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In vivo antibacterial activity of extracts of *Tithonia diversifolia* against *Ralstonia solanacearum* in tomato

Florence Atieno Opondo [a,b,*], Isaac Odhiambo K'Owino [a,b,c], Sarah Cherono Chepkwony [a,b], Viola Jepchumba Kosgei [a,b], Njira Njira Pili [b,d]

[a] *Department of Chemistry and Biochemistry School of Sciences and Aerospace Studies, Moi University, P.O Box 3900-30100, Eldoret, Kenya*
[b] *Africa Center of Excellence II in Phytochemicals Textiles and Renewable Energy (ACEII-PTRE), Moi University, PO Box 3900- 30100, Eldoret, Kenya*
[c] *Department of Chemistry, Faculty of Science, Masinde Muliro University of Science and Technology, PO Box 190-50100, Kakamega, Kenya*
[d] *Department of Biological Sciences, School of Sciences and Aerospace Studies, Moi University, PO Box 3900- 30100, Eldoret, Kenya*

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ABSTRACT

Currently, losses in crop yields caused by *Ralstonia solanacearum* have led to the overuse of synthetic pesticides, which are toxic and non-biodegradable hence detrimental to the environment. This study, therefore, investigated the *in vivo* antibacterial activity of ethanolic and hexane extracts from *Tithonia diversifolia* against *R. solanacearum* in tomatoes. Extraction was performed using the maceration method followed by evaluation of the extraction yields. During the *in vivo* greenhouse experiments, the antibacterial activity of the hexane and ethanolic extracts against *R. solanacearum* was performed using the root inoculation method while pathogenicity of the *R. solanacearum* strains to the susceptible Rio Grande 7-day-old tomato seedlings was investigated using root inoculation method under gnotobiotic conditions. The highest extraction yield of 2.8 ± 0.22 % w/w was observed in the ethanolic extracts of *T. diversifolia*, based on a dry weight basis. *In vivo* greenhouse experiments revealed that ethanolic extracts of *T. diversifolia* gave a percentage disease severity of 24 ± 2.6 % in the leaves and 32 ± 3.1 % in the flowers as compared to metham sodium (positive control), which gave a disease severity of 16 ± 2.2 % ($p < 0.01$). Based on these findings, utilization of *T. diversifolia* ethanolic extracts is hereby fronted as efficient, cheap, and potent biopesticides for the effective control of *R. solanacearum* in tomato plants.

Introduction

Bacterial wilt disease caused by *Ralstonia solanacearum* is a major constraining factor in the production of tomatoes worldwide [1], leading to an overreliance on synthetic pesticides for control of the phytopathogen. Several methods have been developed for the control of *R. solanacearum* including biological, physical, chemical, and cultural measures [1,2]. However, amongst the aforementioned methods, using synthetic chemicals is preferred by tomato farmers due to advantages such as efficacy, reliability, rapidity of action, and quick knockdown effect [3–5]. However, overreliance on synthetic pesticides has negative effects such as the development of resistant pests and pathogens, persistence in the environment, contamination of food crops, environmental degradation,

* Corresponding author.
E-mail address: florence.opondo@mu.ac.ke (F.A. Opondo).

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bioaccumulation, and bioconcentration of toxic residues in humans and non-target organisms [6,7] On the contrary, biopesticides are readily available, easy to use, nontoxic, and biodegradable hence safe to the environment [4,8,9].

Tithonia diversifolia also known as the Mexican sunflower is an invasive weed native to North and Central America [10,11] and has now been naturalized in Asia and Africa where it has become an ecological, agricultural, and economic burden due to its invasiveness [12]. In Nigeria, for example, *T. diversifolia* aggressively suppresses common weeds such as *Chromonaela odorata* [13], while in Kenya, it was introduced as an ornamental plant but has now escaped from cultivation and grows wildly on fields, hedges, along roadsides, and on fallowed land in Western and Central Kenya [14,15]. Despite the negative effects of this invasive shrub, it has been exploited in folkloric medicine as a remedy for *Diabetes mellitus* [11]. It is also used as a remediation for heavy metals from the soil [10,12] and has been found to have antibacterial, antifungal, antiviral, antiemetic, insecticidal, anticancer, antioxidant, anthelmintic, and antiplasmodial activities [5,16,17]. In Uganda and Kenya, *T. diversifolia* is locally known as Akechakech, Kinyula Ngaro, Itano, and Komanyoko (Banyankole community), maruru and amalulu (Luhya tribe), maua makech (Luo), amaua amaroro (Kisii), mula (Kamba), and mauat ne ng'wan (Kalenjin community), all implying the plant has a bitter taste, has been effectively used for the treatment of snake envenomation, malaria, and diarrhea [18–20].

The bioactive compounds in *T. diversifolia* have been demonstrated to possess antibacterial activity against gram negative and gram positive bacteria [21–23]. In a previous study, Oso *et al.*, [24], determined antibacterial activity of *T. diversifolia* extracts against clinical isolates of *Staphylococcus aureus, Klebsiella pnuemoniae, Pseudomonas aeruginosa* and *Staphylococcus aureus* and reported that the methanolic extracts showed good activity against clinical isolates of *P. aeruginosa*. In a recent study, Sumatra et al., [25] investigated the antimicrobial properties of medicinal plants from Indonesia and reported that the extractives from *T. diversifolia* leaves exhibited potential inhibitory activity against *Candida albicans, Bacillus subtilis, Salmonella typhi*, and *Escherichia coli*. The broad-spectrum antibacterial activity of *T. diversifolia* could serve as a good source of new antimicrobial agents and lead compounds for the formulation of biopesticides [21,26]. This research therefore, performed solvent extraction of *T. diversifolia* leaves and flowers and investigated the antibacterial activity of its extractives *in vivo* in a greenhouse set up as a safer, sustainable, readily available, and viable biopesticides for control of *R. solanacearum* in tomato.

Experimental

Collection and preparation of plant materials

Plant material 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, which consisted of leaves and flowers, were taken to the Chemistry laboratory at Moi University and voucher number given at the Biological Sciences laboratory of Moi University. The *T. diversifolia* plant materials were air-dried at room temperature for two weeks and the dried samples were then ground into fine powder using a laboratory grinder. After the size reduction process, powdered samples were sieved with a 200 mm sieve and stored in airtight sample bottles at room temperature awaiting extraction.

Extraction

The ground leaves and flowers of *T. diversifolia* were weighed and subsequently macerated at room temperature for 72 h using a solvent-to-liquid ratio of 1:4 with hexane and ethanol as solvents. The extracts were then filtered using Whatman filter paper no.1 (250 mm) and concentrated to dryness using a Hanshin rotary evaporator under vacuum at 40 °C and reduced pressure of 12 mg cm^{-3}. The obtained crude extracts were weighed by difference and kept in a desiccator awaiting further analyses.

Isolation and characterization of R. solanacearum strains

Highly virulent *R. solanacearum* strain race 3 biovar III were isolated from ten diseased potato tubers from Timboroa, Ainabkoi Subcounty, Uasin Gishu County GPS location, 40°44′30.8″N 73°59′21.5″W, in Kenya. Collected diseased potato tubers were deposited at the Biological Sciences Laboratory at Moi University [19]. The samples 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 Khasabulli et al. [27], with a few modifications. Briefly, 0.5 cm tuber sections were placed inside test tubes containing distilled water, and then 20 μL of 1×10^8 colony forming units (CFU) bacterial suspensions were plated onto 2, 3, 5 triphenyl tetrazolium chloride (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 of TZC solution filter-sterilized was then added to autoclaved medium to give a final concentration of 0.005 % (v/w). The plates were then incubated at 28 °C for 48 h at 65 % humidity. The virulent colonies in the medium were further streaked on TZC medium to get pure colonies of the bacterium suspension which were serially diluted by a 10^3 dilution factor, and subsequently, an aliquot of 20 μL of *R. solanacearum* colonies were plated on agar plates containing Nutrient agar (NA) amended with Kelman's Tetrazolium Chloride (TZC). Characterization of the pathogen was then performed using the Gram staining, potassium hydroxide, catalase oxidase, gas production, and starch hydrolysis tests according to the methods Chaudhry and Rashid, [28] as described by Khasabulli et al. [27] The isolated *R. solanacearum* bacteria were further identified according to their ability to utilize maltose, lactose, cellobiose, mannitol, sorbitol, and dulcitol as described by [29], and stored at 20 °C awaiting further *in vivo* experiments.

Root inoculation and pathogenicity of R. solanacearum strains in tomato seedlings

Pathogenicity of the *R. solanacearum* isolates was performed using the root inoculation methods of Singh *et al.* [30] Briefly, tomato seeds (Rio Grande cultivar) were presoaked in sterile distilled water for 2 days followed by spreading the seeds on sterile wet tissue paper in a petri dish and allowed to germinate in the incubator for 7 days at 28 °C. Preparation of *R. solanacearum* bacterial inoculum was performed by streaking pure colonies of virulent bacterium onto Bacto agar glucose medium supplemented with 0.5 % glucose. The freshly grown *R. solanacearum* virulent colonies were then added to 50 mL of Bacto agar broth with a sterile loop and allowed to grow in the incubator maintained at 28 °C. After 24 h, bacterial cultures were centrifuged at 4000 rpm (3155 × g*)* for 15 min at 4 °C. Root inoculation of tomato seedlings with *R. solanacearum* was performed *in vivo* using the previously germinated 7-day-old tomato seedlings. An aliquot of 20 mL of *R. solanacearum* inoculum (1×10^8 CFU) was taken in a sterile container. The roots of 7-day-old tomato seedlings were washed with distilled water to remove soil and dipped in the bacterial inoculum (up to the root-shoot junction) and transferred to an empty 5 ml sterile microfuge tube. The inoculated seedlings were then transferred to centrifuge tubes and subjected to air exposure for 5 min prior to the addition of 2 mL of sterile water to each tube. Plants exhibiting bacterial wilt symptoms were recorded.

In vivo growth experiments

In vivo growth inhibition assay was conducted in a greenhouse set up using tomato Rio Grande seedlings. Briefly, *R. solanacearum* disease control experiments, pots (0.4 L) were filled with 400 g of dry soil previously sterilized using an autoclave at 121 °C for 20 min, and 50 mL of sterile water was added to each pot to moisten the soil. Tomato seeds were sown in Petri dishes containing tissue paper and placed in an incubator at 25°C. After 7 days of growth in the incubator, 30 tomato seedlings were transplanted in plastic pots containing 400 g of sterile soil in greenhouse experiments. After six weeks of growth in the greenhouse, tomato seedlings were inoculated with 10 mL of 1×10^8 CFU/mL *R. solanacearum* bacterial suspension at optical density 600 nm and applied via non-injured root inoculation methods of Chen et al. [31] with a few modifications. Briefly, two hours after inoculation with *R. solanacearum* bacterium, treatments were applied by drenching the soil with 50 mL of *T. diversifolia* ethanolic extracts (250 mg/mL), and hexane extracts (500 mg/mL) applied by root irrigation. An aliquot of 50 mL 1 % DMSO and 125 mg/mL concentration of metham sodium were used as the negative and positive controls respectively. All inoculated tomato seedlings were monitored and watered once every two days. To reduce experimental errors, all the treatments were performed on 5 pots totaling 30 experimental units performed in a randomized complete block design. The disease severity was assessed for 28 days and based on the proportion of wilted leaves [32] the disease index was calculated using the following equation.

$$\text{Disease Index } (\%) = \frac{\sum(ni\ x\ vi)}{V\ x\ N}\ x\ 100 \quad (1)$$

Where,

ni - is the number of plants with the respective disease levels
vi - is the disease level
V- is the highest disease level (5)
N - is the number of test plants per treatment

Statistical management and data analysis

All quantitative data obtained from extraction and disease severity (%) from the *in vivo* experiments were analyzed statistically using Minitab version 17 software at 99 % confidence interval. Descriptive statistics were performed, and the results were expressed as a mean ± standard error of the mean (SEM) of the replicate experiments. Data from *in vivo* studies from five replicates on the four treatments bioassayed were analyzed and the standard error of the mean was computed. The difference between the means was analyzed using One Way Analysis of Variance (ANOVA). Disease severities (%) were separated using Tukey's honesty significant difference test. p-values < 0.01 were considered statistically significant.

Table 1
Percentage extraction yields of ethanolic and hexane extracts from both the leaves and flowers of *T. diversifolia.*

Solvent	*T. diversifolia* leaves (%) w/w		P*T. diversifolia* flowers (%) w/w
Ethanol	2.8 ± 0.22*	1.2 ± 0.15	0.01*
Hexane	0.92 ± 0.08*	0.74 ± 0.11	0.01*

Results and discussion

Extraction yields

The ethanolic extracts of *T. diversifolia* obtained by maceration yielded 2.8 ± 0.22 % w/w in the leaves and 1.2 ± 0.15 % w/w in the flowers, based on a dry weight basis. The hexane extracts yielded 0.92 ± 0.08 % and 0.74 ± 0.11 % in the leaves and flowers respectively. Overall, a higher percentage yield was reported in the ethanolic extracts as compared to the hexane extracts and the difference was statistically significant ($p < 0.01$) as shown in Table 1 below:

Values are the mean of three replicates ± standard error of the mean. Values within rows followed by asterisks are significantly different at P < 0.01 according to ANOVA test.

The effect of extraction solvents has been reported to influence the nature and extraction efficiency of the bioactive compounds from plants [33]. In this context, selecting an appropriate extraction solvent is essential for obtaining the required bioactive compounds from a plant matrix. A previous study by Pavela *et al.*, [34], highlighted the importance of extraction solvent with good yield rate of 5.28 % from methanolic extracts of *T. diversifolia* leaves. In a recent study, Gitahi et al. [35], investigated the effects of selected organic solvents on toxicity of *T. diversifolia* and reported extraction yields of 1.69 % in dichloromethane and 1.44 % in ethyl acetate, based on a dry weight basis. In this current study, the use of ethanol as extraction solvent is accentuated as evidenced by the good extraction yield of 2.8 ± 0.22 (leaves) and 1.2 ± 0.15 % (flowers), as compared to hexane solvent which afforded extraction yields of 0.92 ± 0.08 % and 0.74 ± 0.11 % in the leaves and flowers respectively. Other factors known to affect extraction yields in *T. diversifolia* include temperature of extraction and method of extraction [36]. Furthermore, the type of solvent used has to be able to afford large quantities of the extractant, possess ease of subsequent handling of the extractant, have less or no toxicity in addition to having less potential health hazards to human beings and non-target organisms [36]. Considering the aforementioned properties of a good solvent for extraction of bioactive compounds, this current research fronts the use of ethanol as solvent of extraction and maceration as the best extraction method for thermally labile compounds.

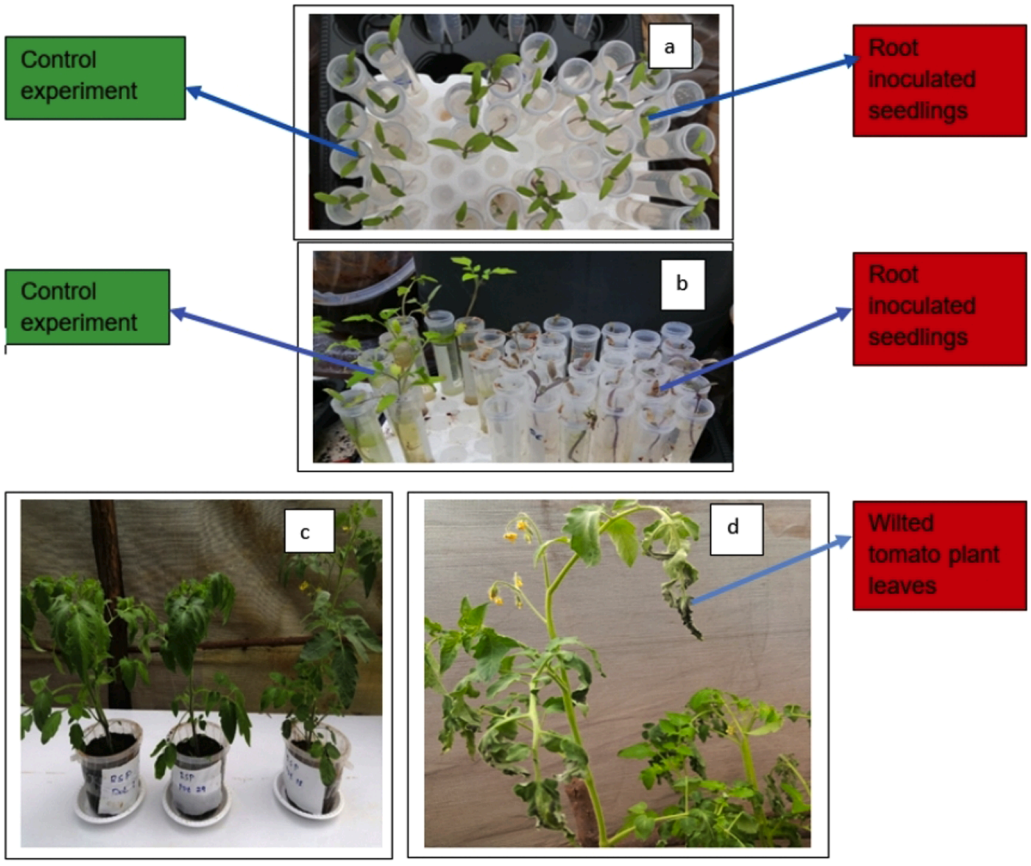

Fig. 1. (a) Root inoculation of 7-day-old tomato seedlings in centrifuge tubes (b) 14-day-old root-inoculated tomato seedlings growing in centrifuge tubes (c) 42 days old tomato plants before inoculation (d) 46 days old tomato plants 4 days post inoculation.

Biochemical tests and characterization of bacterial strains

The biochemical characterization revealed that the bacterial strains gave positive biochemical results for the oxidase reaction, catalase reduction, gas production, and potassium hydroxide test. The isolated bacterial strains were found to be gram-negative and were rod-shaped indicating a characteristic feature of *R. solanacearum* pathogenic bacteria. Similar results were obtained by Opondo et al. and Khasabulli et al. [19,27]. During the biochemical tests, the bacterial strains gave positive tests for utilization of disaccharides including cellobiose, lactose, and maltose. Additionally, positive alcohol utilization was also recorded for dulcitol, mannitol, and sorbitol. Based on the biochemical tests and phylotypic characteristics, the isolated *R. solanacearum* strains were classified as Race III biovar III [37].

Pathogenicity test and in vivo green house experiments

The virulence of the isolated *R. solanacearum* strains were confirmed using their pathogenicity to the susceptible Rio Grande tomato variety under greenhouse conditions. During the pathogenicity test carried out in the greenhouse, 7-day-old tomato seedlings were root inoculated using the isolated *R. solanacearum* strains, while the control experiments were seeded with 1 % dimethylsulphoxide (Fig. 1a).

Pathogenicity of the isolated *R. solanacearum* strains to tomato was confirmed by wilting and final death of the tomato seedling seven days post inoculation (Fig. 1b). Control experiments having seedlings growing in centrifuge tubes seeded with 1 % Dimethyl sulphoxide remained green and healthy as illustrated in Fig. 1b. During *in vivo* greenhouse experiments, random pots were selected for treatment with *T. diversifolia* extracts, totaling 30 pots. Each treatment had five pots while 1 % DMSO and synthetic fumigant (metham sodium) served as negative and positive controls respectively. Fig. 1c shows healthy 42-day-old tomato plants growing inside the greenhouse while Fig. 1d shows 46-day-old tomato plants with leaves showing symptoms of *R. solanacearum* as evidenced by the green and wilted leaves at 4 days post-inoculation (4 dpi).

Data from the *in vivo* studies were analyzed and the results tabulated as shown in Table 2 and illustrated in Fig. 2. As depicted in the figure, a disease severity index of 24 $\pm$ 2.6 % was observed in *T. diversifolia* leaf ethanolic extracts (TDL-EE) and 32 $\pm$ 3.1 % in *T. diversifolia* flower ethanolic extracts (TDF-EE), as compared to the lowest disease severity index of 16 $\pm$ 2.2 % reported in the pots treated with the positive control. Statistical analysis showed that there was a statistical significant difference between the bioactivity of ethanolic extracts of *T. diversifolia* leaves and flowers and the bioactivity of metham sodium against *R. solanacearum in vivo* ($P < 0.01$) as tabulated in Table 2 and illustrated in Fig. 2. It is noteworthy that both hexane extracts and the negative control gave the highest disease severity index of 80 $\pm$ 4.0 %, 80 $\pm$ 3.8 %, and 88 $\pm$ 4.1 % respectively, as evidenced by the percentage of wilted leaves that were on the potted plants as illustrated on Fig. 2 below.

Overall, high disease severity was observed in the hexane extracts of *T. diversifolia* leaves and flowers as compared to the ethanolic extracts, which could be attributed to the absence of hydrophilic constituents. The *in vivo* antibacterial activity of the hexane extractives revealed that the activity was comparable to the negative control (1 % DMSO), as evidenced by the observed disease severity of 88 $\pm$ 4.1 %, which revealed that the extracts had no activity against *R. solanacearum* phytopathogen, causing bacterial wilt disease in the tomato plants during the *in vivo* assays. The present study also revealed that ethanolic extracts of *T. diversifolia* leaves and flowers possess bioactive compounds which could be responsible for the reported antibacterial activity of this species during the *in vivo* greenhouse experiments. This phenomenon explains the low disease index and disease score levels observed in the leaves as compared to the flowers, which could be responsible for the observed antibacterial activity of this species during the greenhouse experiments in this current research. Similar observations were reported by Narasimhamurthy et al., [38], while evaluating *in vivo* antibacterial activity of salicylic acid and *Amomum nilgiricum* and Alemu et al., [5], while investigating the effect of invasive alien species including *Eichhornia crassipes, Mimosa* diplotricha, *Lantana camara* and *Prosopis juliflora* against tomato bacterial wilt caused by *R. solanacearum*.

In this current research, during the *in vivo* greenhouse experiments, ethanolic extracts of air-dried leaves and flowers of *T. diversifolia* exhibited remarkable antibacterial activity against *R. solanacearum* with low disease severity of 24 $\pm$ 2.6 % and 32 $\pm$ 3.1 %, in the leaves and flowers, respectively. On the contrary, the hexane extracts gave a high disease severity of 80 $\pm$ 3.8 % in the leaves and 80 $\pm$ 4.0 %, in the flowers. The reported antibacterial activity could be attributed to the presence of bioactive compounds in the ethanolic extracts of *T. diversifolia* leaves and flowers. It is therefore evident that ethanolic extracts of *T. diversifolia* have potent antibacterial activity against *R. solanacearum* and is envisaged to provide a safe alternative to synthetic pesticides in the management of bacterial wilt disease in tomato and other solanaceous crops.

Table 2
Comparison of disease severity of *R. solanacearum* in tomato plants.

Treatment	% Disease severity of *R. solanacearum* in tomato plants	p
Metham sodium (positive control)	16 $\pm$ 2.2	–
1 % Dimethylsulphoxide (negative control)	88 $\pm$ 4.1*	0.0001*
T. diversifolia leaf ethanolic extract	24 $\pm$ 2.6*	0.01*
T. diversifolia flower ethanolic extract	32 $\pm$ 3.1*	0.01*
T. diversifolia leaf hexane extract	80 $\pm$ 3.8*	0.0001*
T. diversifolia flower hexane extract	80 $\pm$ 4.0*	0.0001*

Values are the mean of five replicates $\pm$ standard error of the mean. Values within rows followed by asterisk are significantly different compared to the positive control at $P < 0.01$ according to ANOVA test.

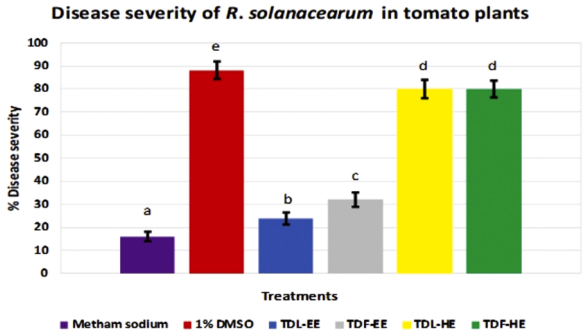

Fig. 2. Disease severity percentage of *R. solanacearum* in tomato plants showing that there was a significant difference in disease severity amongst the four treatments, the positive control and the negative control assayed. Values are plotted as mean ± SEM for five replicates; bars denoted by different letters are significantly different ($P < 0.01$; one-way ANOVA using Tukey's honesty significant difference test). DMSO: Dimethylsulphoxide. TDL-EE: *T. diversifolia* leaf ethanolic extracts. TDF-EE: *T. diversifolia* flower ethanolic extracts. TDL-HE: *T. diversifolia* leaf hexane extracts. TDF-HE: *T. diversifolia* flower hexane extracts.

Conclusion

The findings from the *in vivo* greenhouse studies elaborated the remarkable antibacterial activity of the ethanolic extracts of *T. diversifolia* against *R. solanacearum* in tomato-potted plants, as evidenced by the low disease severity of 24 ± 2.6 % and 32 ± 3.1 %, in the leaves and flowers respectively as compared to metham sodium, positive control (16 ± 2.2 %). The reported antibacterial activity in this study was attributed to the presence of polar bioactive compounds in the ethanolic extracts of *T. diversifolia*. Based on the results, this study gives credence to *T. diversifolia* ethanolic extracts as natural, readily available, and potent biopesticides for sustainable control of *R. solanacearum* in tomato plants. Future studies on isolation, purification and characterization of the bioactive compounds will be considered.

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Data availability

All the data used to support the findings of this study are included in the article. Additional data is available from the authors upon request.

CRediT authorship contribution statement

Florence Atieno Opondo: Conceptualization, Data curation, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. **Isaac Odhiambo K'Owino:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing. **Sarah Cherono Chepkwony:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Visualization, Writing – review & editing. **Viola Jepchumba Kosgei:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing. **Njira Njira Pili:** Investigation, Resources, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that there is no conflict of interest whatsoever regarding the publication of this research work.

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