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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.  $\alpha$ -terpineol (20.3%), eucalyptol  
17 (14.6%), camphor (14.3%) and  $\alpha$ -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 (IC<sub>50</sub>) 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  $\mu$ g/mL (*Xanthomonas oryzae* pv.  
22 *oryzae* and *Pseudomonas fuscovaginae*) and MFC of 5,000  $\mu$ 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.

34 **Code availability.** Not applicable.

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

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

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

## 78 **Materials and methods**

### 79 **Plant material and media**

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

86 Culture media (Potato dextrose agar, Nutrient sucrose agar, and the reference antibiotic  
87 (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<sup>®</sup> 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  $48 \pm 2$  °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% Na<sub>2</sub>CO<sub>3</sub> 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% NaNO<sub>3</sub> (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



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

#### 149 **Antimicrobial activity *in vitro***

##### 150 **Bacterial and fungal strains**

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

##### 159 **Antibacterial activity**

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

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

#### 175 **Antifungal activity**

176 The antifungal activity of the aqueous extract and the essential oil from *T. diversifolia* were  
177 checked *in vitro* by measuring the inhibition of the mycelial growth on PDA, supplemented  
178 with increasing concentrations of the extract and essential oil. Three increasing  
179 concentrations of the aqueous extract were used: 10, 50, and 100 mg/mL; five increasing  
180 concentrations were considered for the essential oil: 625; 1,250; 2,500; 5,000; and 10,000  
181  $\mu$ g/mL. The positive control used was Banko plus<sup>®</sup>, the most common fungicide indicated for  
182 rice (Chlorothalonil 550 g/L - Carbendazm 100 g/L as active substances) at following  
183 concentrations: 62.5; 125; 250; 500; and 1,000  $\mu$ g/mL; the negative control was represented  
184 by PDA plates supplemented with sterile distilled water. Each agar plate was then inoculated  
185 with a 5 mm mycelium plug taken from the margin of a 6-days old culture of each fungus and  
186 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 (IC<sub>50</sub>) was  
195 determined by plotting the growth inhibition percentage as a function of final plant extract  
196 concentration (base-10 logarithm). IC<sub>50</sub> 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 10<sup>6</sup> 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  $\mu$ 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 a 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 a 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  $\alpha$ -pinene (13.5%); considering that another detected molecule was camphor (14.3%), a  
261 cyclic ketone derivative from the oxidation of  $\alpha$ -pinene, both ( $\alpha$ -pinene and camphor)

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

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

267 The aqueous extract and the essential oil from *T. diversifolia* were tested *in vitro* for their  
268 putative antibacterial activity. Both extracts showed remarkable antibacterial activity against  
269 the two bacteria tested: such activity was revealed by the formation of a clear and large  
270 inhibition halo around the paper discs soaked with the extracts. The antimicrobial activity of  
271 both extracts against both phytopathogenic bacteria was comparable, since the inhibitory  
272 haloes showed a similar area in any replicate plates. Interestingly, the tested tracts proved a  
273 superior antibacterial activity than the antibiotic gentamicin, at the given concentrations (Fig.  
274 2).

275 The results obtained from the activity of the aqueous extract and the essential oil on targeted  
276 bacteria are quantitatively illustrated in figure 3. Histograms show that the two bacterial  
277 strains were strongly inhibited by both the aqueous extract and the essential oil of *T.*  
278 *diversifolia*. The inhibition diameters of the aqueous extract and the essential oil ranged from  
279 21 to 24 mm. Interestingly, bacterial sensitivity to both extracts was higher than that of  
280 gentamicin, for which the inhibition diameters vary from 12 to 15 mm for both bacteria. Such  
281 inhibition was reached using the concentration of 100 mg/mL for both extracts, compared to  
282 a concentration of gentamicin of 1 mg/mL.

#### 283 **Determination of Minimum Inhibitory Concentration and Minimum Bactericidal** 284 **Concentration**

285 The inhibition parameters of aqueous extract, MIC and MBC, were not assessed, but those of  
286 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 (IC<sub>50</sub>) 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 IC<sub>50</sub> > 50%. A total growth

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

313 Figure 6 shows the mycelium growth inhibition (%) obtained by using the essential oil of *T.*  
314 *diversifolia* leaves against *B. oryzae* and *F. moniliforme*. The mycelium growth inhibition  
315 ranged from 58.28% at 625 µg/mL to 100% at 5,000 µg/mL of essential oil concentrations on  
316 *B. oryzae* and from 56.87% at 625 µg/mL to 100% at 5,000 µg/mL of essential oil  
317 concentrations on *F. moniliforme*. Therefore, the sensitivity of both fungi to the essential oil  
318 was quite similar. Inhibitory concentration 50 (IC<sub>50</sub>) was then determined and resulted to be  
319 625 µg/mL for both *B. oryzae* and *F. moniliforme*, with a percentage inhibition of 58.29%  
320 and 56.87%, respectively. According to the scale of Nyegue et al. (2006), the essential oil  
321 showed a very strong antimicrobial activity, with an IC<sub>50</sub> > 50%. The minimal inhibition  
322 concentration was 5,000 µg/mL. Also, we obtained complete fungal inhibition at 5,000  
323 µg/mL, which corresponds to the MFC (Fig. 6).

#### 324 **Determination of Minimum Inhibition Concentration and Minimum Fungicidal** 325 **Concentration**

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

#### 332 **Discussion**

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



336 sustainable strategies to control phytopathogenic fungi and bacteria (Reignault and Walters  
337 2007; Martinez 2012). The present study showed that the aqueous extract of *T. diversifolia*  
338 and its essential oil possess a pronounced antimicrobial activity and may be considered  
339 sources of bioactive phytochemicals. Its leaves are very rich in phenols, flavonoids,  
340 terpenoids, alkaloids, glycosides, saponins, and tannins; these results are in agreement with  
341 the findings of Olutobi and Olasupo (2012), who reported the presence of similar  
342 phytochemical compounds in the methanolic extract of *T. diversifolia* leaves. Some authors  
343 have demonstrated their biological activity, among which antibacterial (Desi et al. 2017) and  
344 antifungal (Saini et al. 2009; Mekam et al. 2019).

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

353 The gas chromatographic profile of *T. diversifolia* essential oil showed a total of 19  
354 compounds; terpenes and terpenoids were the main constituents, accounting for 95% of the  
355 composition. In the present study, the main constituents were  $\alpha$ -pinene (13.5%), camphor  
356 (14.3%), eucalyptol (14.6%) and  $\alpha$ -terpineol (20.3%): these results are different from those  
357 obtained by Wanzala et al. (2016) in Kenya, Adebayo et al. (2008) in Nigeria and Ingrid et al.  
358 (2018) in Brazil, who showed that the essential oil of *T. diversifolia* is mainly rich in  $\alpha$ -  
359 pinene in the proportion 63.64%, 4.4% and 45%, respectively. These differences could be due  
360 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,  $\alpha$ -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);  $\alpha$ -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 <sub>10</sub> H <sub>16</sub>	α-Pinene	917.7	13.5
2	C <sub>6</sub> H <sub>6</sub> O	phenol	968.9	0.4
3	C <sub>10</sub> H <sub>14</sub>	p-cymene	1022.6	2.2
4	C <sub>10</sub> H <sub>18</sub> O	eucalyptol, (cineole)	1034.7	14.6
5	C <sub>10</sub> H <sub>16</sub>	γ-terpinene	1072.4	0.5
6	C <sub>10</sub> H <sub>18</sub> O <sub>2</sub>	Epoxy linalool	1093.1	1.3
7	C <sub>10</sub> H <sub>18</sub> O <sub>2</sub>	Linalool, oxide	1111.9	1.5
8	C <sub>10</sub> H <sub>18</sub> O	Linalool, hydrate	1121.8	0.7
9	C <sub>10</sub> H <sub>16</sub> O	α-campholena ldehyde	1153.2	2.6
10	C <sub>10</sub> H <sub>16</sub> O	Pinocarveol	1168.4	5.1
11	C <sub>10</sub> H <sub>18</sub> O	Camphor	1194.7	14.3
12	C <sub>10</sub> H <sub>18</sub> O	Terpinen-4-ol	1203.5	1.8
13	C <sub>10</sub> H <sub>18</sub> O	α-Terpineol	1220.6	20.3
14	C <sub>10</sub> H <sub>16</sub> O	Myrtenol	1225.6	0.3
15	C <sub>10</sub> H <sub>14</sub> O	Carvacrol	1324.7	0.8
16	C <sub>15</sub> H <sub>24</sub> O	Spathulenol	1615.4	3.3
17	C <sub>15</sub> H <sub>26</sub> 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

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558

559

560 **Table 2** Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration  
 561 (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

562

563

564 **Table 3** Minimum fungicidal concentration of essential oil and Banko Plus<sup>®</sup>

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 <sup>®</sup> (µg/mL)	MIC	1,000	500
	MFC	1,000	500
	MFC/MIC	1	1

565