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In vitro and greenhouse evaluation of botanical extracts for antifungal activity against *Phythopthora infestans*

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ABSTRACT

The aim of this study was to evaluate plant extracts for antifungal activities against *Phythopthora infestans* (Mont.) de Bary, an important pathogen of potato which causes late blight disease. Acetone, ethyl acetate and water extracts of garlic (*Allium sativum* L.), Pawpaw (*Carica papaya*), Neem (*Azadirachta indica*), Mexican marigold (*Tagetes minuta*) and peri –winkle (*Vinca rosea*) were screened *in vitro* for their antifungal activities against *Phythopthora infestans* using the disc agar infusion and microtitre double-dilution techniques. The same extracts were then tested for antifungal activity *in vivo* in the greenhouse on inoculated potato plants. The water extracts of *Allium sativum* and *Azadirachta indica* were active against *Phythopthora infestans* and had a minimum inhibitory concentration (MICs) of 1.65 mg/ml. MICs of *Allium sativum*, *Azadirachta indica* and *Tagetes minuta* acetone extracts were 0.78, 3.13 and 6.5 mg/ml respectively against *Phytophthora infestans*. *Allium sativum* and *Azadirachta indica* water and acetone extracts performed well and they effectively reduced potato late blight in the greenhouse. These extracts compared well with the reductions due to application of Ridomil, a synthetic fungicide applied at 42.5g/l (active ingredient) and also with levels in the non-inoculated control. *Allium sativum* and *Azadirachta indica* were effective both *in vitro* and *in vivo* in controlling late blight have a potential to be used as fungicides against the disease. The plants are readily available and the extraction method is also simple and could lead to high adoption as fungicides by resource poor farmers.

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INTRODUCTION

The late blight disease of potatoes is the most devastating disease of potatoes in the world. The disease is caused by the pathogen *Phytophthora infestans* (Mont.) De Bary which may also infect other solanaceous plants. Late blight may kill the foliage and stems during the growing season. It also attacks potato tubers in the field, which may rot either in the field or in storage. Late blight may cause total destruction of all plants in the field within a week or two (Agrios, 2005).

The use of chemicals or synthetic chemical fungicides are being used successfully but indiscriminate use of these chemicals led to have development of fungicides resistance and more important environmental pollution, posing a potential risk to animal and human health such as undesirable side effects due to their carcinogenic properties (Alkhail, 2005; Lyon *et al*., 1995).Considering the adverse and alarming effects of synthetic pesticides on environment and natural habitats and the promotion of environmentally sustainable and organic agriculture, fungicide alternatives such as the use of natural plant products is needed (Slusarenko *et al*., 2008; Rice *et al*., 1998).

Plants contain antimicrobial compounds which can be toxic to pathogens and these natural plant products can be used to control plant diseases. Contrary to the problems associated with the use of synthetic chemicals, botanical extracts are environmentally non pollutive, renewable, inexhaustible, indigenously available, easily accessible, largely non phytotoxic, systemic ephemeral, thus readily biodegradable, relatively cost effective and hence constituted as suitable plant protecting agents in the strategy of disease management (Kuberan *et al*., 2012). The objective of this study was to screen botanical plant extracts

for antimicrobial activity against the *Phytophthora infestans*, a pathogen of potato.

MATERIALS AND METHODS

Extraction of botanical extracts

The plant materials used in the present study were leaves of Peri-winkle (*Vinca rosea*), Mexican marigold (*Tagetes minuta*), Neem (*Azadirachta indica*), sand pawpaw (*Carica papaya* L) and garlic (*Allium sativum* L) bulb. Neem was collected from Chiredzi, pawpaw, Mexican marigold and peri-winkle were collected in areas around Harare. The garlic bulbs were bought at Mbare Musika in Harare. The plants used in the experiment were collected a week prior to the commencement of the experiment. All the plant materials were air dried in the shade and ground to powder using a pestle and mortar. Sequential extractions were performed on 1 kg of each plant powder by soaking them in 2 litres of 27·06 moles acetone, 20·30 moles ethyl acetate and sterile distilled water (24 h in each). The plants were soaked in that order contingent to the polarity of the solvents. The organic solvents were removed using a Büchi Rotavapor (Model R-200, Switzerland) and water plant filtrates were concentrated to powder using a freeze drier (Edwards High Vacuum International, Sussex, England). Different quantities of crude extracts were harvested from different solvents as shown in Table 1. All the extracts were then stored in the refrigerator at 4 °C. Potato plants with blight and stem rot symptoms were collected from farms around Harare. Stems and leaves were surface disinfected in0.15 mg/mL sodium hypochlorite for 3min and rinsed three times in sterile distilled water, and then placed on potato dextrose agar (PDA) under continuous ultraviolet light for 7 days at 24±2 °C. *Phytophthora infestans* cultures were morphologically identified using a stereomicroscope, subcultured and stored in a refrigerator (4 °C) until needed.

Table 1. The quantities (g) of crude extracts harvested from 1 kg plant material using acetone, ethyl acetate and water as solvents.

Plant	Solvent		
	Acetone	Ethyl Acetate	Water
Azadirachta	7	13	53
indica	14	4	414
Allium sativum	33	15	103
Carica papaya	Nd	Nd	Nd
Vinca rosea	20	18	125
Tagetes minuta			

Antifungal activity of plant extracts

The antifungal activity of the extracts was determined using the method described by Kritzinger *et al*. (2005) and stock solutions of the crude extracts were prepared. Correct quantities of each crude extract was added to 100 mL of PDA before pouring into Petri dishes to give final concentrations of 0·5, 1·0, 2·5 and 5·0 mg/mL. The PDA containing the different botanical extracts was poured into 65mm Petri dishes with preset *diametrical lines* drawn on the bottom plate to identify the centre of the plate. Fungal plugs (5 mm diameter) of *P. infestans* from 7-day-old fungal cultures were placed at the centre of the Petri dishes containing PDA amended with either water extracts, acetone extracts0·05 mL/mL (v/v), ethyl acetate extracts 0·05 mL/mL (v/v) or the fungicide Ridomil (42.5 gai/l).The Petri dishes were then sealed with Parafilm® and incubated at 25 °C. Acetone, ethyl acetate and unamended PDA represented the negative controls. Ridomil represented the positive control. The treatments were arranged in a completely randomized design and each treatment was replicated four times. The experiment was repeated twice. The diameter of the growth inhibition zones were measured after 3, 6 and 9 days of incubation (DAI).

Microtitre double-dilution assay

Malt extract broth was inoculated with fungi and incubated for 5 days at 25 °C. The broth was adjusted to 0·2 optical density (thus 1×10^5spores/mL) using a spectrometer just before inoculation. In order to yield a series of 50 mg/mL solutions of extracts, 50 mg of each of the plant extracts were dissolved in 1000 µL of 121·7 mg/mL dimethyl sulphoxide (DMSO). The 96-well microtitre plates were used and 100 µL of the broth was added to all the wells as follows: 100 µL of stock solution (50 mg/mL) of acetone plant extracts were added to the first three wells of row A, ethyl acetate plant extracts were added to the wells 4–6 and water plant extracts were added to wells 7–9. Nutrient broth was added to well 10 as a negative control, while Ridomil (100 µL of 4.2 mL/mL (v/v) was added to well 11 as a positive control. To well 12, 100 µL of 121·7 mg/mL DMSO was added as a negative control. A series of dilutions of plant

extracts, Ridomil and 121·7 mg/mL DMSO were carried out to row H. Six-day-old malt broth cultures (100 µL) of fungi were added to each well. The plates were then covered with lids, sealed with Parafilm® and incubated at 25 °C for 48 hrs. Thereafter, 40 µL of 0·2 mg/mL iodonitrotetrazolium (INT) chloride was added to all the wells, with the exception of columns 3, 6 and 9 as a growth indicator to determine the MIC values for the plant extracts. The microtitre plates were incubated for 24 hrs 25 °C and evaluated. The first clear wells from A to H were regarded as the MICs of extracts. The experiment was repeated three times and the final MIC for the extracts was calculated as described by Fawole *et al*. (2009).

Inoculation of soil and greenhouse evaluation of plant extracts

This experiment was performed in the University of Zimbabwe greenhouses in October, 2013. Steam sterilized soil was thorough by mixed with fungal inoculums adjusted to a concentration of 2.5 x 10^4 spores mL^{-1}. After a 5 day incubation period, soil was treated by incorporating 160 mL of formulated extracts into 5 L of soil. For the positive control soil was treated with 160 mL of Ridomil and water was used in the negative control. Treated soil was placed in plastic bags which were closed tightly and incubated for a further period of 7 days. After 7 days, the soil from each treatment was placed in six 20-cm-diameter plastic and one sprouted tuber of variety Amethyst was planted in each pot. The pots were placed randomly on the greenhouse bench. Plants were assessed for symptoms starting from 2 weeks after crop emergence (WACE) and then weekly thereafter. Each treatment was replicated three times and treatments were arranged in a randomized complete design. Water was applied daily in order to maintain soil moisture at field capacity. Each experiment was repeated twice. Data collected included blight and stem rot disease incidence and severity.

Data Analyses

The inhibition data were arc sine transformed and then analysed statistically using the Genstat computer package (VSN International 2008). The untransformed (Table 1) quantities (g) of crude extracts harvested from 1 kg plant material using acetone, ethyl acetate and water as solvents and transformed data led to the same conclusion; hence the untransformed analysis data have been presented.

RESULTS AND DISCUSSION

Acetone plant extracts inhibited *in vitro* growth of *P. infestans* at all concentrations 6 days after inoculation (DAI) (Figure 1). All concentrations of *Allium sativum* extract completely inhibited the growth of *P. Infestans* while *Azadarachta indica* extract completely inhibited *P. infestans* at 5·0 mg/mL. The general trend observed showed that the inhibition increased as the concentrations of all extracts increased, with the exception of *Vinca rosea* which appeared to have a stimulatory effect on the growth the pathogen as the concentration increased. In fact, *V. rosea* extracts stimulated fungal growth as the concentrations increased.

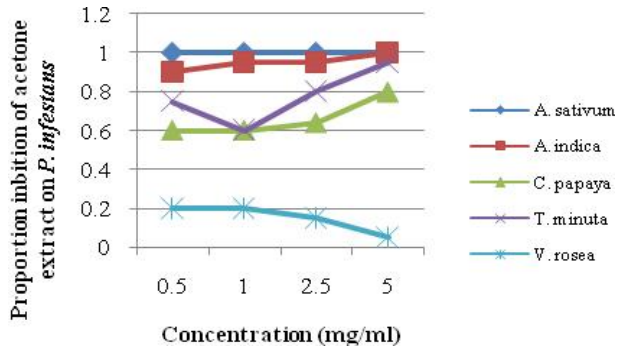

Fig 1. The effect of acetone crude plant extracts on the colony growth of *P. infestans* after 6 days of inoculation.

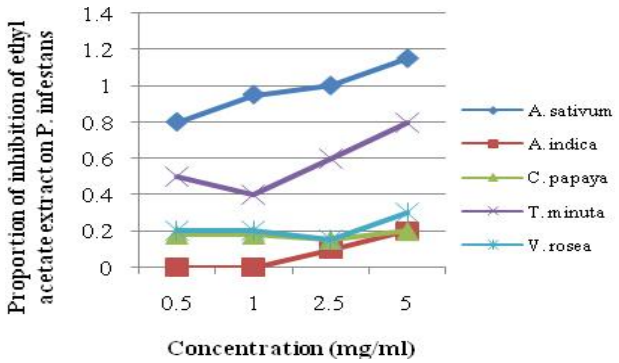

Fig 2. The effect of ethyl acetate crude plant extracts on the colony growth of *P. infestans* after 6 days of inoculation.

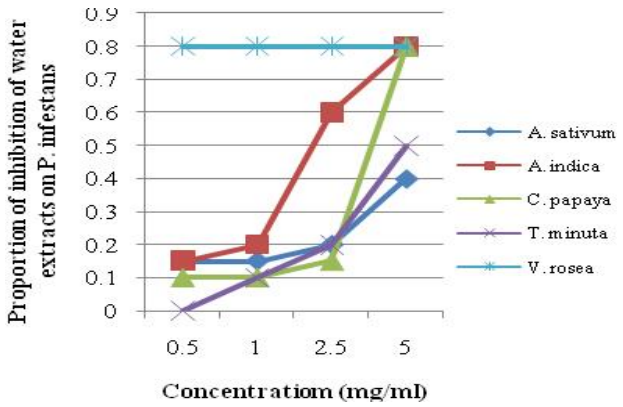

Fig 3. The effect of water crude plant extracts on the colony growth of *P. infestans* after 6 days of inoculation.

P. infestans was completely inhibited by *Allium* ethyl acetate extracts at concentrations of 2·5 and 5·0 mg/mL (Figure 2), while the ethyl extract of *Tagetes minuta* inhibited *P. infestans* growth by 80% at 5·0 mg/mL compared with the control. All other ethyl acetate plant extracts (*Azadirachta*, *Carica* and *Vinca*) failed to inhibit *P. infestans* at all concentrations. The *Carica* water extract was effective at 5·0 mg/mL as it completely inhibited the growth of *P. infestans*. This was followed by water extracts of *Vinca* and *Azadirachta* which inhibited growth of *P. infestans* by more than 90% (Figure 3).

The potato plants treated with water extracts of *A. indica* (5 and 15 mg/mL), acetone extracts from *Carica* (15 mg/mL) and *Tagetes minuta* (5 mg/mL) extracts showed no blight disease incidence (Figure 4a). The above extracts and 5mg/mL water extracts from *Allium* had low blight disease incidence when compared with the non-inoculated control and Ridomil. All the acetone plant extracts, 5mg/ml *Allium* and *Tagetes* water extract-treated plants showed significantly ($P < 0·05$) lower blight disease severity than water treatments and compared well with Ridomil and the non-inoculated control (Figure 4b).

Table 2 shows the MIC for botanicals extracted with acetone, ethyl acetate and water extracts from different plant species. All the *Tagetes* extracts were active against *P. Infestans. Allium* extracts were also active against the pathogen. However, the water extract failed to inhibit the growth of *P. infestans*.

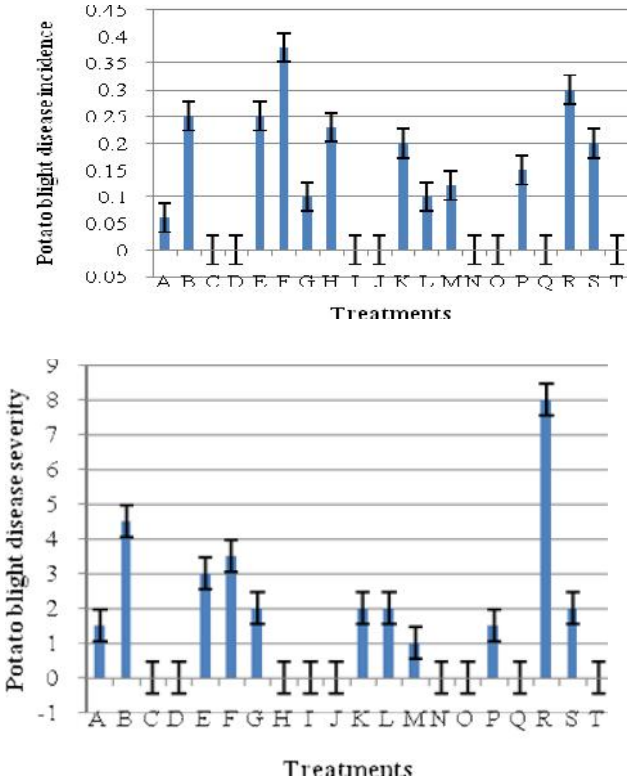

Fig 4. (a) Proportion of potato plants affected with blight: disease incidence. (b) blight disease severity of potato plants treated with plant extracts. Treatments A–H=water extracts and I–P=acetone extracts (A=5 mg/mL garlic extracts; B=15 mg/mL garlic extracts; C=5 mg/mL *Azadirachta* extracts; D=15 mg/mL *Azadirachta* extracts; E=5 mg/mL *Carica* extracts; F=15 mg/mL *Carica* extracts; G=5 mg/mL *Tagetes* extracts; H=15 mg/mL *Tagetes* extracts; I=5 mg/mL garlic extracts; J=15 mg/mL garlic extracts; K=5 mg/ml *Azadirachta* extracts; L=15 mg/mL *Azadirachta* extracts; M=5 mg/mL *Carica* extracts; N=15 mg/mL *Carica* extracts; O=5 mg/mL *Tagetes* extracts; P=15 mg/mL *Tagetes* extracts). Q=non-inoculated control; R=inoculated control;S=DMSO and T=Ridomil.

Azadirachta acetone and ethyl acetate extracts were active against both fungi with MICs of 1·56, 3·13 and6·25 mg/mL, respectively, but the range of concentrations of water extracts did not reveal a MIC. *Carica* and *Vinca* water extracts were active against *P. infestans*, but their organic extracts were inactive against the pathogen. DMSO was inactive against *P. infestans*. Ridomil had a MIC of 0·09 mg/ml, the highest inhibitory activity when compared with all the plant extracts. *Allium* Acetone and ethyl acetate extracts were as effective as Ridomil inhibiting completely the growth of *P. Infestans* in the agar infusion technique.

Table 2. The MIC of selected plants extracts on *Phythopthora infestans* (mg/mL).

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Plant Name	Solvent		
	Acetone	Ethyl Acetate	Water
A. sativum	0.78	0.78	0
A. indica	6.25	3.13	0
T. minuta	3.13	0.78	1.56
C. Papaya	0	0	1.56
V. rosea	0	0	12.5
Ridomil	0.09	0.09	0.09
DMSO	0	0	0

DMSO, dimethyl sulphoxide

Vinca rosea water extracts had higher inhibition values on *P. infestans* than acetone or ethyl acetate extracts and it can be speculated that the antifungal chemical present in *Vinca* is only soluble in water and not in acetone or ethyl acetate. *Carica* leaf water extracts at 5·0 mg/mL performed well and was comparable to Ridomil, by completely inhibiting the growth of *P. infestans* in the agar infusion technique. The performance of *Carica* water extracts on *P. infestans* was superior to that of organic extracts in both *in vitro* techniques used in the present study and these results are in agreement with the findings of Anibijuwon and Udeze (2009), who reported that *Carica* leaf water extracts gave higher inhibition against bacteria than organic extracts. However, *Carica* water extracts failed to suppress the disease *in vivo* by permitting a higher incidence and severity of potato blight than other extracts.

All *Tagetes* extracts were active against *P. infestans* in the microtitre double-dilution experiment and the *in vivo* experiment where it recorded the lowest disease incidence and severity for the pathogen. Generally, there was an increase in inhibition of fungal colony growth by the other plant extracts as concentrations increased and this could have been due to the increased concentration of the antifungal chemicals in the media. Cao and van Bruggen (2001) reported the same trend of increase in inhibition of the growth of *Phytophthora infestans* in media when *A. sativum* extract concentrations were increased. Acetone plant extracts showed the highest inhibitory activity among the solvent extracts *in vitro* and also gave good results *in vivo*. Masoko *et al*. (2005) also found that acetone extracts performed better than extracts of other solvents such as hexane, dichloromethane and methanol. The extraction method could have a contributory factor towards the poor performance of ethyl acetate extracts compared with acetone extracts because most of the organic soluble chemicals in the plants had already been extracted in the acetone and little remained in the residues. The water extracts gave poor results *in vitro*, and this poor performance *in vitro* could be due to the lower concentration (5–5mg/mL) used *in vitro* compared to the higher concentration (5–15 mg/mL) used *in vivo*. The water extract results could also have been influenced by the solubility of compounds in water as speculated by Masoko *et al*. (2005), who stated that water fails to extract non-polar active compounds in plant materials. This explains a need for higher plant water extracts concentration to achieve fungal toxic level. The present study has contributed an addition to the number of plants that can potentially be used by smallholder farmers in controlling blight diseases of potato. All the plant extracts except *Carica* water extracts were effective in reducing disease severity on potato. Extracts from the plant species used in the present study can be adopted easily as fungicides by farmers as they can be obtained easily by the farmers. The smallholder farmers can therefore use *A. sativum*, *A. indica* and *C. papaya* extracts to spray potato plants in order to control *P. infestans* infections. The use of these botanical extracts can reduce the use of synthetic fungicides and this can also reduce pollution of the environment. The plants can also reduce mammalian toxicity caused by synthetic fungicides (Ngowi *et al*., 2007). There is, however, a need to optimise and refine the extraction methods and the performance of water and acetone as solvents in extractions involving *A. sativum*, *A. indica*, *C. papaya* and *V. rosea* as foliar fungicides which can be used in the control of potato diseases, as *A. sativum* and *C. papaya* leaf extracts can be used effectively in the control of late blight in potato.

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