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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Abstract

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

Keywords: *Titonia diversifolia*, Seed-borne pathogens, Biopesticides, Secondary metabolites.

Declarations

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Introduction

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

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

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

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

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

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

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

Phytochemical Screening

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

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

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

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

The Folin-Ciocalteu's assay was used for the quantification of total soluble phenols, using the method described by Siddhuraju and Becker (2007), and gallic acid as a standard. Briefly, 15 µL extract at 1%, 3%, 5%, and 10% concentrations were each mixed with 3 mL of distilled water, 250 µL of Folin-Ciocalteu's reagent, 750 µL of 70% Na_2CO_3 and vortexed thoroughly. The mixture was then incubated at room temperature (18-25 °C) for 10 min and allowed to stand for 2 h at room temperature, after adding 950 µL of distilled water. The optical density was measured at λ = 765 nm. The experiments were performed in triplicate and the total phenol content was expressed as gallic acid equivalents (mg of GAE/g of dry powder) through the calibration curve [OD = f (weight of gallic acid)].

The total flavonoid content was evaluated using the aluminium chloride protocol as described by Enujiugha (2010). Briefly, 0.25 mL of aqueous extract (as prepared above) was mixed with 1.25 mL distilled of water (1:5, v/v) and 50% $NaNO_3$ (75 µL) and allowed to stay for 6 min at room temperature. Then, 150 µL of a 10% aluminium chloride solution was added in the mixture and incubated for 5 min at room temperature. The reaction solution was brought to 5 mL with distilled water and 0.5 mL of 1M sodium hydroxide was added. After homogenization, the absorbance was measured at λ = 510 nm using a spectrophotometer (UV 160, Shimadzu, Japan). The total flavonoid content of each treatment was expressed as catechin equivalents (mg of CE/g of dry powder) using a calibration curve [OD = f (weight of catechin)].

Determination of the chemical composition of essential oil

The composition of the essential oil from *T. diversifolia* leaves was determined by using an analytical gas-chromatography (GC-FID) and gas-chromatography coupled with mass 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 250º C and a flame ionization detector (FID) at 240º C. The oven temperature was programmed as follows: initial temperature 50º C for 1.50 min, increase 10º C/min up to 180º C, 2 min at 180º C, and then increase by 6º C/min up to 280º C, 10 min at 280º 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 µL of essential oil and 30 µL 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 µL 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 ≥ 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 µg/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 µg/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

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

Mycelium growth inhibition (%) = (D-d)/D×100

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

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

Determination of the Minimum Inhibitory Concentrations, Minimum Bactericidal Concentration and Minimum Fungicidal Concentration

The modified microdilution method described by CLSI (2007) was used for the determination of the minimum inhibitory concentrations (MIC). The MIC was defined as the lowest concentration of aqueous extract or essential oil visibly inhibiting bacterial growth after 48 h of incubation at 27 °C. Into each well, 100 µL of broth enriched with 5% red phenol was added. Then, 100 µL of aqueous extract or essential oil were added in every first well of the microplate. Geometric dilutions ranging from 50 to 0.781 mg/mL were carried out and subsequently, 100 µL of media containing 10^6 CFU/mL of the target strain was added to all wells to yield 25 to 0.0152 mg/mL of concentration. The plates were then incubated at 27 °C for 48 h. For both extract and essential oil, the experiment was done in triplicate. A colour change from red to yellow indicated a bacterial growth. To obtain the minimum bactericidal

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

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

Statistical analyses

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

Results

Preparation of aqueous extract from *T. diversifolia* leaves

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

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

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

Quantitative phytochemical content of phenols and flavonoids

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

Extraction and characterization of essential oil

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

Chemical composition of essential oil

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

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

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

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

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

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

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

311 inhibition of *B. oryzae* and *F. monoliforme* 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_{50}) 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_{50} > 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®), 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

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

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

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

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

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

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

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

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

The powerful antibacterial and antifungal activities of *T. diversifolia* extracts opens new 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	$C_{10}H1_6$	α-Pinene	917.7	13.5
2	C_6H_6O	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-campholenaldehyde	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

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	
		X. oryzae* pv. *oryzae	***P. fuscovaginae***
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®

Tested product	Inhibition parameters	Pathogens	
		B. oryzae	***F. moniliforme***
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