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In Vitro Antibacterial Activity of Essential Oils from *Tithonia Diversifolia* Leaves and Flowers Against *Ralstonia Solanacearum*

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ABSTRACT

Bacterial wilt disease caused by *Ralstonia solanacearum* is a major constraining factor in the production of tomatoes in Kenya, leading to an overreliance on synthetic pesticides. As a result, there is increased research on bio-pesticides as safer alternatives. The present study, therefore, characterized and evaluated *in*

vitro **antibacterial activity of essential oils from *Tithonia diversifolia* leaves and flowers against *R. solanacearum.* Hydrodistilled essential oils were characterized through Gas Chromatography-Mass spectrometry (GC-MS) while functional groups were confirmed using Fourier Transform Infrared Spectroscopy (FT-IR). Antibacterial activity was performed using the disc diffusion method and minimum inhibitory concentration evaluated using broth dilution. Hydrodistilled leaves of *T. diversifolia* yielded 0.18 ± 0.08 % (v/w); 0.10 ± 0.07 % w/w as compared to 0.15 ± 0.09 % (v/w); 0.08 ± 0.03 % w/w in the leaves . GC-MS profiling revealed the major compounds were (Z,Z,Z)-9,12,15- Octadecatrienoic acid ethyl ester(18%), palmitic acid (16%), spathulenol (12%), Cis- 9,12,15- Octadecatrienoic acid (8.14%), tetrateracontane (6%) and 1-Octen-3yl-acetate (5.22%) in the leaves ad α-linolenic acid trimethylester(33%), Z,Z Hexadecadienoic acid(26%), octadecanoic acid trimethylester (9%), palmitelaidic acid (8.49%), Germacrene D(5.45%), azelaic acid (5.02%) and caryophyllene oxide (5.00%) in the flowers. Antibacterial activity showed that T. *diversifolia* essential oils had mean inhibition zones of 12.61± 0.22 mm and 11.82 ± 0.76 mm from the leaves and flowers respectively, in comparison to metham sodium which gave inhibition zone of 25.78 ±0.29 mm (p = 0.001). Based on the results, this study gives credence to *T. diversifolia* essential oils as viable antibacterial agents.**

Keywords: In vitro; antibacterial activity; Spathulenol; Tithonia diversifolia; Ralstonia solanacearum

INTRODUCTION

Bacterial wilt caused by soil-borne bacterium *Ralstonia solanacearum* is one of the most devastating bacterial disease limiting tomato production in tropical, subtropical, and warm temperate regions of the World [1-4]. The bacterium is classified as the World's most destructive phytopathogenic bacteria causing tomato yield losses due to its lethality, persistence, wide host range in solanaceous crops, ability to grow endophytically, survive in soil, broad geographic distribution, and versatile methods of transmission [1-6]. Additionally, *R. solanacearum* can survive in plant debris, infected plants host weeds, and spread from one field to another by irrigation or floodwater, soil, farm equipment, and remaining crops from the previous seasons, hence it is difficult to manage [3-4]. Despite the availability of several methods for the management of bacterial wilt disease in tomatoes including chemicals, biological agents, cultural and physical practices [3], the disease has not been successfully managed in Sub-Saharan Africa [2,7]. Emerging trends in physical practices for control of *R. solanacearum* include solarization, hot water treatment of infected soil, planting in the cold season, soil fumigation, and soil drenching [3]. Some of the aforementioned methods including planting tomato, potato and tobacco crops during winter when the temperatures are low and the bacterium is inactive[6], may not be viable in Sub-Saharan Africa where temperatures are usually high all year round. In addition, wet conditions and moderate temperatures in Sub-Saharan Africa usually favor the survival of the bacteria [7]. As a result, synthetic pesticides continue to be overused for plant disease management and pest control due to their efficacy, reliability, rapidity of action, and quick knockdown effect [8-9]. However, control of *R. solanacearum* using synthetic pesticides has far-reaching implications such as environmental pollution, contamination of groundwaters, accumulation of toxic residues in food, and elimination of non-target organisms [9-12]. Biopesticides on the other hand are less toxic, less

persistent, environment-friendly, safe to humans and non-target organisms, economically affordable, highly effective, and target specific [8-13].

Application of biopesticides includes the use of bio-control agents like *Bacillus amyloliquefaciens* [5] and *Pseudomonas fluorescens* [14] and plant extracts like *Amomum nilgiricum* [15] for control of *R. solanacearum*. Furthermore, biopesticides have been appraised to be less persistent in the environment as they are often degraded rapidly by sunlight, or moisture, are less phytotoxic, and are consequently potentially less of a threat to the environment and human health [8]. Additionally, they contain a mixture of compounds, which can work together synergistically in reducing a pathogen or pest with varying modes of action [16]. In this context, plant extracts contain Phyto complex of active compounds which could be contributing to the overall biological effect against bacteria through the collective effect of all its components, some of which will cooperate and some might modulate, while others will act on different, distantly connected targets, ultimately generating synergistic activity of the phytoconstituents [16-17]. Essential oils, in particular, have been demonstrated to be effective antibacterial agents because their lipophilic characteristics contribute to their being capable of destroying the cell wall of bacteria [18]. Additionally, they contain a large number of phytochemicals and hence it is most likely that the reported antibacterial activity of essential oils is due to the synergistic effect of all the constituents and not attributed to one mechanism [18]. Therefore, using essential oils as biopesticides could lead to reduced occurrence of pathogen and pest resistance development. As a result, the use of essential oils has been on the increase as an emerging, potential, and alternative approach in disease management for *R. solanacearum* [12, 19-20]. Essential oils are naturally occurring volatile substances obtained from a variety of plants including *T. diversifolia* [21].

T. diversifolia (Hemsl.) A. Gray (Asteraceae) is a shrub and a member of the sunflower family and is native to North and Central America [21-22]. Although this species is native to the lowlands of southeastern Mexico and Central America, it has now been naturalized in different regions of the world including Africa and Asia [22], where they have become an ecological, agricultural, and economic burden [23].

Despite the negative effects of the invasive shrub, ethno pharmacologically, *T. diversifolia* has been exploited in folkloric medicinal practices as well as in remediation of heavy metals from the soil [21, 23].

In Nigeria, *T. diversifolia* has been reported to be used by herbal medicine practitioners in the treatment of menstrual pain, treatment of wounds, and *diabetes mellitus* [22]. In Mexico where this plant originates from, it is used to treat sprains, bone fractures, bruises and contusions [22]. In Kenya, *T. diversifolia* locally known as *maruru*, *maua* and *amalulu* (for Luhya tribe), *maua makech* (Luo), *amaua amaroro* (Kisii) and *mula* (Kamba) all implying that the plant is bitter to the taste are effectively used for treatment of snake envenomation [24-25]. Among the Kalenjin community in Kenya, *T. diversifolia* is commonly known as *mauat ne ng'wan* meaning bitter flowers, and is used to treat diarrhea.

Biological activities of *T. diversifolia* includes anti-inflammatory, analgesic, antimalarial, antiviral, antidiabetic, antidiarrhoeal, antimicrobial, antispasmodic, vasorelaxant, cancer-chemo preventive, insecticidal, antiemetic, and antiamoebic properties [21-22, 26-27]. Most of

the pharmacological activities of *T*. *diversifolia* have been attributed to sesquiterpene, lactones, diterpenes, flavonoids and some chlorogenic acid derivatives present in the leaves of this species [21]. *T*. *diversifolia* essential oils consist of terpenes, terpenoids and lactones [28]. These compounds have been demonstrated to possess antibacterial, antifungal and insecticidal activities [23, 28]. In a previous study, Farias *et al*., [26] demonstrated antimicrobial activity of *T*. *diversifolia* leaf essential oils against *Staphylococcus aureus*, *Escherichia coli,* and *Pseudomonas aeruginosa* and reported that the observed antimicrobial activity was due to the presence of the main chemical constituents including α-pinene, Limonene, (Z)-β-ocimene, p-cymen-8-ol, Piperitone, (E)-nerolidol and Spathulenol. In another study, Agboola *et al*., [28], evaluated the chemical composition and antimicrobial activity of *T*. *diversifolia* essential oils they reported that secondary metabolites present in the oils including monoterpenes, sesquiterpenes, alcohols, and aldehydes proved effective against *Escherichia coli*, *Proteus mirabi*, *Bacillus megaterium*, *Klebsiella pneumonia*, *Bacillus cereus and Streptococcus pyrogens* thereby, unlocking the potential of the oils for bio-pesticide production. In a recent study, Njuguna *et al*., [29], investigated the contact toxicity of essential oils from the T. *diversifolia* leaves against *Thrips tabaci*, *Bemisia tabaci*, and *Aphis gosypii* and reported that the essential oils possessed remarkable insecticidal activity which could be employed as a safer alternative to synthetic pesticides. In this context, the current research evaluated the chemical composition of *T*. *diversifolia* essential oils and investigated their antibacterial potential against *R*. *solanacearum in vitro*, with the aim of developing natural, green, and sustainable biopesticides for application in the management of bacterial wilt disease in tomato.

MATERIALS AND METHODS

Sample collection and preparation

Fresh leaves and flowers from *T*. *diversifolia* were collected from Maseno in Kisumu County GPS location, 0°02'10.2"S34°45'18.8" E, in Kenya. All the plant samples collected were taken to the Chemistry laboratory at Moi University. Voucher specimens of the plant species were deposited at the laboratory of Biological Sciences of Moi University. The fresh samples from *T. diversifolia* were then washed using distilled water to remove dust and immediately chopped into small sizes and placed into 1000 mL clevenger apparatus for hydrodistillation.

Extraction

Essential oils from the fresh leaves and flowers of *T*. *diversifolia* were extracted separately *via* hydrodistillation using clevenger apparatus according to the methods of Liu *et al*., [30], as described by Wanzala *et al*., [31]. Briefly, 1 kg of freshly chopped *T*. *diversifolia* leaves and flowers were separately weighed and hydrodistilled with 1500mL of distilled water using clevenger apparatus for 8 hours. After hydrodistillation was completed, the volatile essential oils were removed from the top of the hydrosol, dried over anhydrous sodium sulfate (Na_2SO_4), and stored in sealed amber bottles at 4 °C awaiting chemical and bioassay analyses.

GCMS Analysis of essential oils and solvent extracts

GC-MS analyses was performed with a Clarus 500 GC gas chromatograph (Perkin Elmer Inc., USA) coupled with a Clarus 500 MS quadrupole mass spectrometer (Perkin Elmer Inc., USA). Gas chromatography was carried out on a fused-silica capillary column (Elite-5 ms, 60 m×0.25 mm, 0.25 µm film thickness, Perkin Elmer Inc, USA). The gas chromatograph was equipped with an electronically controlled split/splitless injection port while the carrier gas was helium with a constant flow of 1.2 mL/min. The GC oven temperature was set at 200 ˚C for

4 min and programmed in the range 200 ˚C -330 ˚C at a rate of 5 ˚C /min and finally held constant at 330 ˚C for 15 min. Ionization was performed in the electron impact mode at 70 eV, while detection was carried out in scan mode from m/z 35- 700 atomic mass units (a.m.u). Relative percentage amounts were obtained directly from GC peak areas while retention time was recorded in minutes. Components present in the essential oils were identified by matching of mass spectral data with MS library NIST 08 (NIST/EPA/NIH) and comparing the MS fragmentation patterns with those reported in the literature.

Fourier Transform Infrared (FT-IR) spectroscopy

The essential oils were characterized to confirm the functional groups in the compounds identified by GC- MS analysis. The essential oils, from *T. diversifolia* leaves and flowers, were analyzed using an Attenuated Total Reflectance- Fourier Transform Infrared (ATR-FTIR) spectrophotometer, NICOLET 6700 Thermo Scientific 2009-27701 Model. The FT-IR spectra were recorded in the spectral range 4000 to 500 cm^{-1} and scanning was performed with a resolution of 4 cm^{-1} for 100 scans. The functional groups of compounds present in the essential oils were determined by comparing the wavenumbers in the spectra with those on an IR correlation chart and comparison of spectral data with those reported in previous studies.

Bactericidal Activity

Isolation and characterization of Ralstonia solanacearum strains

All experiments were performed using highly virulent *R. solanacearum* strain race 3 biovar III which was isolated from ten diseased potato plants from Timboroa, Uasin Gishu County, and deposited at the Biological sciences Laboratory at Moi University. Collected potato tubers were sterilized with 1% Sodium Hypochlorite (NaOCl) solution for 2 min, followed by three repeated washings with distilled water and blot dried according to the methods of Singh as described by [32]. The plant sections (0.5 cm) were then placed inside test tubes containing distilled water and then be plated onto 2, 3, 5 triphenyl tetrazolium chloride (Kelman's TZC agar) medium (glucose 10 g, peptone 10 g, casein hydrolysate 1 g, agar 18 g, distilled water 1000 ml). An aliquot of 5 ml TZC solution filter-sterilized was added to autoclaved medium to give a final concentration of 0.005% v/v, followed by incubation of the plates at 28 ˚C for 48 hours [32]. The virulent colonies in the medium were characterized by dull white color, fluidal, irregularly round with light pink centers which were further streaked on TZC medium to get pure colonies of the bacterium. Isolated pure colonies of *R. solanacearum* were refrigerated at 20 ˚C to maintain their virulence. To revive an isolate, the stored bacteria were streaked on a TZC agar medium and well-separated fluidal colonies were selected. Preparation of *R. solanacearum* bacterial suspension was performed by pouring sterile distilled water over 24hr old bacterial growths on nutrient agar slants, and the suspension was adjusted to an optical density (O.D) 0.5 in Spectrophotometer (Beckham Coulter DU 700) to obtain a bacterial population of 1×10^8 colony-forming unit per milliliter of the suspension (optical density at 600 nm). Profiling of the pathogen was performed morphologically using culture techniques and biochemical tests including Gram staining test, Potassium hydroxide test, Catalase oxidase test, Gas production test, Starch hydrolysis test, and sugar utilization test according to Manual of Systematic Bacteriology as described by [32].

***In vitro* disc diffusion experiments**

The antibacterial activity of the essential oils from T. *diversifolia* leaves and flowers were tested *in vitro* against *R. solanacearum* according to the disk diffusion method described by [33].

Briefly, a single colony of the isolate containing 1x 10^8 Colony Forming Units (CFU) at 0.5 optical density measured at 600nm was grown on casamino acid peptone glucose medium (CPG) at 28 °C for 48 hours. Each bacterial suspension (200 µl) was then spread on nutrient agar plates and 5mm diameter disks containing 50 µL of essential oil were placed on the surface of the prepared agar plates. Sterile 1% dimethyl sulphoxide was used as negative control while Metham sodium (125 µg/mL) a known soil fumigant [1]. was used as a positive control. The plates were then incubated at 28°C for 24 hours after which diameters of inhibition zones were measured in millimeters using Vernier calipers. All experiments were performed in triplicate.

Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was evaluated using the broth microdilution method using a 96 well microtiter plate as described by [34], with a few modifications on the synthetic fumigant used. Briefly, 50µL of essential oil was added from the 1st to the 10th well on each row and diluted two-fold using 1% DMSO in the concentration range 1.953- 1000 µg/mL. Negative control (1% DMSO) and positive control (metham sodium) were also prepared in the same concentration range. Then, 50µL of 1x 10^{-8} CFU of freshly prepared *R. solanacearum* colonies were added to each well followed by incubation at 28 °C for 24 hours. Finally, 50 µL of 0.01% Tetrazolium chloride medium (TZC) was added to each well followed by incubation at 28 °C for one hour after which MIC was evaluated by visual observation of the color change of the Tetrazolium chloride medium.

Statistical analysis

Data on the *in vitro* antibacterial activities of *T. diversifolia* essential oils against *R. solanacearum* were analyzed statistically using Minitab version 17 software at a 99% confidence interval. Data from mean inhibition zones from three replicate experiments on the four treatments bioassayed were analyzed and the standard mean error was computed. The difference between the means was analyzed using One Way Analysis of Variance (ANOVA). Means of inhibition diameters were separated using Tukey's honestly significant difference test [33]. p-values <0.01 were considered statistically significantly different [35].

RESULTS AND DISCUSSIONS

Extraction of essential oils

Essential oils from leaves and flowers of *T.diversifolia,* were hydrodistilled using clevenger apparatus. Data obtained was tabulated as shown below:

Table 1: Percentage yield for essential oils

Plant extracts	Part of the plant	% yield (Volume/ weight)	% yield (Weight/ weight)
T.diversifolia	Leaves	0.18± 0.08	0.10± 0.07
	Flowers	0.15 ± 0.09	0.08 ± 0.03

Percentage yield of the essential oils was calculated in volume by weight and weight by weight (Igwaran *et al*., 2017) as shown in equations1 and 2 below:

$$\% \text{ Essential oil yield (v/w)} = \frac{Volume\ of\ esential\ oil\ (ml)}{weight\ of\ plant\ sample\ (g)} \qquad \text{(Equation 1)}$$

$$\% \text{ Essential oil yield (w/w)} = \frac{Weight\ of\ esential\ oil\ (g)}{Fresh\ weight\ of\ plant\ sample\ (g)} \qquad \text{(Equation 2)}$$

Leaves of *T*. *diversifolia* gave the highest percentage yields of 0.18 ± 0.04 % (v/w); 0.10 ± 0.02 % w/w as compared to the flowers which yielded 0.15 ± 0.07 % (v/w); 0.08 ± 0.01 % w/w on a fresh weight basis, however there was no statistical significant difference between leaves and flowers essential oils yields (p> 0.01). Similar results were obtained by [36], who reported essential oils yields of 0.18% v/w in *T*. *diversifolia* leaves essential oils. Contrary, Moronkola et al., [37], performed hydrodistillation of air-dried plant material from *T*. *diversifolia* and reported essential oil yields of 0.019 and 0.1% w/w in the leaves and flowers respectively. In another study, [38], reported essential oils yields of 0.12% v/w while performing hydrodistillation of air-dried leaves of *T*. *diversifolia*. The low yields reported in the leaves and flowers may be due to the fact that air-dried plant materials were used for hydrodistillation contrary to the fresh leaves and flowers used in this current research. It is noteworthy that studies performed using fresh leaves of *T*. *diversifolia* leaves and flowers reported higher essential oils [26,36], suggesting that the sample preparation process is a significant factor that affects essential oils yields. Other factors known to affect *T*. *diversifolia* essential oils yields include the geographical location, climatic conditions, type of soil, and phenotypic characteristics of the plant [21,39].

GC MS analysis

GC MS analysis of *T*. *diversifolia essential* oils tentatively identified 21 compounds in the leaves and 8 compounds in the flowers as shown in Tables 2 and 3 respectively. The relative amounts were based on the relative percentage area computed directly from the area under the GC spectra (Figure 1 and Figure 2). Compounds were listed according to their elution order on a non-polar fused-silica capillary column (table 2). GC MS profiling revealed that the essential oil constituents of the leaves was dominated by fatty acids (74.2%), oxygenated sesquiterpenes (12%), and oxygenated monoterpenes (10.48%) (Table 4), whereby the major compounds present were found to be (Z,Z,Z)-9,12,15- Octadecatrienoic acid ethyl ester(18%), palmitic acid (16%)(1aS,4aS,7R,7aS,7bS)-1,1,7-Trimethyl-4-methylenedecahydro-1H-cyclopropa[e]azulen-7-ol (spathulenol) (12%), Cis- 9,12,15- Octadecatrienoic acid (8.14%), tetrateracontane (6%) and 1-Octen-3ylcetate (5.22%).The major chemical constituents in the flowers' essential oils were fatty acids (86.73%), sesquiterpene hydrocarbons (5.45%), and oxygenated sesquiterpenes (5%), as evidenced by the presence of α-linolenic acid trimethylester(33%), Z, Z Hexadecadienoic acid(26%), octadecanoic avid trimethylester (9%), palmitelaidic acid (8.49%) and S, 1Z, 6Z) -8 Isopropyl-1 methyl-5- methylenecyclodeca-1,6-diene (Germacrene D)(5.45%) azelaic acid (5.02%) and caryophyllene oxide (5.00%) (Table 3). Minor compounds assayed in the leaf essential oils included α-pinene (1.17%), β-pinene (1.17%), 1-octen-3-ol (2.21%), 2-octen-1-ol (1.17%) and bicycloheptane (0.63%). Similar findings were observed by Farias *et al*., [26], who reported that α- pinene, Limonene, (Z)-β-ocimene, Piperitone, and Spathulenol were some of the major chemical constituents identified in *T*. *diversifolia* leaves. In another study, contrary to our results, Wanzala et al., [31], analyzed the aerial parts of *T*. *diversifolia* growing on the southern slopes of Mount Elgon in western Kenya and reported that, α- pinene, β-pinene, iso caryophyllene, nerolidol, 1-tridecanol, limonene, sabinene, α-copaene, α-gurjunene, and cyclodecene are pre-dominantly distributed. In a recent study, Njuguna *et al*., [29], while evaluating the qualitative and quantitative profiling of essential oils from air-dried leaves of *T*. *diversifolia* collected from Kandara in Murang'a County, Kenya reported that 3-

carene was the most abundant compound in the essential oils. Major differences were observed in the essential oils composition of *T. diversifolia* growing in the Maseno region, Kisumu County, Kenya as compared to other regions in Vietnam [38], and Brazil [39], as reported in previous studies. This could be attributed to environmental conditions, ecological conditions, climatic factors, geographical distribution, extraction method, sample preparation protocols, and part of the plant assayed [21, 26, 39].

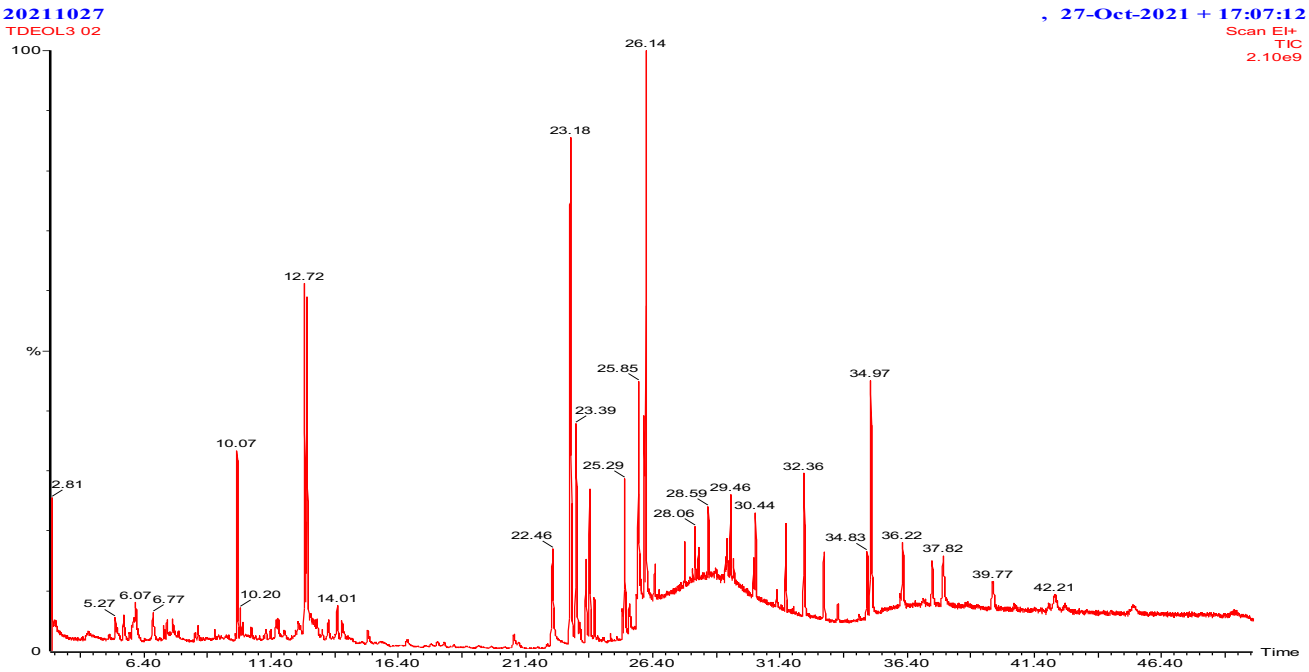


Figure 1: GC spectrum of *T. diversifolia* leaf essential oils

Table 2: GC-MS Analysis of compounds present in *T. diversifolia* leaves essential oils

Peak no	Retention time	Compound	Relative percentage area
1	5.27	alpha.-Pinene	1.17
2	6.07	1-Octen-3-ol	2.21
3	6.65	beta.-Pinene	0.22
4	6.77	2-Octen-1-ol, (Z)-	3.42
5	10.07	1-Octen-3-yl-acetate	5.22
6	10.20	Bicyclo(3.1.1)heptane-2,3-diol, 2,6,6-trimethyl-	0.63
7	12.72	Spathulenol	12
8	14.01	Azelaic acid bis trimethylester	1
9	22.46	n-Hexadecanoic acid	2
10	23.18	Palmitic acid	16
11	23.39	Palmitic acid, ethylester	7.12
12	25.29	(Z,Z,Z)-9,12,15- Octadecatrienoic acid methyl ester	2
13	25.85	Cis- 9,12,15- Octadecatrienoic acid	8.14
14	26.14	(Z,Z,Z)-9,12,15- Octadecatrienoic acid ethyl ester	18
15	28.06	Hexadecanoic acid cyclohexylester	2
16	28.59	Tetracosane	5.94
17	29.48	Heptacosane	2
18	30.44	Hentriacontane	1
19	32.36	Tetratriacontane	1
20	34.97	Tetratetracontane	6
21	37.82	Tetracosanoic acid	2

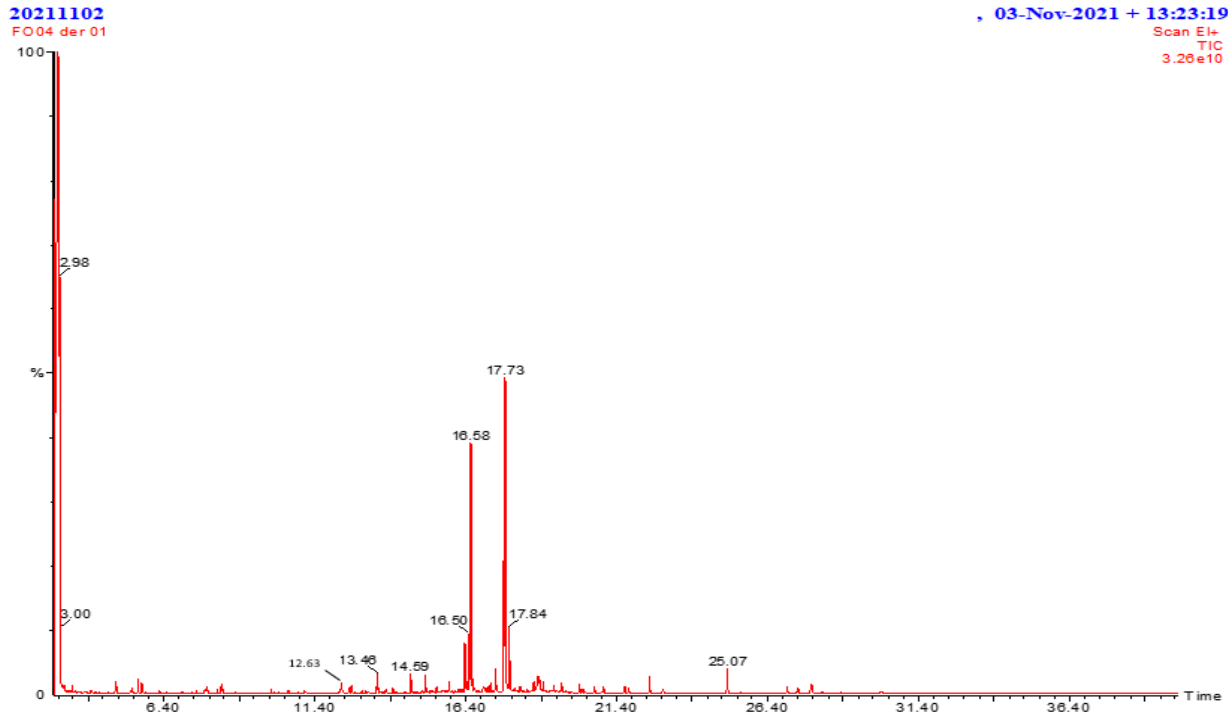


Figure 2 :GC spectrum for *T. diversifolia* flowers essential oils

Table 3: GC-MS analysis of compounds present in *T. diversifolia* flower essential oils

Retention time	Retention time	Compound	Relative percentage area
1	12.63	Caryophyllene oxide	5
2	13.45	Germacrene D	5.45
3	14.59	Azelaic acid bis trimethylester	5.02
4	16.50	Palmitelaidic acid	8.49
5	16.58	(Z,Z)-Hexadecadienoic acid - trimethylester	26
6	17.73	α-Linolenic acid, trimethylester	33
7	17.84	Octadecanoic acid trimethyl ester	9
8	25.07	Hexadecanoic acid cyclohexylester	5.22

Table 4: Analysis of chemical constituents present in *T. diversifolia* leaf and flower essential oils

Chemical compound group	Leaf Area %	Flower Area %
Monoterpene hydrocarbons	1.39	-
Oxygenated monoterpenes	10.48	-
Sesquitrepene hydrocarbons	-	5.45
Oxygenated sesquiterpenes	12	5
Fatty acids	74.2	86.73
Total	**98.07%**	**97.18%**

FTIR analysis

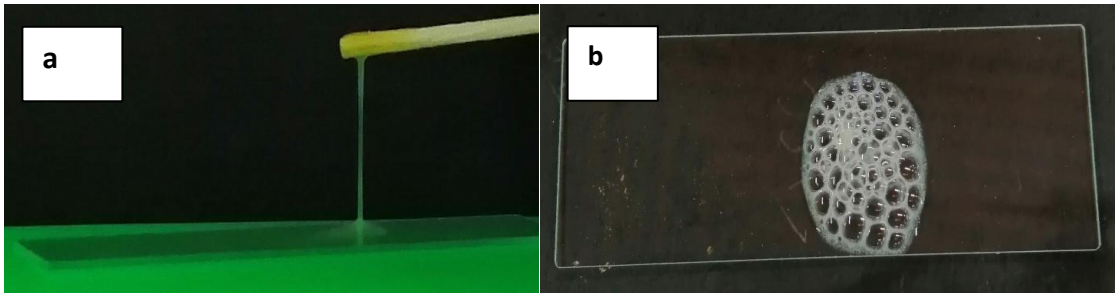
Characterization of *T. diversifolia* leaf essential oils using FTIR-ATR gave significant broadband at 3410.87 cm^{-1} in the region 3450 cm^{-1} to 3300 cm^{-1} representing –OH stretching vibrations of essential oils and two peaks at 2918.78 cm^{-1} and 2848.07 cm^{-1} depicting C-H stretching vibrations (Table 6). There was a significantly strong peak at 1710.63 cm^{-1} in the leaf essential oils which is a typical -C=O stretching bands [40], attributed to the presence of spathulenol which was one of the principal phytoconstituents identified by GC-MS analysis. Other vibrational frequencies were observed at 1466. 11 cm^{-1}, 1317.82 cm^{-1}, 1183.24 cm^{-1} and 1020.51 cm^{-1} as summarized on Table 6 below. In the flowers, significant peaks were observed at 3375.51 cm^{-1}, 2921.58 cm^{-1} , 2851.01 cm^{-1} , 1713.63 cm^{-1},1195.02 cm^{-1} and 1018.23 cm^{-1} (Table 6). Similar results were reported by [41-42].

Table 6: FTIR analysis of T. diversifolia leaf and flower essential oils

Major absorption bands (cm^{-1})		
***T. diversifolia* leaves essential oils**	***T. diversifolia* flowers essential oils**	**Functional groups**
3410.87	3375.51	-OH group
2918. 78	2921.58	-C-H stretching vibrations
2848.07	2851.01	-C-H stretching vibrations
1710.63	1713.63	-C=O stretching
1466.11	1442.54	-CH bending vibrations
1317.82	1309.47	-CH bending vibrations
1183.24	1195.02	C-O stretching vibrations
1020.51	1018.23	C-O-C stretching vibrations

In vitro Antibacterial activity

Highly virulent *R. solanacearum* strain race 3 biovar III were isolated from ten diseased potato plants from Timboroa, Uasin Gishu County, and were characterized using biochemical tests. The wild *R. solanacearum* colonies gave positive results for the catalase oxidase test and potassium hydroxide solubility tests as shown in Figures 5a and 5b below and were found to be gram-negative as depicted in figure 5c and 5d. Similar results were obtained by Khasabulli *et al*., [32], who reported that the positive catalase test could be attributed to the presence of catalase enzyme in *R. solanacearum* because Gram-negative bacteria undergo aerobic respiratory metabolism hence the Production of gas bubbles was observed.



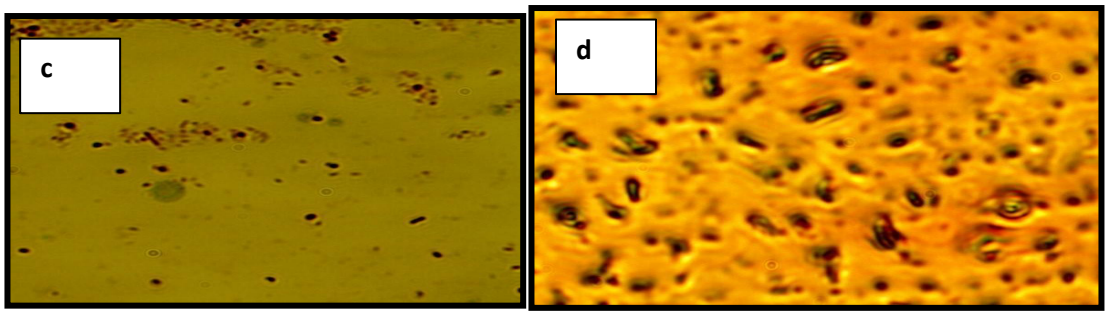


Figure 5 a) Potassium hydroxide solubility test, b) Catalase oxidase test, c) Gram staining of *R. solanacearum,* Magnification x 10, d) Gram staining of *R. solanacearum,* Magnification x 100

R. solanacearum colonies were identified by their large and elevated size, fluidal nature, and color [43], characterized by dull white color, fluidal, irregularly round with light pink centers as shown in Figure 6 a below. The virulent colonies were further steaked on TZC Kelman's medium to get pure colonies of the bacterium as depicted in Figure 6 b. Virulent colonies of *R. solanacearum* were identified by their large and elevated size, fluidal nature, and if they were either entirely white, or with a pale red center while the mutant and non-virulent strains of *R. solanacearum* were uniformly round and dark red, smaller in size, and butyrous or dry on TZC medium (Figures 6a and 6b). Similar results were obtained by [32, 43], who reported that the virulent *R. solanacearum* colonies were characterized by fluidal whitish with a pink center, indicating virulent species.

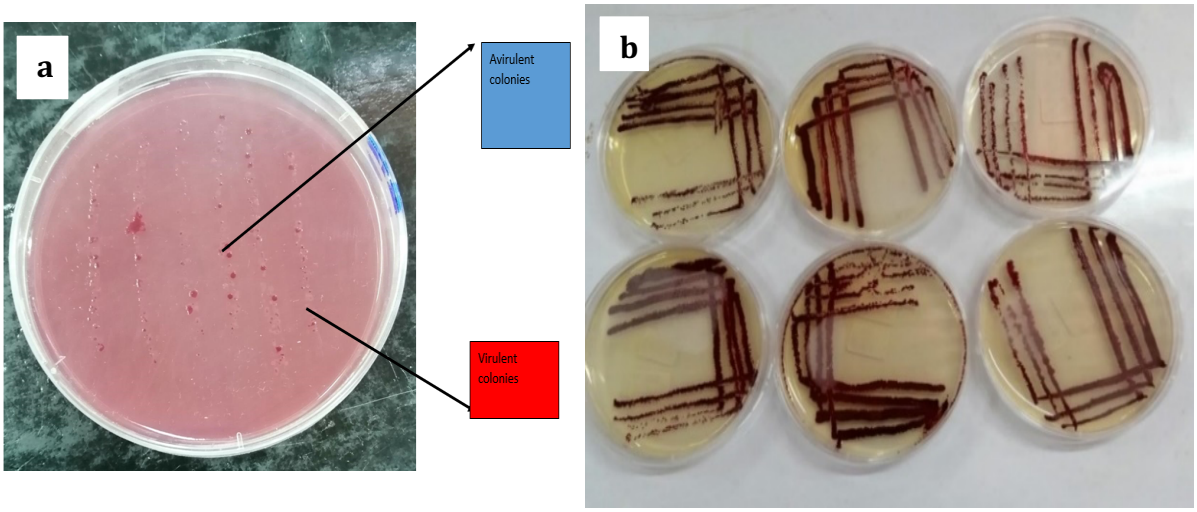


Figure 6: a) Virulent and avirulent colonies of *R. solanacearum* isolates b) Culturing of *R. solanacearum* bacterium on TZC Kelman's Agar medium

In vitro antibacterial activity of the essential oils from *T. diversifolia* leaves and flowers against *R. solanacearum* was analyzed using the disk diffusion quadrat method. The data obtained was tabulated as shown in Table 7 and illustrated in Figure 7.

Table 7: In vitro antibacterial activity of *T. diversifolia* essential oils against *R. solanacearum* after 24 hours of incubation at 28°C

SNO	Treatment	Diameter of inhibition zones in (mm)
1	1% DMSO	5±0.22^{c}
2	TDEOL	11.82 ±0.47^{b}
3	TDEOF	12.61 ±0.22^{b}
4	METHAM SODIUM	25.78 ±0.29^{a}

Values are the mean of three replicates ± standard error.
Values within columns followed by different letters are significantly different at P ≤ 0.01 according to Tukey's honestly significant difference test

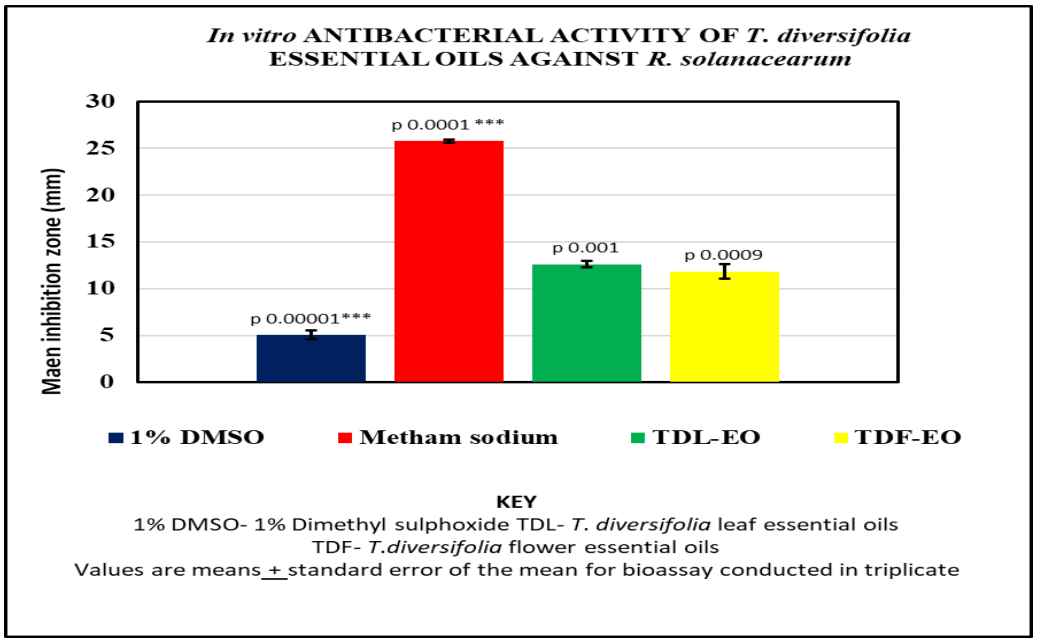


Figure 7: Antibacterial activity of *T. diversifolia* essential oils against *R. solanacearum in vitro*

Mean zone of inhibition results revealed that essential oils from the leaves of *T.diversifolia* exhibited the highest antibacterial activity of 12.61 ±0.22 in the leaves, as compared to 11.82 ±0.76 in the flowers. The MIC for *T. diversifolia* essential oils against *R. solanacearum* was 250 µg/mL in the leaves and 500 µg/mL in the flowers (Table 8) while the MIC for Metham sodium was 125 µg/mL. Statistical analysis of the antibacterial activity of *T. diversifolia* essential oils in comparison to Metham sodium which was the positive control revealed that there was a statistically significant difference between the antibacterial activity of *T. diversifolia* leaves in comparison to metham sodium ($p = 0.001$). Similarly, a statistically significant difference was observed between the antibacterial activity of Metham sodium and the antibacterial activity of *T.diversifolia* flowers essential oils ($p = 0.0009$) as shown in table 7. A comparison of bioactivity between the *T. diversifolia* leaf and flower essential oils and 1% DMSO which was the negative control (Table 7), revealed that there was a significant difference in bioactivity with $p < 0.01$. The observed antibacterial activity of *T. diversifolia* essential oils could be attributed to

phytochemicals including monoterpene hydrocarbons, oxygenated monoterpenes, and sesquiterpene Hydrocarbons, oxygenated sesquiterpenes, and fatty acids as depicted by GC-MS profiling of the major phyto compounds and confirmed by FTIR analysis.

Table 8: Minimum inhibition concentration (MIC) values for *T. diversifolia* essential oils, DMSO, and Metham sodium against *R. solanacearum* after 24 hours of incubation

Treatments	Concentration µg/mL									
	1.953	3.906	7.8185	15.625	31.25	62.5	125	250	500	1000
1% DMSO	+	+	+	+	+	+	+	+	+	+
TDLEO	+	+	+	+	+	+	+	+	-	-
TDFEO	+	+	+	+	+	+	+	-	-	-
METHAM SODIUM	+	+	+	+	+	+	-	-	-	-

KEY

+ bacterial growth appears

- no bacterial growth

Essential oils contain monoterpenes, diterpenes, and sesquiterpenes which serve as defense molecules in different plant parts [19], and hence the bioactivity of *T. diversifolia* essential oils against gram-negative. *R. solanacearum* bacterium *in vitro* could be attributed to the presence of the major chemical constituents including (1aS,4aS,7R,7aS,7bS)-1,1,7-Trimethyl-4-methylenedecahydro-1H-cyclopropa[e]azulen-7-ol, 0Z- Octen-1-ol, (24.22%), (Z,Z,Z)-9,12,15-Octadecatrienoic acid ethyl ester), Cis- 9,12,15- Octadecatrienoic acid(12%), 1-Octen-3ylcetate and (S, 1Z, 6Z) -8 Isopropyl-1 methyl-5- methylenecyclodeca-1,6-diene, which could be causing the synergistic antibacterial effect. Additionally, minor compounds profiled including α-pinene, β-pinene in the leaves, and caryophyllene oxide in the flowers are known antibacterial agents [44] and could be responsible for the observed antibacterial activity against *R. solanacearum in vitro.* These results are in agreement with a previous study by [26], who reported that the main compounds found in *T. diversifolia* essential oils including α-pinene, Limonene, (Z)-β-ocimene, p-cymen-8-ol, Piperitone, (E)-nerolidol, and Spathulenol could be responsible for the antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli,* and *Pseudomonas aeruginosa*. In another study, Li and Yu [45], demonstrated that *R. solanacearum* is susceptible to the presence of essential oils *in vitro*. The findings in this current research revealed that *T. diversifolia* leaf and flower essential oils possess remarkable *in vitro* antibacterial activity against *R. solanacearum* and hence, could be used to make viable formulations to combat the devastating tomato bacterial wilt disease.

CONCLUSION

This study demonstrated that *T. diversifolia* leaf and flower essential oils possess phytochemicals including monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, and fatty acids. Additionally, *in vitro* antibacterial activity showed that the profiled phytochemicals possessed remarkable antibacterial activity against *R. solanacearum*. Based on the observed antibacterial activity of the *T. diversifolia* leaves and flowers essential oils, this study emphasizes the great potential of these essential oils for commercial applications in the management of phytopathogenic bacteria and corroborates the extensive use of *T. diversifolia* in folkloric medicine.

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