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Studies of *Warburgia ugandensis* lyophilized crude extract with *Trichoderma asperellum* on tomato blight pathogens

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ABSTRACT

Early and late blights in tomatoes are among the most destructive diseases. The causative agents are *Alternaria solani* and *Phytophthora infestans* respectively. *Warburgia ugandensis* extracts have been demonstrated to have wide prophylactic and curative use as biocontrol agents against fungal and bacterial pathogens in animals but not in plants. *Trichoderma* species have been used as biocontrol agents for many phytopathogens. *Warburgia ugandensis* stem bark samples were air-dried ground, weighed then soaked in distilled water. The soaked material was then filtered and filtrate lyophilized to obtain crude extract which was screened against *A. solani* and *P. infestans* both in vitro and in vivo. All assays were performed in triplicate. Preliminary phytochemical analysis of the extract and minimum inhibitory concentration (MIC) were determined. Data on the inhibition by the extract was analyzed using ANOVA and the differences between means separated by Tukey's test ($p<0.05$). *Warburgia ugandensis* extract in diffusion wells showed significant inhibition ($P= 0.001$) of the pathogens with no recovery. The MIC for *A. solani* was 1.25 mg/ml while for *P. infestans* was 2.5 mg/ml. Treatment with combined *Warburgia* and *Trichoderma* showed the least disease severity at 27.81 % for *P. infestans* and *A. solani*. This was closely comparable with the commercial fungicide which had disease severity of 33.21 %. The current study suggests that *Warburgia* lyophilized crude extract and *Trichoderma* have potential use as antifungal prophylactic biocontrol against both *Phytophthora infestans* and *Alternaria solani*. Further research is needed to analyze the bioactive compounds responsible for *Warburgia* activity against these pathogens.

Keywords: Biocontrol, Consumers, Safety, Phytochemicals, Blight.

INTRODUCTION

Warburgia ugandensis (Canellaceae) has a long history of ethno-medicinal uses in east, central and southern Africa. It is used to treat and manage a wide range of ailments including gastrointestinal disorders, sore throat, and malaria, respiratory and odontological ailments [1]. Some of the previous research with *Warburgia* has reported that stem bark dichloromethane extracts of *Warburgia* exhibited high antimicrobial activity against *Staphylococcus aureus* and *C. albicans* [2]. Review on antimicrobial activities and phytochemicals present in *Warburgia* indicate that many microorganisms are susceptible to the extracts due to presence of sesquiterpenes [3]. Replacement of chemical pesticides by biological antagonists has safety advantages for consumers and the environment [4]. Commercial fungicides encourage the development of resistance in *Phytophthora infestans* and the pathogen requires higher doses of fungicides in controlling the disease, which increases production costs and has adverse effects on the environment [5].

Trichoderma species are fungi that are naturally present in all types of soils, are antagonistic and parasitic on many plant pathogens [6]. Apart from being a biocontrol agent, the fungus also stimulates plant resistance to diseases, and plant growth [7, 6]. Segarra *et al.*, [8] reported that the use of *Trichoderma asperellum* in pepper plants was able to reduce disease severity of *Phytophthora capsici*. *Trichoderma* species has natural resistance to many toxic compounds [5] and thus can be used together with inorganic compounds without interfering with its activity as a biocontrol agent. The use of *T. asperellum* in the control of *Fusarium oxysporum* was effective due to the ability of *T. asperellum* to grow considerably faster and quickly overwhelming the pathogen [9]. The ability to grow rapidly gives antagonists an important advantage in competition for space and nutrients with pathogen [7]. There is huge amount of literature demonstrating increased research in diverse natural products from plants that can be used as alternative biocontrol chemicals [10].

Yield and quality of tomatoes are greatly affected by early blight and late blight [11]. Early blight of tomato is characterized by the appearance of brown to dark leathery necrotic spots first on leaflets producing target board effect [12]. Early blight poses a great threat to profitable tomato growing. Among the challenges in management of early blight is its variability in pathogenic isolates and prolonged

infectious phase in the disease cycle [12]. The infection causes defoliation, premature fruit drop and reduction in quality and quantity of tomatoes, which may result in up to 50 - 86% yield loss [13]. *Phytophthora infestans* is one of the most threatening pathogenic disease in tomatoes which results in yield losses and in farmers incurring high expenses for disease control and management [14]. Late blight is more prevalent in humid and cool weather. *Phytophthora infestans* effectively produces different types of motile flagellated zoospores that contribute to its severe infection through its successful spread by water and wind [8]. Sporangia are aerially dispersed and deposited on leaf surface, the motile zoospores are released in cool wet conditions and form appressoria which penetrate the leaf epidermis [15]. The pathogen survives for prolonged period and infects on-going cropping season by producing sporangia and zoospores through asexual reproduction while for the next season infection is through sexually formed oospores. The inoculum is harbored in leaves, fruits, debris and in the soil [14]. Late blight symptoms include water-soaked necrotic lesions on the leaves, stems, collar as well as damping off and severe root rot [15, 16]. The aim of this research was to find out the effectiveness of *W. ugandensis* and synergetic effects in combination with *T. asperellum* in management of *A. solani* and *P. infestans* both in vitro and in vivo. Chemical fungicides which are currently used extensively in agriculture cause environmental pollution among other safety challenges [17]. The current research findings could be used to promote the use of organic chemicals in management of blight diseases and reduction of environmental pollution by inorganic chemicals which are used in control and management of these diseases.

MATERIALS AND METHODS

Study site

The study was carried out in Nchiru, Nkomo ward, Meru County in Kenya which lies at 0.05º North longitude and 37.65 º East longitude and 1582 meters above sea level.

Isolation and identification of *P. infestans* and *A. solani*

The early and late blight pathogens of tomatoes were isolated from infected tomato leaves and fruits which were randomly sampled from farmer's farms in Nchiru, Meru County, Kenya. The samples were placed in brown paper bags and transported to the biological laboratory of Meru University of Science and Technology (MUST). The samples were washed under running tap water to remove any surface contaminants. The cleaned samples were then sterilized in 1 % Sodium hypochlorite for three minutes after which they were rinsed three times in sterile distilled water. The cleaned and sterilized samples were blot-dried using sterile blotting paper before being cut into smaller pieces of about 1cm. Direct plating was carried out on sterilized PDA and SDA media amended with antibiotic and incubated at 27 ºC for 5 days. Identification of the *A. solani* and *P. infestans* isolates was carried out according to Barnnet and Hunter [18].

Processing and extraction of *W. ugandensis* stem bark lyophilized crude extract

The *W. ugandensis* stem bark sample was obtained from Kenya Forestry Research Institute (KEFRI) at Muguga Kiambu County. The sample was air-dried at room temperature after which it was grounded into a fine powder using a disk mill. Two hundred grams of the grounded *W. ugandensis* stem bark was weighed and soaked in a 2000 ml conical flask using distilled water and left to stand on an orbital shaker for 24 hours. The soaked material was then filtered using a muslin cloth secured into the Buchner funnel under vacuum using a rotary water pump. The filtrate was put into freeze drying plates and left in a deep freezer for 24 hours until completely frozen. The frozen material was then transferred into the lyophilizer upper chamber and left to run for 28 hours at a temperature of -45 ºC and 1.0 Pascals pressure. The crude extract obtained was put in a sealed sample container and stored at 4 ºC for further analysis.

Phytochemical tests on *W. ugandensis* stem bark lyophilized crude extract

Tests for alkaloid, tannins, saponins, phylobatannins, flavonoids, terpenoids, steroid, phenols, and anthraquinones were carried out according to Yadav and Agarwala [19].

Invitro screening of *W. ugandensis* lyophilized crude extract and *T. asperellum* against blight pathogens

The bioassay screening of *W. ugandensis* extract for its antifungal activity against *P. infestans* and *A. solani* was carried out by well diffusion method on potato dextrose agar (PDA) growth medium as described by Abuto *et al*., [2]. The fungal suspension was standardized to 10^6 conidia/ml in sterile distilled water and 100 µl of each fungal suspension was obtained using a micropipette and spread onto the surface of the petri dishes. The petri dishes were allowed 10 min to solidify after which 5 mm diameter holes were punched on the PDA media in the petri dishes and the holes filled with 100 µl of the previously prepared *W. ugandensis* stem bark crude extract samples. The plates were incubated at 27 ºC for 72 hours. Each assay had 3 replicates. Water and Mancozeb were used as controls. The extract was used at concentrations of 0.025 g/ml and the commercial fungicide was tested at the recommended rate. Sterile distilled water was used as a diluent of the commercial fungicide. The assessment was carried out by measuring the diameter of the zone of inhibition. Dual culture method was used to investigate inhibition of the blight pathogens in vitro. Percentage inhibition of pathogen growth was calculated according to the formula developed Rahman *et al*., [20].

Minimum inhibitory concentration

The initial solutions of *Warburgia* extracts were diluted by two-fold serial dilution. Using a micropipette, 100 µl of broth was dispensed to 11 culture bottles which included both a negative and a positive control. One hundred microliters of the extract from the stock solution whose concentration was 10 mg/ml was transferred to the first culture bottle. This was mixed by using micropipette to draw up and place the mixture back until it was homogenous. Consequently, 100 µl of the contents of the first culture bottle was transferred to the second culture bottle already containing 100 µl of broth. Dilution and mixing were repeated for all the other culture bottles except for the positive control which only had 100 µl of the broth and 100 µl of pathogen only. One hundred µl of the contents of the last culture bottle was withdrawn and discarded, leaving all the culture bottles with uniform volume of 100 µl. A micropipette was then used to add 100 µl of the pathogen to each culture bottle except in the negative control which had 100 µl of the broth and 100 µl of sterile distilled water. All the culture bottles were then incubated at room temperature (25 ± 2 ºC) for 5 days. The concentration of the first culture bottle whose contents had no visible microbial growth was observed and recorded as the minimum inhibitory concentration (MIC). The contents of the culture bottle with MIC was then cultured on sterilized PDA plates.

In vivo* screening of *W. ugandensis* lyophilized crude extract and *T. asperellum* against *A. solani* and *P. infestans

Tomato seeds, Hazera- F1 Hybrid Galilea variety were planted in germination trays using sterilized growth media enriched with DAP fertilizer. Watering was carried out on alternate days untill 28 days in the nursery. The soil for transplanting was mixed with farm yard manure in the ratio of 2:1 before sterilizing in autoclave bags at 121 ºC for 15 minutes. After cooling, 3 kg of the soil was placed into transplanting sleeves. Transplanting was carried out when the seedlings were one month old. Routine management practices were carried out including watering, staking, pruning and top dressing. Inoculation of the plants with blight pathogens was carried out 8 days after transplanting. Sporulation of *A. solani* and *P. infestans* was induced by incubating the isolates in the dark, at 27 ºC for 7 days after which the PDA plates were incubated under continuous lighting for 4 days to induce sporangia formation. Finally, the plates were kept at 4 ºC for 30 minutes to induce cleavage of zoospores, which were released after returning the plates to 27 ºC under continous lighting. The zoospore on the plates were rinsed with sterile distilled water and the suspension was poured into separate beakers for each isolate.

A spore concentration of 1 x 10^6 was obtained using a hemacytometer (Improved Neubauer by ISO LAB) and was used for inoculating the tomato plants 8 days after transplanting. The plants were well watered before inoculating them on upper leaf surface with the help of cotton swab soaked in the inoculum and carborundum. The inoculated plants were enclosed in transparent polythene bag for 24 hrs to maintain humid conditions and improve infection success. Fourty eight hours later, the tomato plants were subjected to the following five treatments;

TW- Treatment with *W. ugandensis*

TT – Treatment with *T. asperellum*

TWT – Treatment with both *W. ugandensis* and *T. asperellum*

TM – Treatment with Mancozeb (+ve control)

TN – No treatment (-ve control).

There were 3 replicates for each treatment. Concentration of *W. ugandensis* that was used was 0.00625 g/ml. The same concentration was used for *T. asperellum.* Experimental design was complete randomized design. The evaluation was done at intervals of 7 days after inoculation for 3 weeks. Phenological assessments carried out were; scoring on disease severity [21], plant height and number of leaves. Percentage severity index (PSI) of the diseases was calculated. The in vivo trials were conducted in a modified greenhouse. Trial 1 was carried out between January and April 2018. The mean temperature was 19.8 ºC, the mean rainfall was 50.50 mm and the humidity was 65.33 %. The second in vivo trial was conducted between April and July 2018. The mean temperature was 19.8 ºC, the average rainfall was 92.53 mm while the humidity was 73.00 %.

Severity index scale

1=Trace - 20 % infection

2=21 % - 40 % infection

3=41- 60 % infection

4=61 - 80 % infection

5=81 - 99 % infection

6 = 100 and/or entire leaf defoliation [21].

PSI = Σ (Individual numerical rating x 100) ÷ (Total number of leaves x 6)

Measurement of plants height was carried out using a tape measure (cm), starting from the stem base at the soil surface to the shoot apex. The number of leaves were also counted.

Data analysis

Two way ANOVA was used to compare means of plant height, disease severity and number of leaves ($P < 0.05$). All the values were expressed as mean ± standard error. Significant mean comparison was conducted using Tukey's test ($\alpha = 0.05$). Percentage disease severity values were log transformed before data analysis. Results were significant if $p < 0.05$. Statistical Analysis Software (SAS) was used to carry out all the analysis.

RESULTS

Isolation and identification of *P. infestans* and *A. solani*

Phytophthora infestans colonies cultured on PDA were white in colour. Observation under the light microscope revealed lemon shaped sporangia borne at end of sporangiophores. *Alternaria solani* culture grown in Sabouraud dextrose agar (SDA) produced a deeply pigmented gray hairy colony or brown colony in potato dextrose agar (PDA). When observed under the light microscope, the mycelium was seen to be haploid and septate with beaked asexual conidia.

Minimum inhibitory concentration (MIC) of *Warburgia* extract

In the culture bottles containing broth and *P. infestans*, there was no growth in the first two culture bottles which had an extract concentration of 10 mg/ml and 5 mg/ml respectively. However, growth of *P. infestans* was observed from the third culture bottle which had *Warburgia* stem bark extract concentration of 2.5 mg/ml. Minimum inhibitory concentration for *P. infestans* was thus 2.5 mg/ml. *Alternaria solani* was found to have no growth in the first 3 culture bottles but growth was observed from the fourth culture bottle which had *Warburgia* stem bark extract concentration of 1.25 mg/ml. The MIC for *A. solani* was therefore 1.25 mg/ml. Growth of the cultures in the bottles containing MIC was observed. Growth of the pathogens was observed in the positive controls which had 100 µl of broth and 100 µl of the pathogen. No growth was observed in the negative controls which contained 100 µl of the broth and 100 µl of water.

***In vitro* screening of *Warburgia ugandensis* lyophilized extract against blight pathogens.**

Warburgia extract gave significant mean inhibition zone (P =0.001) in *A. solani* of 25.67 mm which was significantly different from the inhibition zone in *P. infestans* of 15 mm ($p \leq 0.05$). In this study, *W. ugandensis* extract inhibited *P. infestans* at 15 mm compared to Mancozeb whose inhibition was 11.33 mm (Fig. 1). In addition, *W. ugandensis* showed inhibition of *A. solani* at 25.67 mm compared to inhibition zone by Mancozeb of 24.67 mm (Fig. 2). No inhibition was observed in water control.

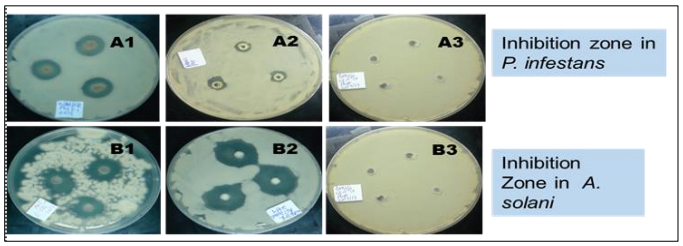

Figure 1: The figure shows inhibition zone of *P. infestans* and *A. solani* by *W. ugandensis* lyophilized crude extract, Mancozeb (+ve control) and water (-ve control). A1- Inhibition Zone of *P. infestans* by *W. ugandensis* lyophilized crude extract. A2 – Inhibition zone by Mancozeb. A3- inhibition by water. B1 – Inhibition zone of *A. solani* by *W. ugandensis* lyophilized crude extract. B2 – Inhibition by Mancozeb. B3- inhibition by water.

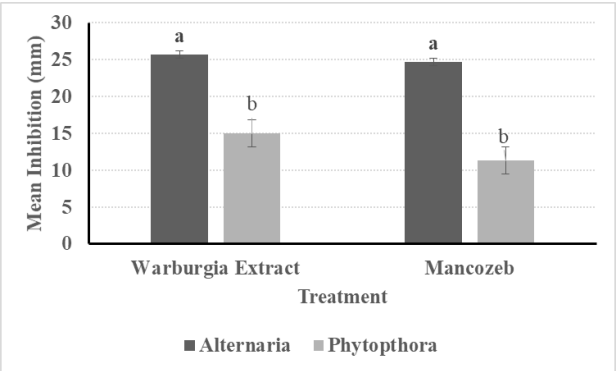

Figure 2: Represents mean inhibition zones of *A. solani* and *P. infestans* by *Warburgia* extract and Mancozeb. Data was analyzed from three replicates of each isolate (n=6). Inhibition zones were measured after 72 hours. Maximum and minimum bars on the bar chart indicates standard error. Mean inhibition zones with the same letter were not significantly different (P≤0.05).

Dual culture inhibition of *T. asperellum* against blight pathogens

Trichoderma colony changed colour as it matured from cream white to green-yellow and finally to green. The results on dual culture between *Trichoderma* and blight pathogens indicated that *T. asperellum* inhibited *P. infestans* by 65.48% and *A. solani* was inhibited by almost 100% on day 8 having grown over the *A. solani* colony as shown in Fig 2.

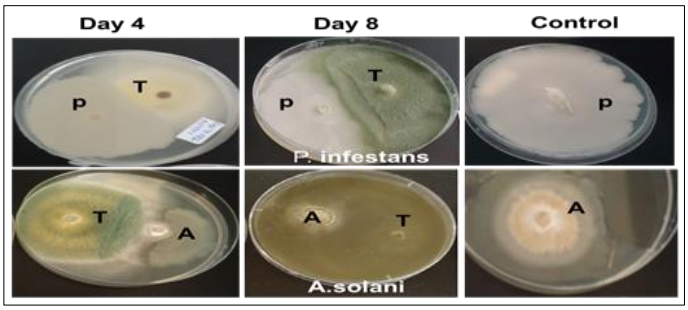

Figure 3: Dual culture inhibition by *T. asperellum* (T) on *P. infestans* (P) and *A. solani* (A) on day 4 and day 8. Results indicated that *Trichoderma i*nhibited growth of *P. infestans* by 65.49% but inhibited *A. solani* by 100% on day 8, [20].

In vivo screening of *W. ugandensis* lyophilized crude extract and *T. asperellum* against *A. solani* and *P. infestans.*

Percentage severity index

The disease severity in the two isolates, *P. infestans* and *A. solani* were significantly different. The highest disease severity in both trials was observed in the negative control and was significantly different from all the others. The lowest disease severity in both trials was observed in the treatment with *Warburgia* combined with *Trichoderma*, and was significantly different from the other treatments. Disease severity for treatments with Mancozeb, *Warburgia* and *Trichoderma* were not significantly different in trial one for both *Phytophthora* and *Alternaria*. In trial two, disease severity for the treatment with Mancozeb was significantly different from all the others while disease severity for treatment with *Warburgia* alone and Trichoderma alone were not significantly different (Table 1). All the p values were significant in trial one. The p values in trial two were also significant except for the interaction between isolate and the treatment which was not significantly different (P≤ 0.05).

Table 1: Percentage disease severity index for trial one and trial two Mean ±SE

	Percentage disease severity index for trial one Mean ±SE		**Percentage disease severity index for trial two Mean ±SE**	
Isolate	**Phytophthora**	**Alternaria**	**Phytophthora**	**Alternaria**
	39.38 ±1.01a	36.69 ± 1.01b	38.89 ± 1.01a	33.14 ± 1.01b
Treatment	**Phytophthora**	**Alternaria**	**Phytophthora**	**Alternaria**
TW	34.41 ± 0.31b	32.25 ± 0.31b	30.11 ± 1.22c	30.11 ± 1.22c
TT	32.99 ± 0.31b	33.74 ± 0.31b	29.54 ± 1.22c	29.54 ± 1.22c
TWT	28.27 ± 0.31c	28.14 ± 0.31b	27.81 ± 1.22d	27.81 ± 1.22d
TM	35.12 ± 0.31b	31.42 ± 0.31b	33.21 ± 1.22b	33.21 ± 1.22b
TN	84.07 ± 0.31a	69.16 ± 0.31a	74.32 ± 1.22a	74.32 ± 1.22a
P values				
Isolate	0.002		0.001	
Treatment	0.001		0.001	
Isolate * Treatment	0.018		0.320	
LSD				
Isolate	1.042		1.037	
Treatment	1.068		1.059	
Isolate * Treatment	1.029			

Data are the percentage disease severity index means ± standard error (SE) from 15 treatments (n=15) consisting of three replicates from 5 different treatments. Treatments: TW – *Warburgia* alone, TT- *Trichoderma* alone, TWT- *Warburgia* + *Trichoderma*, TM- Mancozeb (positive control), TN – Negative control. Mean values followed by the same letter within the same column are not significantly different at p ≤ 0.05.

Plant phenological characteristics

Mean plants height after treatments

In both trials the mean plant height for *Phytophthora* and for *Alternaria* were not significantly different. The treatments with *Warburgia* alone, *Trichoderma*, combination of *Warburgia* and *Trichoderma* and treatment with Mancozeb were not significantly different in trial one. Treatment for the negative control was the lowest and was significantly different from all treatments in both trial one and trial two. In trail two, treatment with *Warburgia* alone was significantly different from all the other treatments. Treatment with *Trichoderma* alone and the combined treatment with *Warburgia* and *Trichoderma and* Mancozeb were not significantly different in trial two (Table 2). The p values were not significantly different in both trials except for the treatments ($p \leq 0.05$).

Table 2: Mean plants height after treatments Mean ±SE (cm).

	Plant Height for trial one Mean ± SE (cm)		**Plant Height for trial two Mean ± SE (cm)**	
Isolate	**Phytophthora**	**Alternaria**	**Phytophthora**	**Alternaria**
	52.16 ± 4.07a	52.14 ± 3.74a	59.61 ± 3.83a	60.78 ± 3.27a
Treatment	**Phytophthora**	**Alternaria**	**Phytophthora**	**Alternaria**
TW	50.80 ± 3.08a	50.80 ± 3.08a	60.11 ± 3.75ab	60.11 ± 3.75ab
TT	60.11 ± 4.71a	60.11 ± 4.71a	66.04 ± 4.64a	66.04 ± 4.64a
TWT	58.85 ± 4.22a	58.85 ± 4.22a	65.16 ± 4.67a	65.16 ± 4.67a
TM	61.35 ± 4.51a	61.35 ± 4.51a	67.73 ± 4.67a	67.73 ± 4.67a
TN	29.63 ± 1.93b	29.63 ± 1.93b	41.91 ± 2.91b	41.91 ± 2.91b
P Values				
Isolate	0.997		0.779	
Treatment	0.001		0.004	
Isolate * Treatment	9.256		0.956	
LSD				
Isolate	7.834		8.581	
Treatment	17.770		19.463	

Data are the plant height means ± standard error (SE) from 15 treatments (n=15) consisting of three replicates from 5 different treatments. Treatments: TW – *Warburgia* alone, TT- *Trichoderma* alone, TWT- *Warburgia* + *Trichoderma*, TM- Mancozeb (positive control), TN – Negative control. Mean values followed by the same letter within the same column are not significantly different at $p \leq 0.05$.

Mean of number of leaves after treatments

The average number of leaves for *Phytophthora* and *Alternaria* were not significantly different in both trials. The mean number of leaves in the negative control was the lowest in trial one and two and was significantly different from the others. In trial one, the mean number of leaves in the other treatments were not significantly different. In trial two, the mean number of leaves in the treatment with *Warburgia* for both *Phytophthora* and *Alternaria* was significantly different from all the other treatments. The treatments with *Trichoderma* alone, combined *Warburgia* and *Trichoderma* and Mancozeb were not significantly different in trial two (Table 3). The p values were not significantly different in both trials except for the p value of the treatments which were significantly different ($p \leq 0.05$).

Table 3: Mean number of leaves after treatments Mean ±SE

	Number of leaves for trial one Mean ±SE		**Number of leaves for trial two Mean ±SE**	
Isolate	**Phytophthora**	**Alternaria**	**Phytophthora**	**Alternaria**
	18.00 ± 1.41a	17.93 ± 1.50a	21.40 ± 1.48a	21.27 ± 1.31a
Treatment	**Phytophthora**	**Alternaria**	**Phytophthora**	**Alternaria**
TW	16 ± 0.80a	16 ± 0.80a	20.67 ± 1.38ab	20.67 ± 1.38ab
TT	21 ± 1.65a	21 ± 1.65a	23.83 ± 1.83a	23.83 ± 1.83a
TWT	21 ± 0.80a	21 ± 0.80a	23.17 ± 1.38a	23.17 ± 1.38a
TM	22 ± 1.67a	22 ± 1.67a	25.00 ± 2.02a	25.00 ± 2.02a
TN	29.63 ± 1.93b	29.63 ± 1.93b	14.00 ± 0.86b	14.00 ± 0.86b
P Values				
Isolate	0.960		0.931	
Treatment	0.001		0.001	
Isolate * Treatment	0.991		0.943	
LSD				
Isolate	7.834		3.168	
Treatment	17.770		7.186	

Data are the number of leaves means ± standard error (SE) from 15 treatments (n=15) consisting of three replicates from 5 different treatments. Treatments: TW – *Warburgia* alone, TT- *Trichoderma* alone, TWT- *Warburgia* + *Trichoderma*, TM- Mancozeb (positive control), TN – Negative control. Mean values followed by the same letter within the same column are not significantly different at $p \leq 0.05$.

Phytochemical tests on *W. ugandensis* stem bark lyophilized crude extract

Table 4: Phytochemical analysis of lyophilized *W. ugandensis* stem bark extract

Parameters	Sample
Flavanoids	++
Terpenoids	+++
Saponins	+
Steroids	++
Phenols	++
Phylobatannins	++
Alkaloids	-
Tannins	+++
Anthraquinones	++

Key: + Faintly present ++ moderately present +++ highly present - Nil

In this study, qualitative preliminary survey of *Warburgia* extract indicated high presence of tannins and terpenoids (Table 4). In addition, flavonoids, steroids, phenols, phylobatannins and anthraquinones were also found to be relatively present. Saponins were also found to have low presence. No levels of alkaloids were detected.

DISCUSSION

Minimum inhibitory concentration (MIC) has been shown to vary in different pathogens as was observed in *Warburgia* inhibition of *A. solani* and *P. infestans*. This is because the two pathogens are different microorganisms which belong to different kingdoms. As a result they respond differently to the chemical substances present in *Warburgia* extracts and show different levels of inhibition. Previous research reported on investigation into leaf extracts of 6 selected South African plant species which were shown to be inhibitive against 7 selected phytopathogenic fungi including *Aspergillus niger, Colletotricum gloeosporioides, Trichoderma harzianum and Fusarium oxysporum*. *Breonadia salicina* leaf extract was found to have MIC values ranging between 0.08-2.50 mg/ml among the different phytopathogenic fungi [22]. The MIC values obtained are comparable to the MIC values of *Warburgia* extract against the blight pathogens. On culturing the contents of the MIC culture bottles for both pathogens, growth of the pathogens was observed meaning that the extract metabolites were fungistatic but not fungicidal. *Warburgia* thus has the potential of being developed as biocontrol agent against blight pathogens.

Results on the in vitro screening of *W. ugandensis* lyophilized extract indicated that the extract was more effective in controlling the growth of *A. solani* than *P. infestans*. This difference could be attributed to the fact that *P. infestans* produces different types of infective motile flagellated zoospores making it a more aggressive pathogen. Comparable results on differences in effectiveness were observed in *Warburgia. ugandensis* hexane extract which was screened against *Leishmania major, L. donovani* promastigotes and amastigotes and was found to be most effective against amastigotes [23]. Apart from *Warburgia*, Other biocontrol agents have also been used to control phytopathogens. These include chitinolytic *Streptomyces rubrolavendulae* S4, which was isolated from termite mounds and was shown to have antagonistic effects on *P. infestans* which causes damping off disease in tomato seedlings [17].

In vitro dual culture tests between *P. infestans, A. solani* and *Trichoderma* isolate showed that the linear growth of the blight pathogen colonies were significantly inhibited and overgrown by *Trichoderma* isolate. This was attributed to the presence of antifungal metabolites which are known to be produced by the *Trichoderma* isolates. In addition, mycoparasitism has been identified as one of the major mechanisms by which *Trichoderma* acts on fungal pathogens [24]. *Trichoderma* species has been used in previous research as a biocontrol agent which has been shown to exhibit different levels of inhibition against various plant pathogens. *Trichoderma* has been reported to be effective as a biocontrol agent against different pathogens just as indicated in this research where it inhibited *A. solani* and *P. infestans* at different levels. Other research has reported on inhibition activity of *Trichoderma* which was exhibited by six *Trichoderma* isolates which were screened against *A. solani*. The results showed that *Trichoderma* affected the growth of *A. solani* both in vitro and in vivo [25] and thus has potential to be used as biocontrol agent against *A. solani* [25]. Previous in vitro study carried out on five *Trichoderma* species against four fungal soil-borne pathogens using dual culture method showed the percentage inhibition of *Sclerotium rolfsii* by three of the *Trichoderma* isolates to be 100%, 62% and 68%. However inhibition of *Sclerotionia sclerotiorum* was 23% [26].

The results on disease severity which was observed in the in vivo trials from the treatments with *Warburgia* and *Trichoderma* showed that the two had reduction in disease severity which was well comparable to the positive control. This could be due to the inhibitive effects of the chemical substances which have previously been shown to be present in *Warburgia* extract and also the antimicrobial metabolites produced by *Trichoderma*. In most cases, the combined treatment with *Warburgia* and *Trichoderma* showed higher reduction in disease severity. This could be caused by the synergistic effects but further research is needed in order to elaborate how this works. The results are similar to results which were obtained from earlier research where field experiments were conducted to assess the effect of four selected *Trichoderma* isolates on suppressing potato late blight. Greenhouse results showed that potato blight severity reduction varied in different isolates [25]. There is increasing research on biocontrol agents in the control of phytopathogens due to their ecofriendly effects. Bahramisharif and Rose [27] reported that effective and consistent plant protection was obtained when *Trichoderma harzianum* and *B. subtilis* was combined with oak–bark compost. They were effective in the control of *P. infestans* and also enhanced growth of potatoes [27]. *Alternaria solani* has also shown susceptibility to various biocontrol agents. In vitro and in vivo trials showed *Bacillus Amyloliquefaciens* and *Trichoderma harzianum* to be effective against *A. solani* of tomatoes. Two of the most effective *Trichoderma* isolates showed percentage interference of 60 % and 48 % against *A. solani* [28].

The results observed on the effect of treatments with *Warburgia* and *Trichoderma* on the tomato plant's height and number of leaves showed a general increase in these phenological characteristics which was closely equivalent to treatment with Mancozed. These could be attributed to the reduction in disease severity. Blight disease mainly affects the plant leaves which are required for utilizing the available nutrients and the products obtained from photosynthesis contribute to plant growth. Since the treatments result in reduced disease severity, the plant becomes better in utilizing the available nutrients thus leading to higher plant growth which is reflected in the growth parameters which were evaluated of height and number of leaves. In addition, *Trichoderma* also has growth promoting potential which

could further contribute to the increase in height and number of leaves observed in the plants. Yao *et al*., [24] showed that apart from reducing disease severity, *Trichoderma* also increased the plant height in potatoes. *Trichoderma* was shown to promote growth of stem height and weight compared to the negative control [24]. Benitez *et al*., [7] also found out that, apart from being a biocontrol agent, *Trichoderma* also stimulates plant growth. Although the mechanisms of *Trichoderma* action were not investigated in this research, *Trichoderma* has been reported to be an effective antagonistic biocontrol agent against various fungal phytopathogens and among its modes of action are mycoparasitism, antibiosis and promoting plant growth [7]. Both *Warburgia* and *Trichoderma* have antifungal properties but *Trichoderma* has also been reported to enhance plant growth. Reports on combination of *Trichoderma asperellum* GDFS1009 and *Bacillus amyloliquefaciens* 1841 demonstrated synergistic interactions by offering protection against *Fusarium graminearum* and enhanced plant growth in wheat [29].

Preliminary investigation into the groups of bioactive compounds present in *Warburgia* revealed the presence of various chemical groups most of which have been shown to have antimicrobial activities. Previous research has indicated that *Warburgia* extract is rich in sesquiterpenes, which contain bioactive compounds such as alkaloids, terpenoids, flavonoids and terpenes all of which are known for their antimicrobial and antiplasmodial activities [30]. The antibacterial and antifungal activities of *W. ugandensis* are reported to be attributed to the abundance of drimane sesquiterpenes in its stem bark and leaves [3].

CONCLUSION

Results presented in this study show that *W. ugandensis* has inhibitory effect against *P. infestans* and *A. solani* both in vitro and in vivo. In both in vivo trials, treatment with *Warburgia* in combination with *Trichoderma* had the lowest disease severity index. In trial one, disease severity in the treatments with *Warburgia* and *Trichoderma* separately were similar with what was observed in Mancozeb while in trial two, the disease severity was even lower for the combined treatment compared to treatment with Mancozeb. The preliminary phytochemical tests showed the presence of several phytochemicals that have been previously reported to be responsible for antimicrobial activities of *Warburgia* extract. The above results show that *W. ugandensis* has the potential to control *A. solani* and *P. infestans* infecting tomatoes both in vitro and in vivo and portrays synergistic effectiveness when combined with *T. asperellum*. Further research is required to investigate antimicrobial activity of *Warburgia* against other phytopathogens and to analyze the specific bioactive compounds responsible for the observed antimicrobial properties of *Warburgia* against *A. solani* and *P. infestans* of tomato.

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Conflicts of interest

The authors declare no conflict of interest.

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