



EVALUATION OF ANTIMICROBIAL ACTIVITIES OF *Tithonia diversifolia* AND *Kigelia africana* AGAINST TOMATO FUSARIUM WILT PATHOGEN (*Fusarium oxysporum* Lycopersci)

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ABSTRACT

Fusarium wilt disease caused by the soil-borne fungus *Fusarium oxysporum* Schlecht is a serious threat to tomato production worldwide. Chemical and cultural methods of management used are either ineffective or toxic to the environment. Plant secondary metabolites; therefore, pose a possible alternative because they are environmentally friendly and have minimal effect on non-target organisms. This study screened phytochemical compounds of two plants; *Tithonia diversifolia* (Hemsl.) A. Gray and *Kigelia africana* (Lam.) Benth and assessed their potency in controlling plant fungal pathogen *F. oxysporum*, a causal agent of Fusarium wilt disease in tomatoes. Leaf extract of *T. diversifolia* and fruit extract of *K. africana* were concentrated in water and screened for phytochemical contents using standard procedures. Concentrations used were 25g/L, 50g/L and 100g/L on the disc. *Fusarium oxysporum* was isolated from infected soil using potato dextrose media, Antifungal activity was evaluated by measuring the zone of inhibition against the test organism. The results showed that the mean inhibitory zones were highest at 100g/l in both plants, although *K. africana* fruit extract portrayed the highest inhibitory activity compared to *Tithonia africana*. The effect of the plant extracts and the negative control was statistically significant ($p < 0.05$). This study indicates that *K. africana* and *T. diversifolia* possess the antifungal activity and can be used as a broad-spectrum fungicide against *F. oxysporum*. These plant extracts may provide an effective measure for the management of Fusarium wilt of tomatoes that may form an integral part of integrated management and it also has prospect as an alternative to reliance only on fungicides.

Keywords: Extracts, antimicrobial, *Kigelia africana*, *Tithonia diversifolia*, *Fusarium*, Phytochemical

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) belongs to the Solanaceae family and is grown and consumed globally. In Kenya, the production of tomato is 14% of the whole vegetable harvest and the whole horticultural crops account for 6.72% (Government of Kenya (GoK), 2016). According to Food and Agriculture Organization, (2018), tomatoes are either grown on fields or in greenhouses and tomato production in the field accounts for 95 % while greenhouse production accounts for 5 % of the entire tomato production in Kenya. Production of tomatoes contributes to economic development and reduction of poverty. Tomatoes are a source of income, job creation and foreign exchange to the farmer leading to earning for economic development (Collins, 2012; Onger, 2014). However, cultivation of it is faced by constraints such as adverse weather and environmental conditions, soil type and pathogenic microorganism causing diseases (Berrueta *et al.*, 2012). *Fusarium oxysporum* is a pathogen that causes Fusarium wilt in tomatoes and it destroys the xylem (Ajillogba and Babalola, 2013). According to Agrios, (2005) control of *Fusarium oxysporum* pathogen has become a problem since it persists in the soil and sustains infection in the farm.

Farmers prefer use of synthetic fungicides to control Fusarium wilt disease in tomato in Kenya (Mwangi *et al.*, 2015). However, synthetic fungicides and cultural practices that include crop rotation are not effective (Chandler *et al.*, 2011; Chellemi *et al.*, 2012; Palti, 2012; Machado *et al.*, 2018). Crop rotation and shifting cultivation is no longer possible due to insufficient land due to increased human population (Bawa, 2016). Excessive uses of chemicals are

environmentally toxic (Koutros *et al.*, 2012; Malkhan *et al.*, 2012; Onkendi *et al.*, 2014). Since farmers maximise fungicide application to limit diseases occurrence of tomato diseases, development of resistant pathogen strains have occurred (Akbar *et al.*, 2009; Malkhan *et al.*, 2012; Gliessman, 2014; Vincelli, 2016). Misuse of fungicides synthetics has rendered them less effective in controlling the pathogen (Hadian *et al.*, 2011). Fungicides have become expensive for farmers to procure (Ngowi *et al.*, 2007; Njoroge, 2014).

Due to limitations of synthetic fungicides and other methods used to control *Fusarium* wilt, there is a need to explore and develop bio-fungicide from plant products (Gilligan, 2008). Plant products may be environmentally friendly with minimal effect on non target soil microorganisms (Njoroge, 2014; Lucia, 2017). *Tithonia diversifolia* and *Kigelia africana* are widely distributed in Kenya, have medicinal value and with high seed production and dispersal (Zarghani *et al.*, 2015). Both are tolerant to harsh environmental conditions and therefore able to reproduce continuously even during the dry season (Munyuli, 2011). The two plants have allelopathic property facilitating their survival by minimising resource competition (Ajayi, 2017). According to Dewole *et al.*, (2013) and Saini *et al.*, (2009) *Tithonia diversifolia* and *Kigelia africana* extracts have been used to control plant pathogens such as *Phytophthora nicotianae* and *Rhizoctonia solani* information on their application in control of *Fusarium oxysporum* sp. lyperseciis scarce. This study was carried out to evaluate the efficacy of the water extract of *Tithonia diversifolia* and *Kigelia africana* against *Fusarium oxysporum* mycelial growth under laboratory conditions.

MATERIALS AND METHODS

Experimental Site

Tithonia diversifolia, *Kigelia africana* and *Fusarium oxysporum* were collected in Gatwiri, Mwea in Kirinyaga County in Kenya where tomatoes production is practiced (Mwangi *et al.*, 2015). The phytochemical analysis and isolation of *Fusarium oxysporum* was conducted at the Technical University of Kenya. Antimicrobial studies were done at Chuka University in the Department of Biological Sciences Laboratories.

Study Design

In determining the antimicrobial properties of the plant extract, a (3×3) complete randomized design was used. Factor A was *Fusarium* pathogen at three levels i.e. three different fungal mycelia concentration at 1×10^3 , 2×10^3 and 3×10^3 and factor B was plant extract at three levels i.e. *Tithonia diversifolia* at 25 g/L, 50 g/L, and 100 g/L replicated 3 times. This design was similarly applied to *Kigelia africana*

Plant Sample Collection

The plants' samples used for the study were *Tithonia diversifolia* and *Kigelia africana*. Purposive sampling was used to locate the sample collection points because the method focuses on the particular characteristics of a population that are of interest. For *Tithonia diversifolia*, the leaves were collected while for the *Kigelia africana* the fruits were collected because they are reported to have the highest concentration of phytochemicals. The samples were then chopped into small pieces on aluminum foil to prevent losses of the phytochemical constituents. The samples were then transferred to a glass bottle to minimize the reaction of the organic phytochemical constituents with the other surfaces. The two plant samples were then placed in a bag and then taken to the laboratory at the Technical University of Kenya for study.

Preparation of Crude Extract Concentration

Preparation of crude extract concentration was done according to Hossain *et al.* (2013). The plant samples were washed and allowed to air dry under shade. After a week, 100 g of fresh sample was chopped and then crushed in a surface-sterilized pestle and mortar by adding 100 ml sterile water and allowed to stand for three days after which filtering was done using Whatman paper. Only the phytochemical constituents soluble in water dissolved to form a solution. Water was removed by freeze drying the solution. Concentrations of 2.5% (25 g/L) 5% (50 g/L) and 10% (100 g/L) were prepared separately by adding 2.5 g, 5 g and 10 g of the extract supernatant respectively with 5 ml of water to enhance dissolution and made up to 100 ml by adding distilled water. The plant extract was then placed into vials and stored in the refrigerator waiting antimicrobial assay

Collection *Fusarium oxysporum* in the farm

Samples containing *Fusarium oxysporum* were collected the soil random sampling was used to obtain the samples.

Preparation of Potato Dextrose Media

Preparation of the media followed the guidelines of commercial manufacture. Thirty-nine grams of Potato Dextrose Agar (PDA) media were placed in 1000 ml distilled water. The media was dissolved in water by heating over the Bunsen burner. Sterilization was done by autoclaving at 15 lbs. pressure (122°C) for 15 minutes. It was then mixed

well before dispensing (Sheringham & Brightwell, 2012).

Preparation and identification of *Fusarium oxysporum* Cultures

Ten grams the soil sample was spread on the surface of the PDA media. The soil sample was then inoculated into the PDA media and then kept in the incubator at 28°C (Latiffah *et al.*, 2010), to allow growth. After growth, the fungus was sub cultured to obtain a pure culture.

Identification of the *Fusarium oxysporum* cultures

Identification and isolation of *F. oxysporum* was done according to De Carolis *et al.*, (2012). This was through observation of growth and microscopic characteristics. For the growth characteristics identification was done through the observation of the colour in the nutrient medium because *Fusarium oxysporum* usually tends to be either violet or purple in colour. For the microscopic characteristics, chlamydospores were observed. First, the formation of either macroconidia or microconidia spores or both were observed. For the microconidia, an observation was based on the presence of unicellular spores with an oval shape.

Preparation of Standard Inoculum of Test Pathogens

This was done according to Alaniz *et al.*, (2011). Spore suspension of *Fusarium oxysporum* was prepared from young 10-day old cultures grown on PDA. The spore suspensions were obtained by using a sterile cork-borer to pick a colony of the fungus on the cultured surface to remove the spores from the surface of the PDA nutrient medium. After the removal of the spore suspensions, the pathogen was estimated using the fungal mycelia of 1×10^3 , 2×10^3 and 3×10^3 for antimicrobial test.

Preparation of Antimicrobial Test Discs

The discs were prepared according to Arunkumar (2009). Test disc was made using a paper punch. Paper discs of 6 mm in diameter were cut off from a sheet of Whatman paper of size three. The paper discs were then sterilized in the oven at 150°C. The bottle containing the paper disc was stored in a cool, dry cupboard until the time for use. Antimicrobial test disc was made by taking two ml of each of the concentration of the stock solution of each plant extract and pipetting on a sterile paper disc of 6 mm diameter in a sterile Petri-dish, this was to give a concentration of 2 mg/disc. The preparation of antimicrobial test disc was replicated three.

Antimicrobial Testing of the *Fusarium oxysporum* Pathogen

The antimicrobial test was done according to Assob *et al.*, (2011). Eighty-one plates of PDA media plates were prepared. Pure isolates of *Fusarium oxysporum* were cultured by transferring a loop of culture into sterile nutrient media on each plate. The antimicrobial test disc soaked with plant extract were placed on the media; the media was placed in an inverted position and incubated at 28°C for 1 week to allow for growth.

Data Analysis

Statistical Analysis Software (SAS) version 9.4 was used to analyze the measured data of zone of inhibition. Two-way ANOVA was carried out to determine if there was a statistical difference in the zones of inhibition between the plant extracts of *Tithonia diversifolia* and *Kigelia africana* exposed to the plant pathogen *Fusarium oxysporum*. Two-way ANOVA was used to determine if there was interaction between the plant extract and the test microorganism, with $\alpha=0.05$ considered significant. Least Significance Difference was also used to determine the difference between the mean.

RESULTS

Isolation of *Fusarium oxysporum*

Fusarium oxysporum was recovered from the soil. The microscopic features that were observed during identification were septation and shapes of microconidia and macroconidia and the structures of the chlamydospores were also noted. *Fusarium oxysporum* grown in the PDA media produced white mycelia, with a cotton appearance that was pink in colour on the reverse side of the plate. Microscopic characteristics for example presence of oval non-septate microconidia and macroconidia with a slight curvature, septate and pointed apical cell confirm that the isolates were of *Fusarium oxysporum*.

Antimicrobial Activity of plant extract

The antimicrobial activity of the plant extract was determined using the disc diffusion assay method. There were observable zones of inhibition for all the concentration of both *Tithonia diversifolia* and *Kigelia africana* plant extract against *Fusarium oxysporum* fungal pathogen.

Antimicrobial Activity of *Tithonia diversifolia* and *Kigelia africana*.

As the plant extract concentration was increased, there was a reduction in the fungal mycelia growth. The effect of the plant extract was statistically significant ($p < 0.05$). The Mean of inhibition ranged from 23.67 to 7.78 for *Tithonia diversifolia* and 23.67 to 11.75 for *Kigelia africana*. The plant extract with the high concentration had more inhibitory activity on the *Fusarium oxysporum* mycelial growth than the lower concentration (Table 1 and 2). The growth inhibition of *Fusarium oxysporum* increased linearly with an increase in the concentration of the botanicals. The zone of inhibition on the mycelial sizes on the control plates were significantly higher ($P = 0.05$) than for the plant extract. The statistical analysis showed that both *Tithonia diversifolia* and *Kigelia africana* extract at different concentrations significantly affected radial growth and sporulation of *Fusarium oxysporum*. From the LSD test, the mycelia growth rate for concentration 100 g/ml was lower than the growth rate of the control. The overall model ($Y_{ijk} = \mu + \alpha_i + \beta_j + E_{ijk}$) was found to be statistically significant at 0.05% level of significance at $P < 0.05$

Table 1: mean mycelia diameter of *Fusarium oxysporum* recorded on Petri dishes treated with different concentrations of *Tithonia diversifolia*

Treatment	Zones of inhibition
p1ridomil	23.67 ^a
p2ridomil	23.00 ^a
p3ridomil	21.67 ^b
c3t3	10.63 ^c
c2t3	10.40 ^{cd}
c1t3	10.22 ^{cd}
c2t2	9.90 ^{cde}
c1t2	9.67 ^{de}
c3t2	9.25 ^e
c2t1	8.2 ^f
c3t1	7.88 ^f
c1t1	7.78 ^f
LSD	0.44
Mean	10.68
CV	6.18

^aMeans followed by the same letter are not significantly different by two way ANOVA followed by LSD test at 5% probability level. Pridomil was pathogen and ridomil negative control, C3T3, C1T1 and C2T2 was *Fusarium oxysporum* pathogen and *Tithonia diversifolia*

Table 2: Mean mycelia diameter of *Fusarium oxysporum* recorded on Petri dishes treated with different concentrations of *Kigelia africana*

treatment	Zones of inhibition
p1ridomil	23.67 ^a
p2ridomil	23.00 ^{ab}
p3ridomil	21.67 ^b
c1k3	16.00 ^c
c3k3	15.38 ^c
c2k3	15.30 ^c
c3k2	13.50 ^d
c1k2	13.22 ^{de}
c2k2	13.10 ^{de}
c2k1	12.30 ^{de}
c1k1	12.13 ^{de}
c3k1	11.75 ^e
LSD	0.75
Mean	14.56
CV	7.71

^aMeans followed by the same letter are not significantly different by two way ANOVA followed by LSD test at 5% probability level. Pridomil was pathogen and ridomil negative control, C3k3, C1k1 and C2k2 was *Fusarium oxysporum* pathogen and *Kigelia africana*

Comparison for the Best Performance between the Plant Extract and Negative Control

The Mean of inhibition ranged from 22.78 to 7.93 (Table 3). The minimum reduction in sporulation was recorded in the negative control, which was significantly lower than the rest of the treatments. The *Fusarium oxysporum* fungal

mycelial diameters on media with the control (Ridomil) were significantly lower compared with the plates that contained *Kigelia africana* and *Tithonia diversifolia*. Higher inhibition of fungal hyphal growth was recorded in media treated with Ridomil. Hyphae on media incorporated with *Kigelia africana* had a bigger diameter than in media treated with *Tithonia diversifolia* though there was no significant difference ($P < 0.05$). The findings showed that the fungicides tested against *Fusarium oxysporum* were significant in inhibiting the fungal mycelial growth. Ridomil was significantly superior to the plant extract. The negative control was effective thus producing zones of inhibition on the plate cultured with *Fusarium oxysporum*, however, *Kigelia africana* was more effective at a high concentration whereas, *Tithonia diversifolia* was comparatively less effective compared to *Kigelia africana*. The sporulation of the *Fusarium oxysporum* varied greatly with plant extract used at different concentrations. The sporulation decreased linearly with an increase in concentrations and the type of plant extract. The effect of the plant extracts and the negative control was statistically significant ($p < 0.05$).

Table 3: Comparison for the best performer between the plant extract and the negative control

Treatment	Zone of inhibition
ridomil	22.78 ^a
K3	15.56 ^b
K2	13.30 ^c
K1	12.07 ^{cd}
T3	10.44 ^{cd}
T2	9.63 ^e
T1	7.93 ^f
LSD	0.59
Mean	12.08
CV	8.05

^aMeans followed by the same letter are not significantly different by two way ANOVA according to LSD test at 5%. Ridomil was the negative control, (K3, K2, K1) was *K. africana* and (T1, T2, T3) was *T. diversifolia*.

DISCUSSION

Isolation of *Fusarium oxysporum*

The colony had a mass of white colour from the upper part of the plate. Cultural characteristics of the pink colour also confirm that the isolate was *Fusarium oxysporum*, these findings were similar to those of Shobha and Kumudini., (2012). The dark purple colour was observed on the under surface of the PDA plate. This finding is consistent with that of El Kichaoui (2016) and Bedasa, (2018)

Antimicrobial Activities of the Plant Extract

Antimicrobial Activities of *Tithonia diversifolia* and *Kigelia africana*

The findings revealed that *Tithonia diversifolia* had the lowest antifungal activity. The study also revealed that as the concentration of *Tithonia diversifolia* extract was increased, the mean mycelia diameter decreased. This was attributed to the various phytochemical constituents for instance; tagitinin C sesquiterpene and a guaianane that contributed to antifungal activity, these phytochemical components of *T. diversifolia* are the ones that either have direct inhibitory effects on pathogens, exhibiting bio fungicidal or bio fungistatic properties (Tagne *et al.*, 2018). However in the study, *T. diversifolia* showed the least zones of inhibition. This could be due to the variation on the quantity of each of the phytochemicals. Enyiukwu *et al.*, (2014) also reported a similar finding that the leaf extracts of *T. diversifolia* is toxic to *F. oxysporum* sp. *spelaeidis* showing a complete inhibition of mycelial growth and spore germination. According to Enyiukwu *et al.*, (2014), he did report similar findings that *T. diversifolia* plant extract was very effective in inhibiting the growth and the sporulation of selected fungal pathogen causing leaf spot disease.

Tagne *et al.*, (2018) also reported that the fungicidal spectrum of *Tithonia diversifolia* has been attributed by various compounds such as stigmasterol and sitosterol. Spore yield among fungicides treatments was as a result of the antimicrobial activities on the mycelia growth. He further reported that *Tithonia diversifolia* antimicrobial activity was very high on some fungal pathogens and the mycelia count was slow compared to the other plates in which *Tithonia diversifolia* was not used thus the fungus was able to sporulate on the plates actively.

The finding revealed that the crude extract of *Kigelia africana* resulted in a significant inhibition in radial mycelia growth. The study revealed that as the concentration of *Kigelia africana* extract was increased, the mean mycelia diameter decreased. This was attributed to the various phytochemical constituents for instance; kigelinone, ferulic acid and iridoid that could be causing the zone of inhibition (Rahmatullah *et al.*, 2010). These findings were similar to the one reported by Rejeki & Addy, (2017) and Zofou *et al.*, (2013) who reported the anti-fungal activity of *Kigelia*

africana against *Fusarium oxysporum* sp. cubense which is a pathogen that attacks bananas. Similar results were also reported by Itonga, (2011) who reported that *Kigelia africana* extract was effective against eighteen different fungi species.

This study was similar to the one reported by Al-Mujamma'a, (2008) in which he reported that *Kigelia africana* extracts induce the disruption in fungal cell metabolism, increased permeability of fungal plasma membrane and destruction of the conidial wall structure. The more the *Kigelia africana* extract is concentrated the higher the inhibition level. The positive Control plates treated with fungus alone had the highest mycelial diameter (no zone of inhibition) which confirms that *Kigelia africana* has antifungal compounds that inhibited *Fusarium oxysporum* mycelial growth. According to Ibrahim *et al.*, (2015), he also reported a similar finding that the antifungal activities of acetone extract of *Kigelia africana* were effective against various fungal pathogens. Moreover, he reported that the antimicrobial activity of the plant extract was due to the phytochemical constituents for instance tannins and other polyphenols present in the extracts.

Comparison of the Best Performance between the Plant Extract and Negative Control.

Fungicides are an effective and straight method for fungal disease management because they are easily obtained in the agro vet shops. The study revealed that the fungicide (ridomil) had the largest zone of inhibition. This could be a result of the interaction of Metalaxyl and Mancozeb contained in Ridomil making it more effective. However, the pesticide could be more effective but the consequences of debris of chemicals have caused problems to human beings, livestock and the environment. In a study carried out by Kesavachandran *et al.*, (2009), he did report that about one million persons die due to the chronic diseases caused by pesticide poisoning. Although, the pesticide is a source of employment to those who formulate and manufacture the chemical (Tornero & Hanke, 2016). It poses a threat to their lives because they handle harmful solvents and chemicals during the manufacture of these chemicals. Farmers are also at risk because they inhale the toxic substances during spraying and mixing of the pesticide.

The inhalation of the pesticides is likely to cause implications on human health. The pesticides disrupt the endocrine system by antagonizing the production of the hormones from the endocrine glands, exposure of the pesticide to different dose rates has implications to the immune system, reproductive gametes and eventually resulting into cancer. Similar findings were also recorded by Iyer & Makris, (2010) who reported that there was increased death rate due to the destruction of the cardiovascular and respiratory diseases caused by pesticides in human beings. He also reported that cancer cases and the rate of death cases had increased. He did observe gastrointestinal and the lymphatic tissues upon exposure to pesticides were at risk of having mutation thus leading to cancer.

This study was similar to the one reported by Amini & Sidovich, (2010) in which he reported that bromuconazole and Prochloraz proved to be antimicrobial and were effective against the fungal *Fusarium oxysporum* in an in vitro study done under the glasshouse conditions. According to Yin *et al.*, (2011), he also found similar findings that different fungicide was more efficient against the control of various fungi and inhibited multiplication of the cells (cell division) and mitosis in fungi. However, Singh *et al.*, (2017) reported that the bromuconazole and Mancozeb component of ridomil was found to be present in the pawpaw fruit. Ridomil was sprayed to the pawpaw to reduce the postharvest losses. Due to the lack of awareness from the consumer and the farmer, they are exposed to these synthetics when they consume food crops that pesticides are used in the management of pest.

Previous research revealed the fungicides inhibitory property usually is a result of polymerization of β -tubulin in microtubules reducing their proliferation and dynamic instability (Ding *et al.*, 2016). Further, methylbenzimidazole carbamate fungicides suppress the meeting of spindle microtubules owing to disturbed chromosomal alignment and microtubule-kinetochore interactions at the metaphase plate causing chromosome loss and chromatid loss, in pathogenic fungi (Lebeda *et al.*, 2010). Difenoconazole fungicide exhibits antimicrobial activities against different plant fungal pathogens for instance ascomycetes, basidiomycetes and deuteromycetes (Kuck *et al.*, 2012). However, this pesticide has caused more implications to the environment. In a study carried out by Close, (2018) he reported that the difenoconazole fungicide that was used to control disease in grapes greatly polluted the environment, in addition it also killed some insects, birds, fish and other non target organisms.

The plant extract had a variation in the zone of inhibition. The differences might be due to the difference in nature, quality and quantity of the inhibitory substances present in the botanicals (Ceylan & Fung, 2004). It is evident from results that the zones of inhibition of the *Fusarium oxysporum* pathogens by the plant extracts depend upon plant species and extracts concentration. Veresoglou *et al.*, (2013); Kumaran, (2003) did report comparable findings that the fungal susceptibility toward a plant extract was due to plant species, the solvent used for extraction and extract concentration, as well as the organism tested. The activity of plant extracts and essential oils as anti-sporulant agents have been revealed against a large number of fungal diseases as reported by several other workers (Singh *et al.*, 2017).

CONCLUSION

There is a lot of implication of the pesticide to the flora, fauna and environment. Therefore adopting these plants products could provide an alternative method of pest and disease control because they are environmentally friendly and that do not affect livestock, human health, aquatic and the environment. The two plant extracts when combined in different ratios also slightly increased the antifungal activity when used against the test microorganism. Plant extracts used as an antimicrobial agent are relatively economical, safe and non-hazardous and show antifungal activity against a large no of fungal diseases. These plant extracts may provide an effective measure for the management of Fusarium wilt of tomatoes that may form an integral part of integrated management and it also has prospect as an alternative to reliance only on fungicides.

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