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In vitro antibacterial activity of selected plant extracts against potato bacterial wilt (*Ralstonia solanacearum* Smith) in Rwanda

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Abstract

Bacterial wilt caused by *Ralstonia solanacearum* Smith is the most severe potato disease in Rwanda because there is no known pesticide for it and cultural control methods seem almost impossible to implement. Therefore, use of plant extracts with antibacterial activities which are locally available, economically affordable and environmental friendly could be an alternative in the management of the disease. This research focused on *in vitro* screening of the antibacterial activity of methanol, water and chloroform extracts of ten local plant materials against the pathogen. From this screening, methanol and water extracts of three promising plant species, *i.e.*, tobacco, wild marigold and garlic were also used for determination of minimal inhibitory concentration (MIC). The results showed higher inhibition zone of methanol extracts (16.85 mm) against bacteria followed by water (14.42 mm) and chloroform (14.19 mm) extracts. All ten plant extracts inhibited the growth of the pathogen. Higher antibacterial activity was found in tobacco, wild marigold and garlic extracts (19.61, 18.56, and 18.3 mm inhibition zones, respectively). Minimal inhibitory concentration (MIC) of methanol extracts from tobacco and wild marigold was 6.25 mg mL^{-1} whereas, garlic methanol extract was 12.5 mg mL^{-1}. Furthermore, MIC of water extract was 12.5 mg/mL^{-1} in all three plant species. The findings revealed that tobacco, garlic and wild marigold extracts are the best in the control of potato bacterial wilt. Moreover, methanol extracts are the most efficient in management of potato bacterial wilt in comparison to water and chloroform extracts.

Key words: Antibacterial activity, botanicals, growth inhibition zone, minimum inhibitory concentration, potato, *Ralstonia solanacearum*.

Introduction

Bacterial wilt or brown rot disease caused by *Ralstonia solanacearum* (Smith) is the second most serious potato disease in sub-tropical and tropical regions and even in some cool temperate areas of the world (Muthoni *et al.*, 2013) after late blight caused by *Phytophthora infestans* (Mont De Bary) (Strange and Scott, 2005; Muthoni *et al.*, 2012; CIP, 2017). In Africa, it is the most serious potato disease throughout central and southern regions mainly in Uganda, Rwanda, Ethiopia, Kenya, Burundi, Nigeria, Madagascar, and Cameroon. Its infection of tubers restricted potato exports to European markets (Priou *et al.*, 2001; Hammes, 2013). Bacterial wilt is the most problematic disease due to the fact that its management using chemicals seems to be nearly impossible or more complicated since there are no known chemicals that can control it effectively (Wagura *et al.*, 2011; Masengesho *et al.*, 2012; Guchi, 2015). Beside the lack of effective chemicals, pesticide resistances as well as negative effects of chemicals on consumer health and natural enemies limit their use at global level (REMA, 2011; Yuliar *et al.*, 2015).

Currently, farmers tend to use mainly cultural practices such as crop rotation, planting in non-infected soils, use of disease free planting materials, growing resistant varieties, removal of infected or suspect plants and destroying them, control of nematodes, use of clean water for irrigation, sanitation of farm implements, and application of quarantine measures (Pal, 2006; Yuliar *et al.*, 2015). However, all these methods have individual practical, technological or economic limitations (Wagura *et al.*, 2011; Yuliar *et al.*, 2015). For example, crop rotation has some limitations because of long survival of the pathogen in the soil even in the absence of host plants (REMA, 2011; Wagura *et al.*, 2011; Hammes, 2013; Yuliar *et al.*, 2015). In addition, small farm size poses challenge for crop rotation program (Wagura *et al.*, 2011; Muthoni *et al.*, 2012; Guchi, 2015). Quarantine measures are also either expensive or difficult to apply and may limit production and commercialization of ware potatoes (Muthoni *et al.*, 2012; Muthoni *et al.*, 2013).

Furthermore, it is not easy to find clean seeds because potato is mainly propagated vegetatively, and this method favors disease spread from mother tubers to next offspring (REMA, 2011; Wagura *et al.*, 2011). The use of resistant varieties is also limited because there is no high level of resistance in potato to bacterial wilt (Priou *et al.*, 2001; Muthoni *et al.*, 2012; Muthoni *et al.*, 2013). In addition, some tolerant potato varieties with no visible symptoms especially in cool regions, harbor latent infection in tubers and can spread the disease via progeny tubers (Priou *et al.*, 2001; Muthoni *et al.*, 2013). Furthermore, some of these varieties are not appreciated by farmers, for instance 'Cruza' in Rwanda (REMA, 2011) or show some defects such as high glycoalkaloid content and sensitivity to temperature conditions (Fock *et al.*, 2001).

It has been reported that some plants contain secondary metabolites with antimicrobial properties and can be used to control plant pathogens by either inducing systemic resistance

or antibacterial activity (Wagura *et al.*, 2011; Malkhan *et al.*, 2012; Rahman *et al.*, 2012; Körpe *et al.*, 2013). Therefore, the use of locally available, economically and environmentally friendly plant extracts with antimicrobial properties could be an alternative in the management of potato bacterial wilt (Körpe *et al.*, 2013; Yuliar *et al.*, 2015). Plant antimicrobial metabolites may inhibit pathogens either by their natural bioactive compounds (phytoanticipins) or compounds synthesized *de novo* in response to pathogen attack or other stress conditions (phytoalexins) (Cowan, 1999; Ribera and Zuñiga, 2012).

Most of the studies have been mainly limited to the assessment of the antifungal and antibacterial activities of plant extracts in medical and clinical microbiology but exploitation of their importance in plant bacteriology is still in its infancy (Yuliar *et al.*, 2015). In addition, the efficacy of botanicals on bacterial wilt (*R. solanacearum*) has not been studied exhaustively (Pradhanang *et al.*, 2003; Deberdt *et al.*, 2012; Arnault *et al.*, 2013; Alamshahi and Nezhad, 2015; Guchi, 2015; Yuliar *et al.*, 2015). Moreover, it has been reported that the yield and composition of bioactive compounds of each species is affected by diverse factors such as environmental conditions, genetic factors (species, varieties), plant organs, stage of growth, extraction techniques and even the extraction solvents (Senatore *et al.*, 2003; Kukrić *et al.*, 2012; Arnault *et al.*, 2013; Alamshahi and Nezhad, 2015). Therefore, *in vitro* study was carried out to determine the inhibitory effect of methanol, chloroform and water extracts of ten selected plant materials from Rwanda on *R. solanacearum*.

Materials and methods

Plant materials: Ten plant species (Table 1) were collected randomly from Northern and Southern regions of Rwanda and identified by a botanist from the University of Rwanda, School of Forestry and Biodiversity Conservation, Department of Forest and Nature Conservation (FNC). The plants were selected based on their active compounds with antimicrobial properties, local availability, and low cost. For onion and garlic, the bulbs were used, whereas for the remaining plants only leaves were collected for extraction of bioactive compounds**.**

Extraction of bioactive compounds: Collected plant materials were shade-dried in a room at ambient temperature for four weeks and then placed in oven at 40 ºC for two days for complete drying. Water and methanol solvents were chosen to extract polar metabolites, whereas chloroform was used for extraction of non-polar active compounds (Cowan, 1999; Ncube *et al.*, 2008; Sasidharan *et al.*, 2011; Malkhan *et al.*, 2012; Rahman *et al.*, 2012). The extraction was performed by macerating 50 g of dried powdered plant material in 200 mL of water, methanol, and chloroform (ratio of 1: 4) and left to stand for three days on a rotary shaker. Thereafter, the mixture was filtered using Whatman filter papers. The solvents were evaporated by RotarVapor at 40 ºC and 280 rpm until complete drying. Subsequently, extracts were diluted to 50 mg mL^{-1} (w/v) concentration with 1 % dimethylsulfoxide (DMSO) and stored at 4 ºC until use (Mwitari *et al.*, 2013).

Table 1. Collected plant materials for screening against *R. solanacearum*

English name	Scientific name	Family	Organ used
Onion	*Allium cepa* L.	Alliaceae	Bulbs
Garlic	*Allium sativum* L.	Alliaceae	Bulbs
Lemongrass	*Cymbopogon citratus* Stapf	Poaceae	Leaves
Castor bean	*Ricinus communis* L.	Euphorbiaceae	Leaves
Rosemary	*Rosmarinus officinalis* L.	Lamiaceae	Leaves
Lion's ear	*Leonotis nepetifolia* R.Br.	Lamiaceae	Leaves
African basil	*Ocimum gratissimum* L.	Lamiaceae	Leaves
Tobacco	*Nicotiana tabacum* L.	Solanaceae	Leaves
Wild marigold	*Tagetes minuta* L.	Asteraceae	Leaves
Stinging nettle	*Urtica massaica* Mildbr.	Urticaceae	Leaves

Preparation of bacterial inoculum: A virulent isolate of *Ralstonia solanacearum* from potato 'Gikungu' was used in this study (Mutimawurugo *et al.*, 2019). From infected materials, a vascular flow technique was used to get bacterial suspension of *R. solanacearum* (Fig. 1A). Furthermore, cultural, morphological, and physiological diagnoses of bacterial colonies were performed on Kelman's Triphenyl Tetrazolium Chloride (TTC) and Casamino peptone glucose (CPG) agar media to distinguish *R. solanacearum* from other bacteria (Fig. 1B and 1C) and to differentiate virulent colonies from non-virulent ones (Mutimawurugo *et al.*, 2019). Gram staining was also performed to distinguish *R. solanacearum,* which is a gram-negative bacterium, from gram-positive strains (Fig. 1D, Mutimawurugo *et al.*, 2019). Pure culture colonies were transferred into a sterile glycerol stock and kept at -20 °C until use (Mutimawurugo *et al.*, 2019).

Screening for antibacterial activity of plant extracts: Initial screening was done by agar disc diffusion method as described by Ncube *et al.* (2008). 50 µL bacterial suspension at 4.8 x 10^7 CFU

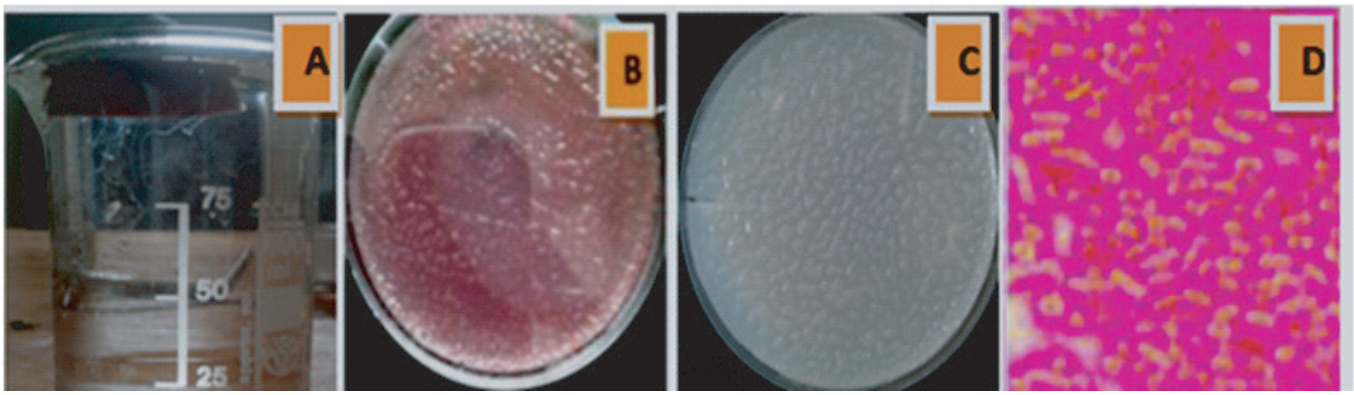

Fig. 1. Characterization of *R. solanacearum* (A) isolated from Gikungu potato cv. by stem vascular flow test, (B, C) Morphological features of the colonies on TTC and CPG culture, respectively, and (D) Microscopic observation of gram stained *R. solanacearum*. Source: Mutimawurugo *et al.* (2019).

mL^{-1} was inoculated on the surface of Mueller-Hinton agar plates. Afterward, filter paper discs of 5 mm diameter saturated with 20 µL (at the concentration of 50 mg mL^{-1}) of methanol, chloroform or water extracts from the ten plant materials were placed on the surface of each plate. Sterile water, absolute methanol, chloroform, and 1 % DMSO served as negative controls while streptomycin was used as positive control. The plates were kept in the fridge at 4 °C for 1 h to allow plant extract diffusion and then incubated at 37 °C for 24 h. After this period, growth inhibition zone around each disc was measured. A completely randomized design (CRD) with factorial arrangement was used and each treatment (methanol, water and chloroform extracts from each plant species) was replicated three times. Analysis of variance (ANOVA) was performed using SAS software, to determine the difference in growth inhibition zone due to the antibacterial activity of the plant extracts. The treatments means were separated using Tukey's honestly significant difference test at $P \leq 0.05$.

Determination of minimal inhibitory concentration (MIC): The methanol and water extracts from three plant species (tobacco, wild marigold, and garlic) that showed the highest antibacterial activity were tested to determine the minimal inhibitory concentration (MIC). This test was done by broth micro-dilution method, a potential technique for quantitative determination of MIC (Mounyr *et al.*, 2016). In this method, 96 wells micro titer plates were used and into each of the 12 wells in a row, 50 µL of nutrient broth was added. From the 1st to 10th well, 50 µL of plant extracts initially dissolved in 1 % DMSO were added at two fold serial dilutions (50 to 0.098 mg mL^{-1}). In the 11th and 12th well, 50 µL of positive (streptomycin) and negative (methanol, sterile distilled water, or DMSO) controls as well as positive growth control (nutrient broth) were added. Then, 50 µL of bacterial suspension at concentration of 4.8×10^{-7} CFU mL^{-1} overnight grown in Mueller Hinton broth at 28 ºC was added to each well. The plates were kept at 37 ºC for 18 h, after which 50 µL of 0.01 % of triphenyl tetrazolium chloride (TTC) was added to each well. The TTC was used as a dye reagent to indicate the viability or death of bacteria (Mounyr *et al.*, 2016). Thereafter, the plates were incubated at 37 ºC for 1h, after which MIC was evaluated by visual observation of colour change of the medium (Ncube *et al.*, 2008). Three plates were used for three plant extracts (tobacco, wild marigold and garlic). In each plate, three rows were used for ten serial diluted concentrations of methanol extracts and other three next rows for water extracts. Each concentration of methanol or water extracts from three plant species was replicated three times.

Results and discussion

Screening of plant extracts against *R. solanacearum*: Methanol, water and chloroform extracts of the ten plant materials significantly inhibited the growth of *R. solanacearum* at different levels at $P \leq 0.05$ compared to the negative control (Table 2). It was also observed that all plant extracts inhibited the growth of the pathogen at the same level of streptomycin (positive control) except methanol, water, and chloroform extracts of rosemary, lion's ear, and stinging nettle. Furthermore, methanol, water and chloroform extracts of the ten selected plant materials showed different levels of activity against the pathogen. For methanol extracts, the highest antibacterial activity was obtained with tobacco, wild marigold, garlic, and onion (21.00, 20.67, 20.33 and 18.33 mm inhibition zone, respectively). For water extracts, the highest antibacterial activity was found in tobacco, wild marigold, and garlic. In the case of chloroform extracts, comparable strong inhibitory effect was observed in tobacco (17.50 mm) followed by wild marigold than garlic (17.00 mm) and castor bean (16.00 mm).

In the present study, it was found that all the test extracts inhibited the growth of the bacteria at the same level of the positive control

Table 2. Growth inhibition zone (mm ± SD) of methanol, water and chloroform extracts from ten selected plant materials against *R. solanacearum.*

Plant extract	Methanol extract	Water extract	Chloroform extract	Mean
Tobacco	21.00±2.00 a	20.33±2.89 a	17.50±1.32a	19.61±1.86 a
Wild marigold	20.67±1.15 ab	18.00±3.46 ab	17.00±1.00 ab	18.56 ±1.90 ab
Garlic	20.33±2.08 ab	17.67±2.08 ab	17.00±1.3 ab	18.33±1.76 abc
Onion	18.33±0.58 abc	13.33±5.69 bc	14.50±2.29 bcd	15.39±2.62 cde
Stinging nettle	17.73±2.10 bcd	15.00±2.00 abc	12.33±1.15 d	15±2.67de
Lion's ear	17.67±2.05 bcd	10.67±3.06 c	14.33±0.58 cd	14.22±3.50 e
Castor bean	17.17±0.29 cd	13.67±5.13 bc	16.00±1.00 abc	15.61±1.78 bcde
African basil	17.10±1.00 cd	16.00±3.61 abc	14.67±2.08 bcd	15.92±1.17 bcde
Lemon grass	16.67±3.51 cd	16.67±1.53 ab	14.33±0.58 cd	15.89±1.35 bcde
Rosemary	14.67±1.53 d	14.67±2.89 bc	14.00±1.73 cd	14.45±0.39 de
Mean $_2$	16.85±3.58 a	14.42±1.97 b	14.19 ±1.38 b	*
Controls (+; -)				
(+): Streptomycin	18.67±3.50 abc	17.00±3.46 ab	16.33±0.76 abc	17.33±1.21 abcd
(-): Methanol	1.33±0.12 e	*	*	1.33±0.12 f
(-): Water	*	0.00 ± 0.00 d	*	0.0 ± 0.0 f
(-): Chloroform	*	*	2.33±0.31 e	2.33±0.31 f
(-) DMSO	0.00 ± 0.00 f	0.00 ± 0.00 d	0.00 ± 0.00 f	0.0 ± 0.0 f
P value (α=0.05)	P<0.0001	P<0.0001	P<0.0001	P<0.0001

The values are an average of growth inhibitory zone (mm ± SD) from triplicates of methanol, water, or chloroform extracts from each of ten plant materials as well as the controls. Values within a column followed by the same letter are not significantly different at $P \leq 0.05$ according to Tukey's Studentized Range (HSD) test. (+, -): Positive and Negative controls. Absolute methanol, sterile distilled water, and absolute chloroform were used as negative control in methanol, water and chloroform extracts respectively. Streptomycin and 1 % DMSO was used as positive and negative control in all extracts. SD: Standard deviation. (*: Not applicable).

(streptomycin), with the exception of lion's ear. Although almost all the plant extracts exhibited the same antibacterial property as the synthetic antibiotic, they showed different levels of activity against the pathogen. The highest antibacterial activity was exhibited by tobacco, wild marigold, and garlic: 19.61, 18.56, and 18.3 mm, respectively. African basil, lemongrass and castor bean extracts displayed strong efficacy on the management of the pathogen with growth inhibition zone of 15.92, 15.89, and 15.61 mm, respectively. Additionally, onion, stinging nettle, and rosemary had a moderate inhibitory effect (15.39, 15 and 14.45 mm, respectively). However, the lowest inhibitory effect was obtained with lion's ear (14.22 mm) (Table 2).

It has been reported that some plant species contain bioactive compounds with antimicrobial activities (Cowan, 1999; Alabouvette *et al.*, 2006; Abo-elyousr and Asran, 2009; Stangarlin *et al.*, 2011; Wagura *et al.*, 2011; Malkhan *et al.*, 2012; Rahman *et al.*, 2012; Ribera and Zuñiga, 2012; Körpe *et al.*, 2013; Yuliar *et al.*, 2015; Sharma *et al.*, 2016). A large number of species in the family *Solanaceae* are rich in biochemicals of medicinal values (Sharma *et al.*, 2016). Tobacco extract was mainly reported to have insecticidal activities, but it also has antibacterial and antifungal properties against human diseases (Singh *et al.*, 2010; Bakht *et al.*, 2012; Sharma *et al.*, 2016). Phytochemical analysis of leaf aqueous and methanol extracts of tobacco revealed it contains flavonoids and alkaloids, which contribute directly to antibacterial activity against different strains of gram-positive and gram-negative bacterial strains (Singh *et al.*, 2010; Sharma *et al.*, 2016).

Apart from tobacco, many authors also confirmed that wild marigold (*Tagetes minuta*) belonging in Asteraceae family has antimicrobial activities against plant pathogens (Zeller and Ullrich, 2006; Irum and Mohammad, 2015; Gakuubi *et al.*, 2016). It was found that flavonoids, flavonols, and essential or volatile oils from *T. minuta* had a suppressive biological activity against pathogens and some insects (Irum and Mohammad, 2015; Yuliar *et al.*, 2015; Senatore *et al.*, 2003; Gakuubi *et al.*, 2016). These bioactive compounds inhibited the growth of different gram-negative bacteria and gram-positive bacteria (Gakuubi *et al.*, 2016). For instance, antibacterial activity of *T. patula* against *R. solanacearum* was confirmed in *in vitro* experiment carried out by Yuliar *et al.* (2015). They concluded that the *Tagetes* residues controlled the pathogen through their possible mechanisms of action of antimicrobial activities and by the indirect suppression of the pathogen through improved chemical, physical, and biological soil properties.

Allium plants contain volatile substance allicin which is the basis of antimicrobial action of those species against a broad range of plant pathogenic fungi, gram-negative and gram-positive bacteria, and Oomycetes (Benkeblia, 2004; Curtis *et al.*, 2005; Slusarenko *et al.*, 2008; Borlinghaus *et al.*, 2014). It has been reported that *Allium* plants contain antimicrobial properties against bacterial wilt of tomato (*R. solanacearum*) and other soil-borne pathogens as well as nematodes (Abo-elyousr and Asran, 2009; Rongquan *et al.*, 2011; Deberdt *et al.*, 2012). *Allium* byproducts can be used as an alternative to the phased out methyl bromide, a pesticide which was widely used for soil fumigation to kill the soil-borne diseases and pests (Arnault *et al.*, 2013; Mwitari *et al.*, 2013). In addition, Allicin was found to be as effective as other antibiotics namely kanamycin, penicillin, and ampicillin (Curtis *et al.*, 2005; Borlinghaus *et al.*, 2014). All these findings support the results from the present *in vitro* assay in which tobacco, wild marigold and garlic extracts highly inhibited the growth of potato bacterial wilt pathogen.

Antibacterial activity of solvent extracts against *R. solanacearum*: Antibacterial activity of methanol, water and chloroform extracts (mean of growth inhibition zone of the ten test plant materials from each solvent extract) against *R. solanacearum* was determined. From an *in vitro* assay, the results showed that all the methanol, water and chloroform extracts inhibited growth of *R. solanacearum* compared to positive and negative controls at $P \leq 0.05$. In general, the average of antibacterial activity from ten plant materials was significantly higher in methanol extracts (16.85 mm) than both water (14.42 mm) and chloroform (14.19 mm) extracts (Table 3). Methanol extracts controlled *R. solanacearum* to the same extent as the positive control streptomycin, a synthetic antibiotic. In addition, methanol extracts had a significant difference in growth inhibition of bacteria in comparison with water and chloroform extracts while water and chloroform extracts were similar.

Table 3. Growth inhibition zone (mm ± SD) of solvent extracts (methanol, water and chloroform) from ten selected plant materials against *R. solanacearum*

Solvent extract	Growth inhibition zone (mm)
Methanol	16.85±3.58 a
Water	14.42±1.97 b
Chloroform	14.19±1.38 b
Controls (+; -)	
(+) Streptomycin	17.33±1.21 a
(-): Methanol	1.33±0.12 c
(-): Water	0.00±0.00 c
(-): DMSO	0.00±0.00 c
P value (α=0.05)	*P*<0.0001

The values are an average of growth inhibitory zone (mm ± SD) of methanol, water or chloroform extracts from ten plant materials. Values within a column followed by the same letter are not significantly different at $P \leq 0.05$ according to Turkey Studentized Range (HSD) test. (+): Positive control (streptomycin), (-): Negative control (methanol, sterile water, chloroform, and 1 % DMSO).

In this research, methanol plant extracts inhibited the growth of *R. solanacearum* at the highest level among the other extracts (water and chloroform). They also inhibited the growth of bacteria to the same level as the synthetic antibiotic streptomycin which was used as a positive control. Similar effectiveness of methanol plant extracts from nettle compared to water extracts in the control of test gram-positive and gram-negative bacteria was confirmed by Körpe *et al.* (2013). In another *in vitro* experiment, methanol leaf extracts from castor bean were found to be more active against gram-positive bacteria as well as gram-negative bacteria than ethanol and water extracts (Naz and Bano, 2012). All these findings support observations in the present study that methanol extracts had a higher performance in the control of *R. solanacearum* than water extracts.

It was reported that the extraction solvent is one of the factors that affect the yield and composition of natural compounds (Kukrić *et al.*, 2012; Arnault *et al.*, 2013). Usually, methanol and water are organic solvents which are mainly used to extract polar compounds, whereas, chloroform is used for non-polar metabolites (Cowan, 1999; Ncube *et al.*, 2008; Mwitari *et al.*,

2013). Methanol has been revealed to be the best solvent to extract a high range of polar metabolites (Cowan, 1999; Kukrić *et al.*, 2012; Malkhan *et al.*, 2012; Arnault *et al.*, 2013). Apart from polar compounds, chloroform was used for extracting non-polar compounds (Cowan, 1999; Rahman *et al.*, 2012). In general, the results from this study showed lower antibacterial performance in chloroform than methanol extracts. This suggests that all or most of the ten selected plant materials may contain much more polar antibacterial metabolites than non-polar compounds.

Minimal inhibitory concentration (MIC) of plant extracts: From *in vitro* experiment, it was found that the MIC of methanol extracts from tobacco and wild marigold which completely restrained the growth of *R. solanacearum* was 6.25 mg mL^{-1} whereas methanol extract from garlic inhibited the growth of bacterium at 12.5 mg mL^{-1}. All water extracts tested killed the target bacterium at 12.5 mg mL^{-1} (Fig. 2, explanation for values are in Table 4). These findings showed that both methanol extracts from tobacco and wild marigold killed the pathogen at the low concentration (6.25 mg mL^{-1}) while their water extracts inhibited the growth of *R. solanacearum* at high concentration (12.5 mg mL^{-1}). Both methanol and water extracts from garlic controlled the bacterium at high concentration of 12.5 mg mL^{-1}.

Table 4. Minimal inhibitory concentration (MIC) (mg mL^{-1}) of test three plant extracts against *R. solanacearum*. (Explanation of values in Fig. 2)

Plant material	Solvent extract	MIC (mg $mL^{-1)}$
Tobacco	Methanol	6.25
	Water	12.5
Wild marigold	Methanol	6.25
	Water	12.5
Garlic	Methanol	12.5
	Water	12.5

From this experiment, the MIC of methanol was lower than the one of water extracts especially in tobacco and wild marigold extracts. This means that methanol extracts had higher antibacterial effect than water extracts against *R. solanacearum*. Similar results were found in the initial screening carried out to evaluate the efficacy of three selected solvent extracts (methanol, chloroform, and water) in management of the pathogen in Rwanda. In addition, two out of three test plant materials (tobacco and wild marigold) had the same MICs in methanol extracts. All three plant extracts inhibited the growth of the target bacterium at the same MIC in water extracts.

In previous studies, it was found that the efficacy of some plants extracts against different pathogens is dose-dependent (Curtis *et al.*, 2005; Gakuubi *et al.*, 2016; Sharma *et al.*, 2016). This supports the findings from the present study in which *R. solanacearum* was killed at higher concentrations and remained viable at the low concentrations both in methanol and water extracts from all three tested plant materials. In *in vitro* assay to determine the effectiveness of tobacco extracts at different concentrations (6, 12, 18 and 24 mg mL^{-1}) against different pathogenic bacteria, it was observed that all extracts had antibacterial activities against the test bacterium at the highest concentration (24 mg mL^{-1}) (Bakht *et al.*, 2012). This was higher than the MICs that inhibited *R. solanacearum* in the present study in both methanol and water extracts from tobacco. Various researchers listed different factors that affect the yield and composition of antimicrobial compounds for each species such as climatic conditions under which the plant has grown, organ used, stage of growth, extraction techniques and even the extraction solvents (Senatore *et al.*, 2003; Kukrić *et al.*, 2012; Arnault *et al.*, 2013; Alamshahi and Nezhad, 2015; Gakuubi *et al.*, 2016). The difference in MICs may be due to one or combination of some of these factors.

Similar concentration-dependent effect of plant active metabolites was also found in garlic and onion against bacteria and fungi (Curtis *et al.*, 2005; Borlinghaus *et al.*, 2014). In *in vitro* study, plant extracts were assessed at different concentrations and all of them controlled the pathogens at the highest concentration and garlic had higher antimicrobial activity than onion extracts (Curtis *et al.*, 2005; Borlinghaus *et al.*, 2014). All these results demonstrate that all the three plant extracts which were tested in the present study have strong antibacterial activity, which is also dose-dependent in the control of potato bacterial wilt. In general, methanol extracts inhibit the growth of this pathogen at low dose compared to water extracts.

In addition, Gakuubi *et al.* (2016) reported that gram-negative bacteria are usually more resistant to bioactive compounds than gram-positive ones. For instance, it has been found that MIC of *T. minuta* was 6.25 to 25 µg mL^{-1} for test gram-positive bacteria and 25 to 50 µg mL^{-1} for gram-negative bacteria (Senatore *et al.*, 2003). From another study, it was also observed that antibacterial activity of *T. minuta* increased with increasing concentration levels and gram-positive bacteria are less resistant to volatile

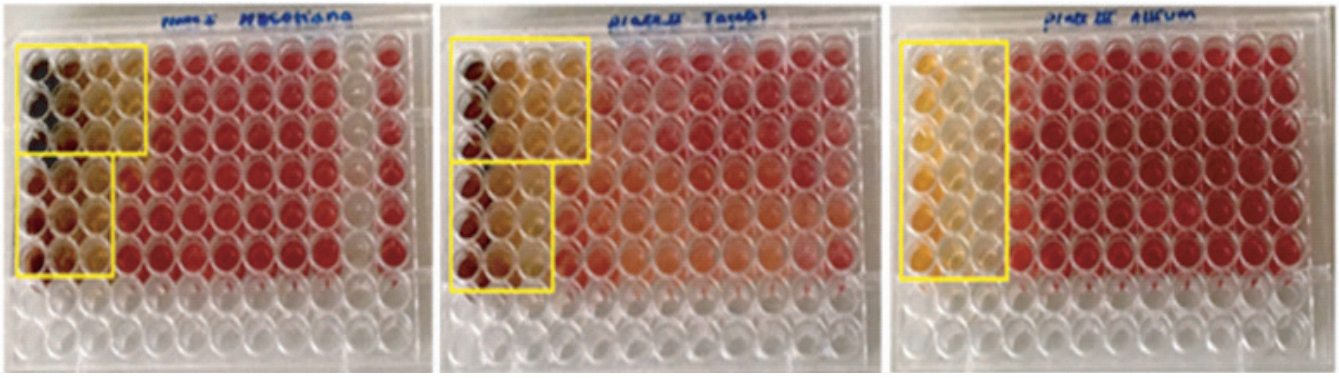

Fig. 2. Micro-dilution assay for minimal inhibitory concentration (MIC) of methanol and water extracts from active plant species against *R. solanacearum* on 96-well micro titer plates. Tobacco, wild marigold, and garlic extract (1st, 2nd, and 3rd plate, respectively. In each plate, the first three rows were used for methanol extracts and the next three rows for water extracts. The first to tenth wells of each row were used for serial diluted concentration (from 50 to 0.098 mg mL^{-1}). The eleventh and twelfth wells were used for the controls (streptomycin and nutrient broth in plate 1; sterile water and DMSO in plate 2; sterile water and methanol in plate 3). The presence of viable bacterial cells was indicated by the reduction of TTC into a red color, otherwise it retains the same color of initial medium (wells encircled by yellow line).

oils than gram-negative bacteria (Gakuubi *et al.*, 2016). The researchers reported that the MIC of the essential oils from *T. minuta* on gram-negative bacteria was 16.5 ± 0.9 mg mL^{-1}, whereas, the one against gram-positive ones was 6.7 ± 0.8 mg mL^{-1}. The *T. minuta* affects the multiplication and growth of gram-positive bacteria strongly than gram-negative ones (Gakuubi *et al.*, 2016). The results confirmed that natural compounds led to higher sensitivity on gram-positive than on gram-negative bacteria (Senatore *et al.*, 2003; Gakuubi *et al.*, 2016). It is already known that *R. solanacearum* is a gram-negative bacterium (Priou *et al.*, 2001; Mutimawurugo *et al.*, 2019) and this could explain why the MIC of *T. minuta* in the control of this pathogen may be higher than the one which inhibited the growth of other bacterial strains.

From this study, it is concluded that methanol extracts have higher potential against bacterial wilt of potatoes followed by water and chloroform extracts. Furthermore, minimum inhibitory concentration (MICs) is lower in methanol extracts than in water extracts. Thus, methanol extract has stronger antimicrobial properties than water extracts and is the most recommended in management of *R. solanacearum*. Moreover, all the tested plant extracts are effective in growth inhibition of *R. solanacearum* of potato under *in vitro* conditions. All the plant extracts tested except lion's ear are able to control bacterial wilt to the same extent as the synthetic antibiotic streptomycin. The use of the botanicals which are locally available, economically affordable, easy to prepare, nontoxic to non-target organisms and also environmentally friendly is a good management strategy for the potato bacterial wilt. The best performing plant materials were tobacco, wild marigold and garlic extracts. Hence, these promising plant extracts from this study can be used to produce natural compounds against bacterial wilt with non-toxicity to the environment. The antibacterial activity of these extracts should be confirmed *in vivo* in greenhouse as well as field experiments under Rwandan climatic conditions. The active ingredients of bioactive plant extracts should also be identified as well as their modes of action against *R. solanacearum*.

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Abbreviations

CFU: Colony Forming Unit; CIP: International Potato Center; CPG: Casamino Peptone Glucose DMSO: Dimethylsulfoxide; MIC: Minimal Inhibitory Concentration; RAB: Rwanda Agriculture board; REMA: Rwanda Environment Management Authority; TTC: Triphenyl Tetrazolium Chloride; UR: University of Rwanda.

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