi were determined directly on PDA supplemented with aqueous extract
217 and essential oil. MICs were the lowest concentrations of aqueous extract or essential oil
218 inhibiting visible growth of the target fungi on the agar plate after 7 days of growth. To
219 determine the minimum fungicidal concentration (MFC), the explants present in the plate
220 considered as MIC were subcultured in non-supplement PDA plates. After 4 days of
221 incubation at 27 °C, the absence of mycelial growth indicates the MFC. The ratio MFC/MIC
222 was then calculated: according to Avril and Fauchere (2002), adapted to fungi by Nyegue
223 (2006), for $MFC/MIC < 4$ the sample is classified as "fungicidal", when the values are in the
224 range $4 < MFC/MIC < 16$, the sample is considered "fungistatic", and finally when MFC/MIC
225 > 32 , it is called "tolerant".

226 **Statistical analyses**

227 Average and standard deviations have been calculated using Excel 2007 software. The graphs
228 were made using SigmaPlot and GraphPad Prism software. Analysis of data variance and
229 comparison of means using the Post Hoc (LSD) test performed at the 5% probability level (p
230 < 0.05), using IBM-SPSS16.0 software.

231 **Results**

232 **Preparation of aqueous extract from *T. diversifolia* leaves**

233 The crude aqueous extract of *T. diversifolia* leaves was obtained by maceration of powdered
234 dry leaves. Dried aqueous extract consisted of a dark green powder; its average yield was
235 29.75% of the dry leaves weight.

236 **Qualitative phytochemical screening of aqueous extract of *T. diversifolia***

237 The qualitative phytochemical screening results showed that phenols, flavonoids, terpenoids,
238 tannins, saponins, anthocyanins, glycosides and alkaloids were all present in the aqueous
239 extract of *T. diversifolia* leaves.

240 **Quantitative phytochemical content of phenols and flavonoids**

241 The calculated amount of phenols and flavonoids in the aqueous extract varied in a
242 concentration dependant manner. For phenols, it ranged from 19.33 to 274.4 mg of GAE/g of
243 dry powder and for flavonoids it ranged from 10.6 to 102.4 mg of CE/g of dry sample (Fig.
244 1). Therefore, the average content of total phenols was 146.9 mg of GAE/g and the average
245 content of total flavonoids was 56.5 mg of CE/g of dry powder. Total phenolics content was
246 approximately three times higher than the content of flavonoids.

247 **Extraction and characterization of essential oil**

248 The mean extraction yield for the essential oil obtained by hydrodistillation was 6% of the
249 initial fresh biomass. The organoleptic and physical characteristics of the essential oil were
250 determined: the oil was liquid and volatile, the colour was pale yellow and the smell was
251 pungent. The density (g/mL) of the essential oil was 0.7.

252 **Chemical composition of essential oil**

253 The chromatographic profile of the essential oil is shown in table 1. The chemical
254 composition of essential oil showed that it was mainly composed of hydrocarbonated,
255 oxygenated monoterpenes and with some sesquiterpenes.

256 The GC profile of the essential oil showed that terpenes/terpenoids are the main constituents
257 (Table 1). Alpha-terpineol, a monoterpene alcohol, was the component detected in highest
258 amount (20.3%); with terpinen-4-ol (1.8%) its isomeric form, they represent almost one-
259 fourth of the chemical composition of this essential oil. Another major component of the oil
260 was α -pinene (13.5%); considering that another detected molecule was camphor (14.3%), a
261 cyclic ketone derivative from the oxidation of α -pinene, both (α -pinene and camphor)

substances may be considered the major component of *T. diversifolia* essential oil. Sesquiterpenes were also found in a not negligible amount: spathulenol, globulol and ledol reach together a percentage of 10.5%. Therefore, monoterpenes and sesquiterpenes represent more than 60% of the essential oil components.

***In vitro* antibacterial activity of aqueous extract and essential oil**

The aqueous extract and the essential oil from *T. diversifolia* were tested *in vitro* for their putative antibacterial activity. Both extracts showed remarkable antibacterial activity against the two bacteria tested: such activity was revealed by the formation of a clear and large inhibition halo around the paper discs soaked with the extracts. The antimicrobial activity of both extracts against both phytopathogenic bacteria was comparable, since the inhibitory haloes showed a similar area in any replicate plates. Interestingly, the tested tracts proved a superior antibacterial activity than the antibiotic gentamicin, at the given concentrations (Fig. 2).

The results obtained from the activity of the aqueous extract and the essential oil on targeted bacteria are quantitatively illustrated in figure 3. Histograms show that the two bacterial strains were strongly inhibited by both the aqueous extract and the essential oil of *T. diversifolia*. The inhibition diameters of the aqueous extract and the essential oil ranged from 21 to 24 mm. Interestingly, bacterial sensitivity to both extracts was higher than that of gentamicin, for which the inhibition diameters vary from 12 to 15 mm for both bacteria. Such inhibition was reached using the concentration of 100 mg/mL for both extracts, compared to a concentration of gentamicin of 1 mg/mL.

Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The inhibition parameters of aqueous extract, MIC and MBC, were not assessed, but those of essential oil were determined. The latter data made it possible to calculate the ratio

287 MBC/MIC. This relationship made it possible to characterize a bactericidal, bacteriostatic
288 action or to determine the “tolerance” of a strain (Table 2).

289 Table 2 shows that the MIC for both phytopathogenic bacteria was 125 µg/mL for the
290 essential oil; the reference antibiotic (gentamicin) gave a MIC of 31.25 µg/mL, again for both
291 bacteria. The measurement of the MBC/MIC ratio, with values of 2 (*X. oryzae* pv. *oryzae*)
292 and 1 (*P. fuscovaginae*) showed that the essential oil of *T. diversifolia* can be considered as
293 bactericidal, according to the scale of Avril and Fauchere (2002). As expected, gentamicin
294 proved its antibiotic effect against both bacteria, with a MBC/MIC ratio of 1.

295 ***In vitro* antifungal activity of aqueous extract and essential oil**

296 In general, aqueous extract and essential oil of *T. diversifolia* showed good inhibitory activity
297 against both *B. oryzae* and *F. moniliforme*. Such inhibitory activity increased in a dose-
298 dependent manner (Fig. 4). A concentration of 100 mg/mL of the plant extract inhibited the
299 mycelial growth of *B. oryzae* and reduced its growth rate by approximately 68.44%. Similar
300 results were observed against *F. moniliforme*: a concentration of 100 mg/mL reduced the
301 mycelial growth by approximately 70.69%. The essential oil demonstrated a more evident
302 antifungal activity against both pathogens. It was active at a concentration of 625 µg/mL, and
303 produced a total fungal inhibition at a concentration of 5,000 µg/mL and above (Fig. 4).

304 Figure 5 shows the quantitative mycelium growth inhibition (%) stimulated by the aqueous
305 extract from *T. diversifolia* leaves against *B. oryzae* and *F. moniliforme*. The mycelium
306 growth inhibition ranged from 25% at 10 mg/mL to 68.44% at 100 mg/mL of aqueous extract
307 on *B. oryzae* and from 20% at 10 mg/mL to 70.69% at 100 mg/mL of aqueous extract on *F.*
308 *moniliforme*. Inhibitory concentration 50 (IC₅₀) was determined and it was calculated at 50
309 mg/mL on *B. oryzae* and *F. moniliforme*. According to the scale of Nyegue (2006), the
310 aqueous extract exhibited a strong antimicrobial activity with IC₅₀ > 50%. A total growth

inhibition of *B. oryzae* and *F. moniliforme* was not reached using aqueous extracts at the dilutions tested (Fig. 5).

Figure 6 shows the mycelium growth inhibition (%) obtained by using the essential oil of *T. diversifolia* leaves against *B. oryzae* and *F. moniliforme*. The mycelium growth inhibition ranged from 58.28% at 625 µg/mL to 100% at 5,000 µg/mL of essential oil concentrations on *B. oryzae* and from 56.87% at 625 µg/mL to 100% at 5,000 µg/mL of essential oil concentrations on *F. moniliforme*. Therefore, the sensitivity of both fungi to the essential oil was quite similar. Inhibitory concentration 50 (IC₅₀) was then determined and resulted to be 625 µg/mL for both *B. oryzae* and *F. moniliforme*, with a percentage inhibition of 58.29% and 56.87%, respectively. According to the scale of Nyegue et al. (2006), the essential oil showed a very strong antimicrobial activity, with an IC₅₀ > 50%. The minimal inhibition concentration was 5,000 µg/mL. Also, we obtained complete fungal inhibition at 5,000 µg/mL, which corresponds to the MFC (Fig. 6).

Determination of Minimum Inhibition Concentration and Minimum Fungicidal Concentration

As shown in table 3 the MFC obtained in our experiments using the essential oil was 5,000 µg/mL; this value was obtained for both fungi tested. The calculated ratio MFC/MIC was 1; according to the scale of Avril and Fauchere (2002), the essential oil of *T. diversifolia* leaves has a fungicidal activity. Therefore, in our experiments, the activity of the essential oil was comparable to the antifungal action of the reference fungicide (Banko plus[®]), since both their calculated MFC/MIC were less than 4 (Table 3).

Discussion

Plant-derived biomolecules have drawn great attention during the last 15 years, due to their general antimicrobial properties; indeed, they have been suggested as prospective compounds to be used during the development of innovative biopesticides and in the implementation of

sustainable strategies to control phytopathogenic fungi and bacteria (Reignault and Walters 2007; Martinez 2012). The present study showed that the aqueous extract of *T. diversifolia* and its essential oil possess a pronounced antimicrobial activity and may be considered sources of bioactive phytochemicals. Its leaves are very rich in phenols, flavonoids, terpenoids, alkaloids, glycosides, saponins, and tannins; these results are in agreement with the findings of Olutobi and Olasupo (2012), who reported the presence of similar phytochemical compounds in the methanolic extract of *T. diversifolia* leaves. Some authors have demonstrated their biological activity, among which antibacterial (Desi et al. 2017) and antifungal (Saini et al. 2009; Mekam et al. 2019).

In this study, the quantitative analysis of phenols and flavonoids yielded 274.47 mg of GAE/g and 102.4 mg of CE/g of dry powder, respectively. While using *T. diversifolia* water extract in their study, Olayinka et al. (2015) obtained a phenols level (64.58 mg of GAE/g of dry powder) and flavonoids (851.67 mg of CE/g of dry powder). This could be explained by the fact that plants under conditions of stress induced by biotic and abiotic factors may show changes in the production of different classes of metabolite or sometime due to the technology used to assay the secondary metabolites (Lapornik et al. 2005; Arbona et al. 2013; Osama 2018; Mekam et al. 2019).

The gas chromatographic profile of *T. diversifolia* essential oil showed a total of 19 compounds; terpenes and terpenoids were the main constituents, accounting for 95% of the composition. In the present study, the main constituents were α -pinene (13.5%), camphor (14.3%), eucalyptol (14.6%) and α -terpineol (20.3%); these results are different from those obtained by Wanzala et al. (2016) in Kenya, Adebayo et al. (2008) in Nigeria and Ingrid et al. (2018) in Brazil, who showed that the essential oil of *T. diversifolia* is mainly rich in α -pinene in the proportion 63.64%, 4.4% and 45%, respectively. These differences could be due to the difference among geographical areas where the plants grew and were harvested

361 (Arbona et al. 2013). These authors gave no clear details on the handling of *T. diversifolia*;
362 thus, other intrinsic factors, such as storage condition and age of plants, could considerably
363 influence the composition (Lapornik et al. 2005).

364 The activity of essential oil is often reduced to the activity of its major compounds, or those
365 likely to be active; however, some minor compounds may act in synergy with the major or
366 other compounds (Sonboli et al. 2006; Lahlou 2004). The antibacterial and antifungal activity
367 observed in this study could be attributed to the presence of the identified major compounds.
368 In fact, α -pinenes destroy the cellular integrity of pathogens, inhibiting both their respiration
369 and the ion transport process, while modifying cell permeability (Andrews et al. 1980);
370 eucalyptol and camphor display antimicrobial effect against phytopathogenic fungi and are
371 widely exploited to control post-harvest diseases and the growth of mycotoxigenic fungi
372 (Rahmouni et al. 2019); α -terpineol was recently shown to possess antimicrobial activity
373 against important phytopathogenic fungi (Song et al. 2019).

374 The essential oil from *T. diversifolia* proved to be active against rice pathogenic bacteria, with
375 MICs of 125 $\mu\text{g/mL}$; according to Tegos et al. (2002), phytochemicals or extracts with MIC
376 values between 100 $\mu\text{g/mL}$ and 1,000 $\mu\text{g/mL}$ are considered as antimicrobials of interest. The
377 differences in sensitivity between the fungal and bacterial species concerning the aqueous
378 extract and the essential oil of *T. diversifolia* leaves observed during our study may be due to
379 intrinsic factors specific to each microorganism (Takeo et al. 2004) or due to the
380 phytochemical profile of the aqueous extract and essential oil; oxygenated molecules like
381 phenols, alkaloids, flavonoids, oxygenated terpenoids are generally more active than lipophilic
382 hydrocarbons (Silva et al. 2012), but the high concentration of the latter makes the essential
383 oil more active.

384 The essential oil was more active as antimicrobial compound than the aqueous extract against
385 the two target bacterial strains (*X. oryzae* pv. *oryzae* and *P. fucovaginae*) and the two rice

386 pathogenic fungi (*B. oryzae* and *F. moniliforme*); the MICs of our essential oil were 125
387 µg/mL and 5,000 µg/mL against bacteria and fungi strains, respectively. These MICs were
388 higher compared to those reported by Ingrid et al. (2018) and Oludare et al. (2016), who also
389 worked with *T. diversifolia* essential oil; they found MICs of 1,000 µg/mL against
390 *Streptococcus mitis* and 72,000 µg/mL against *Fusarium solani*. Thus, the activity of
391 essential oil of *T. diversifolia* is microorganisms dependent (Miranda et al. 2016).

392 In the present study a significant mean inhibition halo of 22 mm for *X. oryzae* pv. *oryzae* was
393 observed with 3 mg/mL of the aqueous extract *T. diversifolia* leaves. This result differed from
394 that reported by Desi et al. (2017) who showed that, up to 10 mg/mL, the aqueous and
395 methanol extracts of leaves of *T. diversifolia* harvested in Nigeria had no effect on *X. oryzae*
396 pv. *oryzae*. The noticed difference in the activity may also be due to the genetic diversity
397 within *X. oryzae* pv. *oryzae* populations, the population structure and the biology of the
398 phytopathogenic bacteria (Lapornik et al. 2005).

399 In this study, no MIC was obtained against the two target bacterial strains with the aqueous
400 extract of *T. diversifolia*. The findings reported by Obafemi et al. (2006) in Nigeria showed
401 that the methanol and ethanol extracts from the leaves of *T. diversifolia* had a significant
402 inhibitory activity against clinical Gram positive bacteria (*Clostridium sporogenes* with MIC
403 of 15.6 µg/mL and *Streptococcus faecalis*, with MIC of 72.5 µg/mL) and Gram negative
404 bacteria (*Pseudomonas aeruginosa*, with MIC of 15.6 µg/mL). Thus, the human pathogenic
405 bacteria seem to be more sensitive to *T. diversifolia* extracts when compared to plant
406 pathogenic bacteria; this may be due to the high concentrated in bioactive constituents (*e.g.*:
407 feruloyl, coniferin) with antimicrobial activity generally present in ethanol and methanol
408 extracts, as compared to aqueous extracts (Mekam et al. 2019).

409 The powerful antibacterial and antifungal activities of *T. diversifolia* extracts opens new
410 chances for African farmers to manage the most destructive rice pathogens and, additionally,

411 may stimulate new opportunities in the development of locally based small/medium sized
412 industries devoted to make use of a common local botanic resource. In order to reinforce this
413 approach towards the production of Traditional Improved Pesticides (TIP) and the discovery
414 of new growth potentiating substances, these results call us to complete this work and consider
415 the evaluation of active fractions on pathogen reductions for future studies. In particular, such
416 plant bioactive extracts might be taken into consideration when developing seed treatments, in
417 order to effectively decrease the primary inoculum of these seed-transmitted pathogens;
418 therefore, the next research step would be to observe their phytotoxicity on the germination
419 and physiology of the rice seedlings.

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555 **List of tables**

556 **Table 1** Formula, name of the compounds, retention index (RI) and percentage of the
 557 compound in the essential oil extracted from *Tithonia diversifolia* leaves

	Formula	Name of chemical compounds	RI	Percentage
1	C ₁₀ H ₁₆	α-Pinene	917.7	13.5
2	C ₆ H ₆ O	phenol	968.9	0.4
3	C ₁₀ H ₁₄	p-cymene	1022.6	2.2
4	C ₁₀ H ₁₈ O	eucalyptol, (cineole)	1034.7	14.6
5	C ₁₀ H ₁₆	γ-terpinene	1072.4	0.5
6	C ₁₀ H ₁₈ O ₂	Epoxy linalool	1093.1	1.3
7	C ₁₀ H ₁₈ O ₂	Linalool, oxide	1111.9	1.5
8	C ₁₀ H ₁₈ O	Linalool, hydrate	1121.8	0.7
9	C ₁₀ H ₁₆ O	α-campholenealdehyde	1153.2	2.6
10	C ₁₀ H ₁₆ O	Pinocarveol	1168.4	5.1
11	C ₁₀ H ₁₈ O	Camphor	1194.7	14.3
12	C ₁₀ H ₁₈ O	Terpinen-4-ol	1203.5	1.8
13	C ₁₀ H ₁₈ O	α-Terpineol	1220.6	20.3
14	C ₁₀ H ₁₆ O	Myrtenol	1225.6	0.3
15	C ₁₀ H ₁₄ O	Carvacrol	1324.7	0.8
16	C ₁₅ H ₂₄ O	Spathulenol	1615.4	3.3
17	C ₁₅ H ₂₆ O	Globulol	1622.7	1.5

18	$C_{15}H_{26}O$	Ledol	1631.7	5.7
19	$C_{15}H_{26}O$	2-Naphthalenemethanol	1689.8	1.2

558

559

Table 2 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of essential oil from *Tithonia diversifolia* and gentamicin

Tested product	Inhibition parameters	Pathogens	
		<i>X. oryzae</i> pv. <i>oryzae</i>	<i>P. fuscovaginae</i>
Essential oil (µg/mL)	MIC	125	125
	MBC	250	125
	MBC/MIC	2	1
Gentamicin (µg/mL)	MIC	31.25	31.25
	MBC	31.25	31.25
	MBC/MIC	1	1

564 **Table 3** Minimum fungicidal concentration of essential oil and Banko Plus®

Tested product	Inhibition parameters	Pathogens	
		<i>B. oryzae</i>	<i>F. moniliforme</i>
Essential oil (µg/mL)	MIC	5,000	5,000
	MFC	5,000	5,000
	MFC/MIC	1	1
Banko Plus® (µg/mL)	MIC	1,000	500
	MFC	1,000	500
	MFC/MIC	1	1

565