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Effects of Selected Plants' Extracts on *in vitro* Growth of *Ralstonia slanacearum* (Smith), the Causal Agent of Bacterial Wilt of Irish Potatoes

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Abstract: The antibacterial effect of crude medicinal plant extracts of *Ocimum gratissimum*, *Brassica oleracea* var. *botrytis* and *Ipomoea batatas* on *Ralstonia solanacearum* (Smith) extracted from infected potato tubers was determined by *in vitro* study using ethyl acetate and methanol solvents. The extracts were used at concentrations of 0.4, 0.2, 0.1, 0.05 and 0.025 mg mL^{-1}. It was found that all the plant extracts used at their different concentrations except methanol extracts of *Ipomea batatas* at 0.025 mg mL^{-1} were effective to varying degrees in controlling the growth of bacterial colonies. The best results were observed with ethyl acetate extracts of *Ipomoea batatas* at concentration of 0.4 mg mL^{-1} giving mean inhibition zone of 4.2 mm followed by ethyl acetate extract of *Brassica oleracea* at concentration of 0.05 mg mL^{-1} that was 4.12 mm.

Key words: Antibacterial effect, *Ralstonia solanacearum*, *Brassica oleracea* var. *botrytis*, *Ipomoea batatas*, *Ocimum gratissimum*

INTRODUCTION

Irish potato (*Solanum tuberosum*) is the second most important staple food crop in Kenya after maize (Felix *et al*., 2010). It is ranked fourth most important food crop in the world after wheat, maize and rice with annual production approaching 300 million tons (Otipa *et al*., 2003). In Kenya the crop ranks second after maize and plays a major role in feeding the ever increasing population and providing source of income to the local people (FAO, 2006). However, production of potatoes has stagnated over the last 10 years with total yields ranging between 600 and 1200 thousand tones though acreage under the crop has been on the increase (Ministry of Agriculture, 2007). The low yields have been attributed to poor agronomic practices, low use of inputs especially fertilizers, low soil fertility, limited access to good quality seeds, diseases (especially bacterial wilt, late blight and viruses) and insect pests (Muthoni and Nyamongo, 2009; Lemaga *et al*., 2001a).

Among the diseases that infect Irish potato is bacterial wilt (also known as brown rot of potatoes) caused by race 3 biovar 2 of *Ralstonia solanacearum* and ranks the second most important potato disease after late blight (Felix *et al*., 2010; Lemaga *et al*., 2001b). The pathogen invades the roots of the host plant and aggressively colonizes the xylem vessels causing a lethal wilting (Tahat *et al*., 2008). In Kenya, the disease has been reported to cause losses ranging between 30-70% at altitudes ranging between 1800-2800 m above sea level. It is considered more problematic than late blight since it has no known chemical control procedures and many farmers do not know how to control it (Muthoni and Nyamongo, 2009). Besides, available cultural control methods such as crop rotation, use of clean seeds, planting in non-infested soils and growing tolerant varieties (Tahat and Sijam, 2010) have individual practical, technological and economic limitations (Lemaga, 2001; Muthoni and Nyamongo, 2009). Crop rotation for example is not feasible because the disease has been noted even in first planting in newly cleared land (Jinnah *et al*., 2002; Khalequzzaman *et al*., 2002). In addition, *R. Solanacearum* can survive in the soil for long periods in the absence of host plants (Tahat *et al*., 2008) and small farm sizes hinder effective crop rotation programmes (Muthoni and Nyamongo, 2009).

Green plants have been shown to represent a reservoir of effective chemotherapeutants and can provide valuable sources of natural pesticides (Dorman and Deans, 2000). Among the plants that have been shown to contain some antimicrobial components include *Brassica oleracea* var. *botrytis* (Kirkegaard, 2005), *Ipomoea batatas* (Graham *et al*., 2000; Kirkegaard, 2005) and *Ocimum gratissimum* (Mbata and Saikia, 2005; Adebolu and Salau, 2005; Amadi *et al*., 2010). Use of agrochemicals is becoming less favorable because of

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environmental pollution and detrimental effects on a variety of non-target organisms (Bonjar *et al*., 2006). Biological methods of control including use of natural plant products have therefore been preferred because most of them are locally available, environment friendly, have no side effects and development of resistance is rare (Okigbo and Ogbonna, 2006; Soytong *et al*., 2001; Khalequzzaman *et al*., 2002). They, however, need to be applied in an integrated manner as little can be achieved when they are solely applied. Fortunately, race 3 biovar 2 of *R. solanacearum* has a narrow host range and can be successfully controlled by Integrated Disease Management (Felix *et al*., 2010).

Very little work has been done to investigate the use of natural plant products to control of brown rot diseases of potatoes. Muthoni and Nyamongo (2009) reported that increased bacterial wilt occurrences can be explained by the lack of appropriate management practices as research on this subject has been at very low scales. This study was therefore carried out to determine the effects of selected plant extracts and their concentrations on the *in vitro* growth and development of *R. solanacearum* colonies.

MATERIALS AND METHODS

The study was conducted at Maseno University between September, 2006 and November, 2007. Fresh leaves of *O. gratissimum*, *B. oleracea* var.*botrytis* and vines of *I. batatas* variety SPK 004 were collected from Maseno region in Western Kenya. They were plucked, washed, shredded and dried as described by Okigbo and Ogbonna (2006). They were then ground into fine powder at Kenya Sugar Research Foundation in Kisumu, Kenya in readiness for solvent extraction.

Sequential extraction: Cold extraction of the powdered plant materials was done sequentially with organic solvents (ethyl acetate and methanol) of varying polarities following the method described by Eaton (1989). Known quantity of dry ground leaf material was soaked in extraction solvents (Table 1) in Erlenmeyer flask and left for four days with occasional shaking. The liquid portion was then filtered using Whatman No.1 filter paper. The filtrate was then concentrated *in vacuo* in a round-bottomed flask using rotary evaporator at 60 and 70°C for ethyl acetate and methanol respectively (Junaid *et al*., 2006). The extracts obtained were weighed and kept in vial bottles in readiness for bioassay tests (Eaton, 1989; Llorach *et al*., 2003). The amount of extracts obtained during the sequential extraction of the test plants were weighed and recorded (Table 1).

Isolation of wilt bacteria from infected potato tubers: Infected tubers were obtained from a test plot at the National Agricultural Research Laboratories (NARL) fields, Nairobi. These were cleaned under running water to remove adhering soil, air-dried, then cleaned using 97% ethanol to remove any microorganism on its surface. The skin at the end of the stolon was removed using a disinfected scalpel to make vascular tissues visible. A bacterial suspension was prepared using the method described by Priou *et al*. (1999). Approximately 0.5 mL of the bacterial suspension was spread on nutrient agar in Petri dishes. The plates were incubated for 48 hours at 28°C and bacterial colonies that were fluidal, flat, pearly white and irregular identified.

Pathogenicity test: The method of Koch's postulates was performed with *Solanum tuberosum* var. Tigoni 381381 as the host. After a 24 h period without water, one side of some potato roots were injured one centimetre from the stem and approximately 20 mL of an aqueous suspension of *R. solanacearum* of 1×10^7 cfu mL^{-1} was poured around the base of the stem. Five days after inoculation (after the wilting symptoms were exhibited), vascular flow test was run by cutting a piece of potato stem (5 cm long) and suspending it in clear water in a glass container. The cut stem was held with a clip to keep it in a vertical position until smoke like threads streamed downwards from the cut stem (Priou *et al*., 1999).

Antibacterial assay: The antibacterial effects of the plant extracts against *R. solanacearum* were evaluated using the method described by Barry *et al*. (1979) and De Souza *et al*. (2005). Inoculation was done by rubbing a sterile cotton swab containing the pathogen on the surface of solidified agar as described by Linnette *et al*. (1974).

Table 1: Weight of raw material and yield of plant extracts

Plant	Ground leaf material (g)	Solvent used	Solvent volume (mL)	Yield of plant extract (g)
O. gratissimum	200	EtOAc	1000	10.93
		MeOH	750	8.70
I. batatas	300	EtOAc	1200	10.00
		MeOH	1200	32.40
B. oleracea var. *botrytis*	300	EtOAc	1100	12.60
		MeOH	1500	47.30

EtOAc: Ethyl acetate, MeOH: Methanol

Experimental design, data recording and analysis: All tests were laid down in Randomized Complete Block Design (RCBD) with four replications. The antibacterial activity was recorded as the width (in mm) of clear zones of inhibition surrounding the diffusion discs after 48 h (Reiner, 1982; Baker *et al*., 1983; Deans and Ritchie, 1987). The data were subjected to ANOVA using SAS version 9.1 (SAS, 2005) and effects declared significant at 5% level. Separation of means was done only for those parameters where the ANOVA was significant, using Least Significant Difference at 5% level of significance ($LSD_{5\%}$) (Steel and Torrie, 1980).

RESULTS

Activity of plant extracts against *R. solanacearum*: The combined analysis of variance (Table 2) showed that the three plant extracts exhibited highly significant ($p<0.0001$) differences on their effects against growth of *R. solanacearum* at different concentrations (Fig. 1a). In addition, the two solvents (ethyl acetate and methanol) used in extraction of plant extracts portrayed highly significant ($p<0.0001$) differences in their suitability as extraction solvents (Fig. 1b). The interactions between plants extracts, their concentrations and the different extraction solvents were also highly significant ($p<0.0001$). This indicated that *R. solanacearum* responded differently to different plant extracts, their different concentrations and to different solvents used.

The antibacterial activity of both methanol and ethyl acetate extracts of *I. batatas* against wilt bacteria increased as their concentration increased. However best activity was observed with ethyl acetate extracts compared to methanol extracts. The best ($p<0.05$) results were observed with ethyl acetate extracts of *I. batatas* at concentration of 0.4 mg mL^{-1} giving a mean inhibition zone of 4.2 mm. Methanol extract of *I. batatas* at concentration of 0.025 mg mL^{-1} were inactive against *R. solanacearum* (Fig. 2a).

Activity of ethyl acetate extracts of *O. gratissimum* against the wilt bacteria increased as concentration increased up to 0.2 mg mL^{-1} beyond which it declined. Statistically, similar inhibitory activity was recorded at extract concentrations of 0.1 and 0.4 mg mL^{-1}. Methanol extracts of *O. gratissimum* at concentration of 0.025 and 0.05 mg mL^{-1} exhibited similar activity where inhibition zone measured 2 mm but the activity then increased as the concentration was increased. At concentrations of 0.05 and 0.1mg mL^{-1}, both the methanol and ethyl acetate extracts of *O. gratissimum* were not significantly ($p>0.05$) different in their inhibitory activity against the wilt bacteria (Fig. 2b).

Table 2: Combined analysis of variance for the three plant extracts

Source	DF	SS	MS	F-value	Pr>F
Blocks	3	0.208	0.069	1.29	0.2828[ns]
Plant	2	6.781	3.391	62.83	<0.0001
Solvent	1	10.563	10.563	195.72	<0.0001
Concentration	5	147.521	29.504	546.69	<0.0001
Plant×Solvent	2	11.656	5.828	107.99	<0.0001
Plant× Concentration	10	22.385	2.239	41.48	<0.0001
Solvent× Concentration	5	7.083	1.417	26.25	<0.0001
Plant×Solvent× Concentration	101	2.885	1.289	23.88	<0.0001

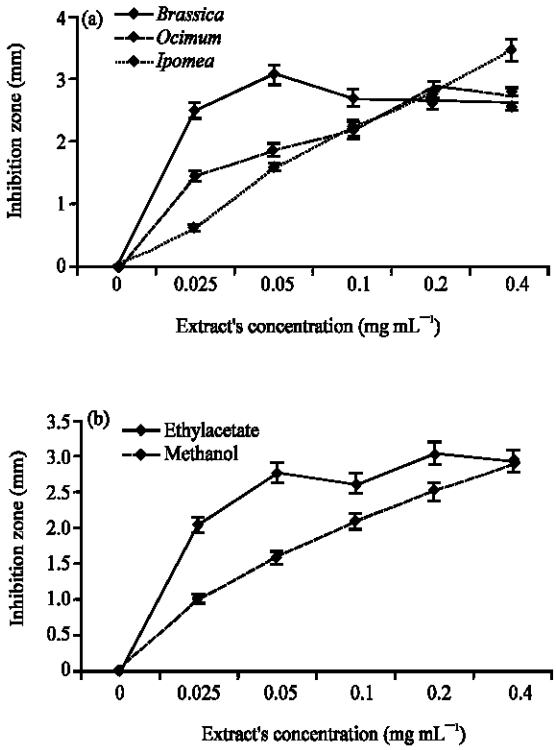

Fig. 1: (a) Comparative suitability of Ethyl acetate and Methanol as extraction solvents and (b) comparative combined antibacterial effects of *O. gratissimum, I. batatas* and *Brassica oleracea* var. *botrytis* extracts against *R. solanacearum*

Inhibitory effects of *B. oleracea* var. *botrytis* are illustrated in Fig. 3. The best results were realized with ethyl acetate extracts of *B. oleracea* at concentrations of 0.025 and 0.05 mg mL^{-1} which recorded an inhibition zone of 3.78 and 4.18 mm, respectively. The two treatments were, however, not significantly ($p>0.05$) different from each other. Inhibitory activity of methanol extracts of *B. oleracea* increased as concentration increased attaining an inhibition zone of 2.94 mm at the highest concentration of 0.4 mg mL^{-1}. Unlike methanol, suitability of ethyl acetate as an extraction solvent appeared to decline at higher concentrations.

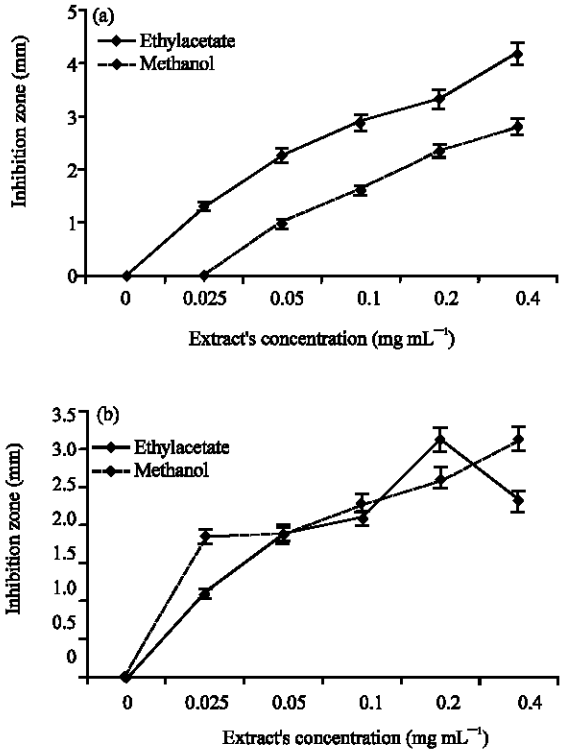

Fig. 2: Antibacterial effects of (a) *I. batatas* and (b) *O. gratissimum* extracts extracted using ethyl acetate and methanol solvents

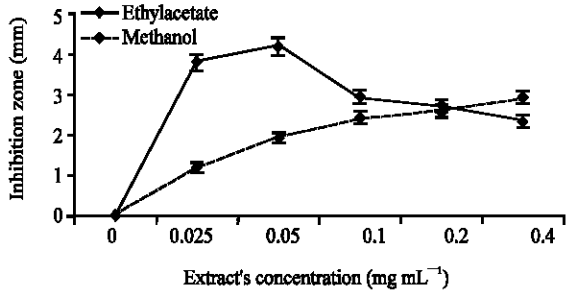

Fig. 3: Antibacterial effects of *B. oleraceae* var. *botrytis* extracts extracted using ethyl acetate and methanol solvents

DISCUSSION

This study demonstrated that compounds extracted from the three plants using the two organic solvents vary in their efficiency in inhibiting bacterial growth. The difference observed in antibacterial activity of the extracts is likely to be due to the solubility of the active compound(s) in ethyl acetate and methanol or the presence of inhibitors to the antibacterial principle. This agrees with the report of Okigbo and Ogbonna (2006). The two solvents have different polar abilities with methanol having higher polarity and thus they tend to dissolve different compounds from the plant materials dipped in them. Polar solvents dissolve polar compounds best and non polar solvents dissolve non polar compounds best (Siddhuraju and Becker, 2003). The presence of antibacterial substances in the different extracts which caused the inhibition of radial growth *in vitro* agree with reports of other studies (Olayinka, 2009; Mbata and Saikia, 2005). The active principles present in plants are influenced by many factors which include the age of plant, extracting solvent, method of extraction and time of harvesting plant materials (Ajalie and Okigbo, 2005; Okigbo *et al*., 2005; Okigbo and Ogbonna, 2006).

***O. gratissimum* extracts:** Antibacterial effects of *O. gratissimum* extracts have also been reported by Ntezurubanza *et al.* (1984), Nakamura *et al.* (1999), Iwalokun *et al.* (2003), Lemos *et al.* (2005), Akinyemi *et al.* (2004), Lemos *et al.* (2005) and Lopez *et al.* (2005) reported that Ocimum oil is active against several species of bacteria (*Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, Shigella, Salmonella and Proteus) and fungi (*Trichophyton rubrum T. mentagrophytes*, *Cryptococcus neoformans*, *Penicillium islandicum* and *Candida albicans*). Although there was a gradual increase in the inhibitory activity of ethyl acetate extracts of *O. gratissimum*, the activity was less consistent compared to the methanol extracts. The significantly higher activity of methanol extracts compared to ethyl acetate extracts could be an indication that methanol absorbed more antibacterial principles from *O. gratissimum* than ethyl acetate. This could therefore mean that the anti microbial principles of African basil against wilt bacteria are polar compounds. Similar findings were reported in a study of Junaid *et al.* (2006) while examining the methanol and hexane extracts of *O. gratissimum* against *E. coli*. They found higher inhibition in methanol extracts and concluded that the active components of the plant could be highly polar.

***I. batatas* extracts:** The study showed that *I. batatas* extracts contains antibaterial compounds against *R. solanacearum*. This agrees with Cevallos-Casals and Cisneros-Zevallos (2002) who reported that sweetpotato extracts caused growth inhibition of *Salmonella enteritidis*. The significantly high activity of ethyl acetate extract for all concentrations used compared to methanol extract is an indication that ethyl acetate extract contained more anti microbial principles against *R. solanacearum*

than methanol extracts. This could probably mean that the active compounds of this plant against the bacteria are less polar compounds. Ishiguro *et al.* (2004) and Islam (2006) reported that the leaves of *I. batatas* contains large amounts of polyphenolics such as anthocyanins and phenolics acids and are rich in vitamins and minerals. When ethanol and water were used in their extractions, higher polyphenols concentration was found with ethanolic extracts. The compounds extracted using the solvent may be similar to those released when the vines were used in a biofumigation study (Kirkegaard, 2005) to control bacterial wilt where the effectiveness of the vines in controlling bacterial wilt increased with increase in the amount used.

***B. oleracea* var. *botrytis* extracts:** There was significantly high activity of ethyl acetate extracts of *B. oleracea* var. *botrytis* at lower concentrations of 0.025, 0.05 and 0.1 mg mL^{-1} compared to methanol extract. This indicates that most antimicrobial principles of ethyl acetate extract of cauliflower are highly effective against *R. solanacearum* in lower concentrations. This could be interpreted to mean that the less polar active compounds of this plant interact best with the bacteria at low concentrations. The activity of methanol extract increased gradually with increase in its concentration surpassing the effectiveness of ethyl acetate indicating that the polar compounds were more effective at higher concentrations. Walters (2009) reported the release of isothiocyanates (ITCs) from Brassica species like cauliflower that suppressed the bacterial wilt of potatoes when used as green manure and that the effectiveness increased as materials incorporated increased. Larkin and Griffins (2007) also reported the release of surfur compounds from Brassica species that suppressed soil borne potato diseases when chopped leaves were incorporated in potato fields.

CONCLUSION

The study demonstrated that *O. gratissimum*, *I. batatas* and *B. oleracea* var. *botrytis* contain antibacterial compounds which can be utilized in preparation of a potential phyto-bactericide to control the pathogenic bacteria that causes brown rot of potatoes. It was apparent that the use of raw plant extracts of the three plants has a potential to substitute the chemical approach of controlling the disease. This kind of biological approach would be economically viable and environmental friendly. The plants are also available to all farmers including those that do not have ready access to other chemicals. The study did not ascertain the specific chemical compounds that were responsible for antibacterial activity. Further research is therefore recommended to identify the active compounds against the wilt bacteria.

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