EVALUATION OF *ACACIA STUHLMANNII* PLANT EXTRACTS FOR THEIR EFFICACY ON MANAGEMENT OF BACTERIAL WILT OF TOMATO CAUSED BY *RALSTONIA SOLANACEARUM*

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I56/PUC/2086/11

A thesis submitted in partial fulfillment of the requirements for the award of Master of Science Degree in Microbiology of Pwani University

October, 2015
DECLARATION

Declaration by the candidate

This thesis is my original work and has not been presented for a degree in any other University or other award.

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I dedicate this research thesis to my dear parents Mr. and Mrs. Chrisantos Nyaboga, my brother Simon and sisters, Christine, Irene and Evelyne, who have been my inspiration towards this achievement.
ACKNOWLEDGMENT

I thank my supervisors Dr. Joseph Mwafaida Mghalu and Prof. Paul Guyo for their guidance in all stages of this research, and for their tireless effort in correcting this thesis. I acknowledge Dr. Rose Kigathi for the statistical advice. I also acknowledge my colleague Mr. Mwadogo Omar who has been supportive towards the development of this thesis. I’m indebted to all technicians for their tireless support while working in the Biology and Chemistry laboratories of Pwani University.

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ABSTRACT

Plant phytoactive compounds are antimicrobial against multidrug resistant bacteria. These compounds can be extracted from plants by use of solvents. The objective of this study was to extract and evaluate crude compounds from *Acacia stuhlmannii* root barks against *Ralstonia solanacearum*. Crude extracts was obtained by single solvent maceration. Polar (ethanol) moderately polar (ethyl acetate) and non-polar (hexane) solvents yielded 7.94, 4.90 and 3.27% respectively, from about 1 kg of powder. The extracts were tested against causative agent of bacteria wilt of tomato in the laboratory and in pots under a black shade net condition. *Invitro* assay was done by disc diffusion sensitivity test at incubation temperatures of 28°C and 35°C using cotrimozazole and 10% v/v Dimethyl Sulfoxide (DMSO) as positive and blank controls, respectively. Hexane extracts performed best at all incubation temperatures. Further assays were done to determine the minimum inhibition concentrations (MIC) of extracts using serial dilution method. Concentrations of 18.3, 19.3, and 25.7 mg ml⁻¹ w/v were determined as MIC for hexane, ethyl acetate and ethanol extracts, respectively. Findings indicated stability in activity of hexane, ethyl acetate and ethanol extracts against changes in temperatures, ultra violet (UV-B) band and pH. *Invivo* assays were done on two (2) weeks old tomato (Cal J variety) seedlings. Application of 20 ml of MIC extracts was done through soil drenching two (2) days before inoculation, at inoculation time and two (2) days after inoculation. About 15 ml of *Ralstonia solanacearum* suspension of 10⁷ CFU ml⁻¹ was inoculated in each 500 ml plastic pot. Sets treated with sterile distilled water were used as controls. All seedlings were pricked twice around the root region to enhance infection after inoculation and watered regularly. Diseases severity was scored on a six-pointed scale at an interval of three (3) days for 12 days. Recorded data was subjected to analysis of variance (ANOVA) and means separated at 95% confidence.
level. Hexane extracts reduced disease development by a maximum of 84.2\%, ethyl acetate extracts by 52.7\% and ethanol extract by 41.5\% after twelve (12) days of incubation. Treatment done two days before and simultaneous with pathogen inoculation were not significantly different (P>0.05) at day 12. Further, hexane extracts were assessed for bioactivity at half MIC, full MIC and double MIC rates. The performances of full and double MIC were not statistically different at P>0.05. Hexane extracts were further evaluated for efficacy in pot trials. The disease severity indices and fruit production were assessed after 75 days from treatment. Treatments applied with hexane extracts simultaneously or before inoculation significantly suppressed disease occurrence compared to controls. However, there was no significant difference (P>0.05) in disease incidence and fruit yield for treatments where the extracts were introduced before or simultaneously with \textit{R. solanacearum} inoculations. Treatments done two days after pathogen inoculation did not (P<0.05) suppress bacterial wilt disease. These findings show that hexane extracts from \textit{A. stuhlmannii} provide a promising biocontrol strategy in the control of \textit{R. solanacearum} disease in tomatoes.

Key words: Biological control, bacterial wilt, disease suppression
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<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>Ø</td>
<td>Diameter</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μl</td>
<td>Microliter</td>
</tr>
<tr>
<td>ºC</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ASAL</td>
<td>Arid and Semi-Arid Land</td>
</tr>
<tr>
<td>ASFM</td>
<td>American Society for Microbiology</td>
</tr>
<tr>
<td>AVRDC</td>
<td>Asian Vegetable Research and Development Centre</td>
</tr>
<tr>
<td>BW</td>
<td>Bacteria Wilt</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeters</td>
</tr>
<tr>
<td>CPGA</td>
<td>Casamino Peptone Glucose Agar</td>
</tr>
<tr>
<td>CRD</td>
<td>Completely Randomized Design</td>
</tr>
<tr>
<td>DAI</td>
<td>Days after Inoculation</td>
</tr>
<tr>
<td>DBS</td>
<td>Department of Biological Sciences</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DSI</td>
<td>Disease Severity Index</td>
</tr>
<tr>
<td>EPPO</td>
<td>European and Mediterranean Plant Protection Organization</td>
</tr>
<tr>
<td>Etnl</td>
<td>Ethanol</td>
</tr>
<tr>
<td>EtOAc/ETAC</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
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<tr>
<td>FAO</td>
<td>Food and Agriculture Organization</td>
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<td>FAOSTAT</td>
<td>FAO Statistical Databases</td>
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<td>GoK</td>
<td>Government of Kenya</td>
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</table>
Ha  Hectares
Hex  Hexane
Hrs  Hours
IZD  Inhibition Zone Diameter
kg  Kilogram
km  kilometer
m  Miter
M  Molar
MIC  Minimum Inhibitory Concentration
MHA  Muller Hinton Agar
MHB  Muller Hinton Broth
mgml$^{-1}$  Milligram per milliliter
MoA  Ministry of Agriculture
PC  Percentage Control
PR  Percentage reduction
Rs2bv1  *Ralstonia solanacearum* race 2 biovar 1
Rs3bv2  *Ralstonia solanacearum* race 3 biovar 2
SDW  Sterile Distilled Water
SE  Standard Error
TTC  Triphenyl Tetrazolium Chloride
USDA  United States Department of Agriculture
WAP  Weeks after Planting
CHAPTER ONE

INTRODUCTION

1.1. Background information

*Ralstonia solanacearum* (Smith) causes economically important disease called bacterial wilt. The pathogen induces wilting in susceptible crops where it multiplies rapidly in xylem vessels blocking water conduction (Hayward, 1991; Denny, 2006). Entry of *R. solanacearum* into the host crops is accelerated by root-knot nematode (*Meloidogyne* spp.) which wound and form galls in tissue (Hayward, 1991). Bacterial wilt disease of tomato (*Solanum lycopersicum L.*) impedes tomato production in open field and greenhouse farming. Bacteria wilt of tomato is caused by either race 1 or race 3 which had been confirmed in Kenya by European and Mediterranean Plant Protection Organization (EPPO, 2004). Pathogenic strains, race 3 biovar 2 (Rs3bv2) of *R. solanacearum* is more adaptable and is therefore distributed worldwide and is the most phytopathogenic with significant yield losses (Plantpath, 2008; Denny, 2006). It is estimated that Rs3bv2 strain is responsible for an estimated $950 million in losses each year (Ephinstone, 2005).

1.2. Tomato production and therapeutic value

Tomato produces edible fruits and is therefore cultivated worldwide as a premium crop. Harvested fruits can be eaten, canned or consumed as sauce (FAO, 2008). Global consumption of tomato has increased significantly with crop cover estimated at over 4 million Ha with a corresponding production estimated at 130 million tons in 2008 (FAO, 2008). Global tomato production increased to 161.8 million tonnes in the year 2012 (FAOSTAT, 2012). Despite of the increased global yield in the year 2012, Kenya managed a production of 397,007 tones which is a drop from 590,000 tones yielded in 2010 (MoA, 2010). Since the year 2010, over 50% annual yield loses in host plants have
been attributed to bacterial wilt disease which is now a country wide menace (Muthoni et al., 2014)

Tomato has anti-oxidants such as, lycopene, vitamin C and beta-carotene that reduce risks of cancer (Polívková et al., 2010), and type 2 diabetes (Shidfar et al., 2011). Well documented studies show tomato consumption is therefore nutritious and of high economic value. Any disease that may compromise its production will have ripple effects to the economy of Kenya and the world over.

1.3. Problem statement

Tomato farming is one of the flourishing and premium solanaceous cultivation. A single tomato plant has a fruit production potential of up to 15 kg in the first harvest, going up to 60 kg by the time it has completed its full cycle (Makunike, 2007). However, tomato farming is compromised by factors such as agronomic constraint, pest and diseases (Geoffrey et al. 2014). These constraints have substantially affected production, marketing of quality tomatoes and steady supply of tomato locally and internationally (Humphrey, 2009).

Tomato is generally highly susceptible to most economically important soil borne pathogens including bacterial wilt considered for 10–20% yield losses reported annually (USDA, 2003). To date there is no single agent that can be applied to successfully control bacterial wilt disease (Yuliar et al. 2015).

Integrated management strategies, including cultural practices, crop rotation and use of resistant cultivars, provide some limited success in the management of this disease (Yuliar et al. 2015). *Ralstonia solanacearum* causes important yield losses that vary from 0 to 91% in tomato cultivars, depending on climate, soil type, cropping pattern, and strain (Ephinstone, 2005). The pathogen cause destructive economic impact by inducing systemic symptoms that cause rapid fatal wilt in host plant (AVRDC, 2005). It
is imperative therefore, to identify alternative systems and agents that can be applied to control BW disease.

1.4. Justification of the study

*Acacia stuhlmannii* Taub plant root concoction has been used in Tanzania in promotion of animal health and as a repellant to snakes (Minja 1994). The plant commonly known as Msaro by Giriami, (mijikenda tribe) people of Kenya’s coastal region is a valuable folk medicine. Root bark concoction has antimicrobial effect against human pathogens. Preliminary work *in vitro* (unpublished) with extracts from *A. stuhlmannii* have shown activity on different human and plant pathogens. Plant extracts of *A. stuhlmannii* may therefore contain active compounds against *R. solanacearum* bacterium. Successful trials may form a basis for determination of the active ingredient that may be used in future to synthesize products for agronomic application against a number of important bacterial diseases. There is currently no single agent that can be applied to successfully control BW disease. It is vital therefore, to identify alternative systems and agents that can be applied to control it. This therefore, calls for development of novel antibacterial agents that are more effective, economically feasible and eco-friendly than the current conventional controls. Extracts from *Acacia* spp have long served as medicinal cure for viral and bacterial infections in human (Richard, 1990). Therefore, there is need for exploring the potential of extracts from *A. stuhlmannii* to manage *R. solanacearum*.

1.5. Hypotheses

Ho: There are no compounds with bioactivity in *A. stuhlmannii* plant extract.

H₁: There are compounds with bioactivity in *A. stuhlmannii* plant extract.

Ho: Extracts of *A. stuhlmannii* cannot inhibit growth of *R. solanacearum*.

H₁: Extracts of *A. stuhlmannii* can inhibition growth of *R. solanacearum*. 
Ho: Extracts of *A. stuhlmannii* are not stable against drastic thermal, UV-Band pH changes.

H$_1$: Extracts of *A. stuhlmannii* are stable against drastic thermal, UV-Band pH changes.

Ho: Extracts of *A. stuhlmannii* cannot suppress bacterial wilt disease in *invivo* trials.

H$_1$: Extracts of *A. stuhlmannii* can suppress bacterial wilt disease in *invivo* trials.

1.6. **Objectives of the study**

1.6.1. **General objective**

To evaluate the efficacy of *A. stuhlmannii* plant extracts against bacterial wilt disease of tomato.

1.6.2. **Specific objectives**

1. Extract bioactive compounds from *A. stuhlmannii* root barks.

2. Evaluate suppressive effect of *A. stuhlmannii* extracts against *R. solanacearum* *invitro* assays.

3. Evaluate the stability of the bioactive compounds from *A. stuhlmannii* root barks

4. Assess the efficacy of *A. stuhlmannii* extracts against bacterial wilt disease in potted trials.
CHAPTER TWO
LITERATURE REVIEW

2.1. Classification of *R. solanacearum*

The complex *R. solanacearum* species is classified mainly in races, biovar and geographically distributed phylotypes (Fegan and Prior, 2005). Race 1(Rs1) biovar (1, 3 and 4) strains, race 2 biovar 1(Rs2bv1), and race 3 biovar 2 (Rs3bv2) are three of the most common and important strains in tropics and sub-tropics EPPO, (2004). *Ralstonia solanacearum* was recently classified as *Pseudomonas* with similarity in most aspects, except that it does not produce fluorescent pigment like *Pseudomonas* (Agrios, 2008). Within the complex *R. solanacearum* species, there are four major monophyletic clusters of strains, termed phylotypes which are geographically distinct, phylotypes I-IV are found in Asia, America, Africa, and Indonesia, respectively (Fegan and Prior, 2005).

2.2. Distribution of *R. solanacearum* of tomato

*R. solanacearum* is a worldwide important etiological agent that causes serious wilt to a wide range of host plants (Monther and Kamaruzaman, 2010). Though race determination in *Ralstonia solanacearum* strains is a formidable task; race 1 and 3 are most important strains that causes bacteria wilt in tomato (Champoiseau et al., 2009; AVRDC, 2005). Race 3 biovar 2 (Rs3bv2) is worldwide and has a wide host range which includes potatoes, tomatoes, and geraniums (Plantpath, 2008). Race 1 has biovar 1 (and 3) reported to cause bacteria wilt of tomato in southern United States (Wicker et al., 2005). Biovar 1 is widely distributed in Asia, Africa, Australia, North America, and South America (Sullivan et al., 2013). Apart from tomato biovar 1 cause loses in eggplant, tobacco, and potato and cucurbits (Fegan and Prior, 2005). Lemessa and Zeller, (2007) collected *R. solanacearum* biovar 1 (19 isolates) and biovar 2A (43 isolates) in Ethiopia.
2.3. Physiology and infectivity of *R. solanacearum*

The bacteria rapidly multiply to produce masses that colonize the xylem vessels of a susceptible host plant, causing a devastating lethality (Momol *et al.*, 2000). Bacterial masses often aided by slime, block water conduction in xylem vessels which result in dying of infected plants even when saturated with moisture (Monther and Kamaruzaman, 2010). Horizontal inoculation of adjacent susceptible plant is possible through *R. solanacearum* reserves in debris and symptomless host that are released in the presence of water (irrigation or run off from rain) to adjacent areas (Monther and Kamaruzaman, 2010; AVRDC, 2005).

2.4. Symptoms of bacterial wilt disease

Infected tomato plants are characterized by stunting, chlorosis and epinasty on tomato foliage especially at the tip. Recently, reported bacteria wilt may lack yellow coloration as in *Fusarium* wilt (AVRDC, 2005). In early stages, top leaves of infected tomato plants, appear flagging which may recover temporally at nightfall when temperature reduces followed by sudden collapse of infected plant at a later stage (Champoiseau *et al.*, 2009). The pith of colonized stems appears dark-brown with a slimy yellow, brown or grey exudate when soaked in water (Brown and Wilt, 2010). This implies that biovar 1 that was initially America has distribution in Africa.

2.5. Transmission of *R. solanacearum* pathogen

The spread and occurrence of *R. solanacearum* in host crops is through agronomic practices such as irrigation practices, transplanting, cultivation, wounding and pruning using infected tools (AVRDC, 2005). Dispersal of *R. solanacearum* bacterium over long-distance was reported by AVRDC, in 2005 where the bacterium was transferred through infested soil via transplanted seedlings and farm implements. Spread of BW disease is also reported through vehicular cultivation and it is also quickened in areas
infested by root-knot nematode (Meloidogyne spp.) which wound and form galls in tissue (Hayward, 1991). Vast geographical spread has been reported through infected seeds, stems and tubers where R. solanacearum remain latent and distributed wide and far (Mwangi et al., 2008). Host with latent infection such as Amaranthus spp. (pigweed) are symptomless and release R. solanacearum to infected soil where it is carried in run off (Sullivan et al., 2013).

2.6. Temperatures and persistence on bacterial wilt

Trivial temperature for bacterial wilt severity is between 24–35°C (EPPO, 2004). In geographical areas with elevated temperature of between 30–35°C latent inoculation are potentially activated into pathogenic strains which are incidentally pandemic (Hayward, 1991). Higher incidence and severity of bacterial wilt disease in host crops has been reported in soil with high moisture content (Momol et al., 2000). Bacterial wilt is seldom in temperate climates where the mean temperature falls below 10°C in any winter month (EPPO, 2004). In soil with temperatures slightly below 40°C with no moisture R. solanacearum has was reported absent after one year (Hayward, 1991). Important strains that cause wilt have majorly been reported significantly in tropics and sub-tropics, and rarely in temperate areas with low temperatures (EPPO, 2004). Important pathogens have persistence and infestation reserves in greenhouse and tunnels where temperatures are often elevated (Momol et al., 2000).

2.7. Control strategies for bacterial wilt disease

The level of bacteria wilt pathogen in infected soil renders the land unproductive to any susceptible solanaceous crop (AVRDC, 2005). Various control strategies that have long been used to reduce the level of this pathogen in the soil include: Use of antagonistic entophytic bacteria of genus Pseudomonas spp. and Bacillus spp. which have biocontrol effect against bacterial wilt (Ramesh and Phadke, 2012); use of developed
resistant tomato varieties such as Hawaii7996 (H7996) which though susceptible to a new hyper-virulent strain T731, remains the most promising resistant cultivar according to united states department of agriculture (USDA, 2010, Zarate et al., 2006); cultural practices often applied as part of integrated disease management (Onduso, 2014) have effective control of level of bacterial wilt. So far, no single system has been developed for total protection of the host plants from BW diseases. Intensive use of biological methods is the most recent strategies due to sustainability, spread after initial establishment and long-term efficacy (Yuliar et al., 2015). Therefore; there it was necessary to explore bioactive extracts from A. stuhlmannii as a potential biocontrol agent against R. solanacearum.

2.8. Application of botanical compounds

2.8.1. Bioactive plant compounds

Plants that produce anti-microbial compounds have antagonistic effect against some multidrug resistant bacteria (Ahmad and Beg, 2001). Antibacterial effect is attributed to single or major groups of anti-microbial phytochemicals such as alkaloids, terpenoids, flavonoids, quinones, essential oils, lectins, polypeptides, phenolics, polyphenols and tannins (Ramar and Ponnampalam, 2010). According to Sangoyomi et al. (2011), use of plant extracts is widely applied control of diseases severity. Studies have shown that anti-microbial properties against some human and plant pathogens is due to phytoactive compounds (Pradhanang et al., 2003; Taiwo et al., 2007; Sangoyomi et al., 2011).

2.8.2. Control of BW disease of tomato using phytoactive compounds

The most important bioactive compounds of plants are alkaloids, flavonoids, tannins and phenolic compounds as reported by Edeoga (2005). Presence of these compounds is the reason for this good antibacterial potency in both gram positive and the gram negative bacteria (Shanab et al., 2010; Aboul-Enein et al., 2011). Similar compounds
have been extracted from *Eichhornia crassipes* (Mart.) Solms (Pontederiaceae) which showed an inhibition zone of 26 mm on *R. solanacearum* spread on the petri-plates (Alemu *et al.*, 2013). Preliminary studies have shown that *A. stuhlmannii* has similar compounds and can therefore be applied as a potential biocontrol agent against *R. solanacearum* in tomato.

Plant essential oils such as thymol have antagonistic activity against bacterial wilt (Paret *et al.*, 2010). Currently, no single extract from *A. stuhlmannii* have been tested against BW pathogen. Preliminary efficacy evaluation has shown *A. stuhlmannii* has antibiotic activity against various human bacteria disease pathogen. It is therefore prudent to test the efficacy of *A. stuhlmannii* against *R. solanacearum* strains of tomatoes.

### 2.9. Therapeutic significant compounds of the *Acacia* genus

#### 2.9.1. Therapeutic benefit of *Acacia* genus

*Acacia* is the second largest genus in the family Leguminosae with about 1380 species. *Acacia* is found majorly in Australia, tropical and subtropical regions of the world (Maslin *et al.*, 2003; Moore *et al.*, 2010) The use of medicinal plants as folk medicines for the treatment of common infections is well known and documented in rural areas especially in villages and native communities of many developing countries (Sandhu and Heinrich, 2005; Rojas *et al.*, 2006). The species *Acacia jacquemontii* Benth is the extensively used folk medicine in Africa and particularly in Kenya for the treatment of pneumonia, malaria, primary inoculation of syphilis, sterility and stomach ache (Kokwaro, 1976). Saini *et al.*, (2008) reported high antibacterial activity in methanol extracts than extracts by hexane. He reported the highest antifungal and antibacterial activity in methanol extracts of *Acacia nilotica* ssp. *indica* and *A. catechu* L. the highest while *A. jacquemontii* showed lowest bioactivity (Saini *et al.*, 2008).
2.9.2. Screened compounds from *Acacia* genus

The phytochemical screening of *A. mellifera* using ethanol, methanol, ethyl acetate and hexane revealed the presence of alkaloids, tannins, anthraquinones, glycosides, saponins, flavonoids, steroids and terpenoids and phenols (Lalitha *et al.*, 2010). These compounds have antagonistic effect against the development of BW disease with a pathogen and disease reduction up to 91.7% compared to the infected control (Alemu *et al.*, 2013). Preliminary screening shows these compounds in *A. stuhlmannii* root extracts. According to Muthaura *et al.* (2007) root bark decoction is used as folk medicine for the treatment of pneumonia, malaria, and primary inoculation of syphilis, sterility and stomach ache in Kwale area in the south coast of Kenya.

2.10. *Acacia stuhlmannii* Taub

2.10.1. Distribution and botany of *Acacia stuhlmannii* plant

*Acacia stuhlmannii* is a flora that belongs to the family leguminosae-mimosoideae, found in Kenya, Tanzania, Ethiopia, Zimbabwe, Botswana, Transvaal (Thulin *et al.*, 2008). The plant grows in varied height that range from shrub to trees up to approximately 6 m (Thulin *et al.*, 2008). Mature *A. stuhlmannii* plant bears seeds enclosed in inflated hyperium pod which is green when unripe and grey-black when dry. The pods are either straight or curved with thick and hard, with a reduced base, indehiscent, of up to 9 x 2.5 cm (Thulin *et al.*, 2008). They are covered with long golden shinny hairy structures, which appear smooth and slimy in the cool day. Pods bear ellipsoid slightly wrinkled seeds of 4.5–9 mm diameter (Thulin *et al.*, 2008). Flowers have white heads with fade pink appearance, attached to pink peduncles (Plate 2.1F). Branches bear sharp thorns that arise in pair in an obtuse angle. Thorns often grow to a length between 3.5–4 cm. While thorns are less thick in branches, they have prominent base and with nail appearance on stem (Plate 2.1A). Branches bear thorns and leaves at
the same points that alternate at an obtuse angle. Leaf arrangement is opposite binate (4–12 pairs) and leaflets (usually 7–25 pairs) are greatly reduced in size (Thulin et al., 2008). They appear pale green when young and turn dark green when mature (Plate 2.1D and E). Stem are olive green with observed golden spots beneath thin and brown barks which appear slime and peeling (Plate 2.1A2).

![Photographs describing the botany of A. stuhlmannii plant.](image)

**Plate 2.1:** Photographs describing the botany of *A. stuhlmannii* plant.

### 2.10.2. Secondary metabolites and preferred part of *A. stuhlmannii* plant

*Acacia stuhlmannii* shrub grows on clay soils and red loam which are fairly fertile for cultivation. Abiotic stress in environment affect the level and type of metabolites produced (Ramakrishna and Ravishankar, 2011). A study done on oil producing plant (*Matricaria chamomile* L.) reported that essential oil content reduce majorly with increased salinity and water stress (Razmjoo et al., 2008). Therefore the present study preferred hinterland collection for sufficient nonpolar extracts.
Root bark is the most commonly used part of common phytotherapeutic remedies (Muthaura et al., 2007). The stem bark and leaves may be alternative harvesting parts since chemical composition is not significantly different from that in the roots (Cunningham, 2001). In this study the root barks were preferred to leaves because they were few and coated with black substance. Appropriate sustainable root harvest strategy was adopted for sustainable utilization of this tree in this study.

2.11. Extraction and evaluation of bioactive compounds

2.11.1. Collection and transport of root barks of A. stuhlmannii plant

The root bark is the most commonly used part of A. stuhlmannii plant. This leads to profound destruction when the whole plant is uprooted (Cunningham, 2001). Conservation and harvesting strategies to facilitate sustainable utilization of these plant resources are therefore necessary (Cunningham, 2001).

2.11.2. Extraction of plant bioactive compounds

Phytoactive compounds can easily be extracted in polar solvents such as methanol, ethanol and moderately polar ethyl acetate (Cos et al., 2006). Methanol and acetone extracted compounds have reported significant antibacterial activity against R. solanacearum as described by Alemu et al. (2013). Cold aqueous and hot decoction of plant leaves have bioactivity against R. solanacearum (Abo-Elyoursr and Asran, 2009). Although water has a high dielectric constant, ethanol is preferred due to its greater capability in dissolving the bioactive compounds and for its higher heating efficiency (Wu et al., 2014). Further studies have reported contents of impurities in highly polar (water) extracts such as organic acids, sugars, soluble proteins (Bandar et al., 2013). Based on these findings, extraction by ethanol was preferred instead of water.
Currently, there are no reports on extraction and application of products from *A. stuhlmannii* against BW pathogen. It is therefore imperative to expand the extraction protocol by using polar, moderately polar and non-polar solvents for these bioassays.

**2.12. Stability of phytoactive compounds**

**2.12.1. UV exposure and bioactivity**

Previous studies have shown that daily exposure of plants to active UV-B band, (280–315 nm) wavelengths, increases plant secondary metabolites (Zhang and Björn, 2009). Anti-microbial phytochemicals are due to single or synergistic effect of secondary metabolites (Ramar and Ponnampalam, 2010). Ultra violet rays (UV-B) may be useful in boosting of the bioactivity and efficiency of medicinal drugs and agrochemical. Plants exposed to ultra violet stress respond by producing metabolites to deal with stress (Zhang and Björn 2009). Effect of UV-B on solvent extracted compounds of *A. stuhlmannii* is not known. There is need for evaluation bioactivity of UV-B exposed extract of *A. stuhlmannii* plant.

**2.12.2. Temperature stability evaluation**

Antimicrobial compound(s) extracted by ethanol from *Solanum sisymbriifolium* Lam. have demonstrated bioactivity stability between 30–80°C and slight drop at 100°C and 12°C (Gupta et al., 2014). Consistent results are expected for ethanol extracts of *A. stuhlmannii* which is water soluble. Preliminary studies have shown better bioactivity in solubilized fatty compounds of *A. stuhlmannii* which positively correlates to increased level of temperature. Evaluation for bioactivity of hexane and ethyl acetate extracts are vital due to hydrophobic compounds yield by the solvents.

**2.12.3. pH adjustment versus bioactivity**

Antimicrobial activities of plant extract can be extremely affected by pH (Morales-cabrera et al., 2013). Dynamic environmental condition such as pH remains the main a
challenge in the field of medicinal drug discovery, because of the rapid change in pH in the gastrointestinal system for oral drugs (Di and Kerns, 2009). Research elsewhere support that antimicrobial activities of plant extract can also be extremely affected by pH variation (Mercedes and Javier, 2013). Depending on solvent type (polarity) yield extract vary on antimicrobial activity (Muthuvelan and Balaji, 2008). This is due to difference on extract polarity, more polar extracts by aqueous and ethanol solvents have wide activity spectrum (Hatil and Moneer, 2006). This may be due to low pH of ethanol extracted compounds, although not supported by experiments done by Abu-Tarboush, 1994. Basic adjustment by calcium does not suppress level of BW as supported by research done by (Gokhan et al., 2010). No research that has been made on the bioactivity of A. stuhlmannii plant extract exposed to adjusted pH levels.
CHAPTER THREE
MATERIALS AND METHODS

3.1. Area of study

The study was conducted in Pwani University located 60 km north of the major coastal town Mombasa in Kenya. *In vivo* bioassays were done within the University farm in black shed net located at 39° 50’E and 03° 37’S. Laboratory experiments were conducted in biological sciences laboratory.

3.2. Extraction and evaluation of bioactive compounds from *A. stuhlmannii* root barks

3.2.1. Collection of root barks of *A. stuhlmannii*

Barks of *Acacia stuhlmannii* plant sample were collected from Danicha area within Ganze Sub-county in Kilifi County. The area (native to Giriama people) centered around 39° 46’E latitude, 03° 28’S longitude and 3 m altitude. Collection was carried out in January, 2014 in the period of no rains and moderately elevated temperatures usually up to around April (Muthaura *et al.*, 2007). Due to temperature stress plants synthesize higher amount of essential oils (Razmjoo *et al.*, 2008) and metabolites important in dealing with environmental (biosphere) related stress (Ramakrishna and Ravishankar, 2011). Harvesting of the root bark was done sustainably as described by (Cunningham, 2001), where only a root of 30 cm or two per plant were obtained. Superficial, extended roots to about a depth of 30 cm were harvested. Root barks were washed with tap water, and then rinsed in sterile water (SDW). Plant materials were cut into small pieces and packed in paper bags for transportation to the University. Plant was positively identified by a taxonomist from the museum of Kenya and a duplicate of dried fertile sample was deposited in the Kenya National Museum for authentication.
3.2.2. Drying and grinding of *A. stuhlmannii* plant sample

Root barks of *A. stuhlmannii* plant were dried in a shade at the room temperature (25°C) for five (5) days. Barks amounting to 6 kg were milled into a fine powder using an electrical miller. Grinding was done to increase surface for the solvent activity. The powder was weighed into batches of 1 kg each, packed in the Ziploc/freezer bags and stored at -4°C until further use.

3.2.3. Extraction process

The powder was extracted under room temperature condition (25°C) using a single solvent maceration method as described in Jansirani *et al.* (2014) with adjustments. About 1 kg of the powdered barks each were soaked in five (5) liters of ethanol (C\textsubscript{2}H\textsubscript{5}OH), hexane (C\textsubscript{6}H\textsubscript{14}) and Ethyl acetate (C\textsubscript{4}H\textsubscript{8}O\textsubscript{2}) solvents in separate bottles. Each batch was then wrapped in black polythene bags and kept at room temperature for seven (7) days. The filtration of the extract was done using filter paper (Whatman® 125 mm Ø Cat No 1001 125, Whatman International Ltd Maidstone, England). Concentration of crude extract was done at a temperature range of 40-50°C using R209 rotary evaporator (Peaken Motor Co., Ltd) at 180 revolutions per minute (rpm). Solvent was removed by evaporation in hot air oven at 40°C (Alemu *et al*., 2013). Extracts were preserved at 4°C in air tight dark bottles until use as described by Savithramma *et al.* (2011).
3.2.4. Phytochemical screening of *A. stuhlmannii* root barks extract

Laboratory screening of *A. stuhlmannii* extract was carried out to determine the presence of the following compounds; tannins, saponins, cardiac glycosides, flavonoids, alkaloids and coumarins.

**Test for tannins:** Tannins were tested as described in Doss *et al.* (2009) with slight modifications. Extract concentrate was mixed with distilled water (50 mg in 2 ml H₂O in a test tube) and brought to boil at 65°C in a water bath. Filtration was done using filter paper (Whatman® 125 mm Ø Cat No 1001 125, Whatman International Ltd Maidstone, England) followed by addition of 0.1% FeCl₃ to the filtered samples and observed for brownish green or a blue color development.
Test for saponins (Foam test): Extracts were tested for saponins using the procedure described in Journal (2012) with little adjustment. Powdered samples (2 g) of extract were boiled together with 20 ml of distilled water in a water bath (65°C) and filtered. Sample filtrate was mixed with distilled water (2:1) in a test tube and agitated vigorously. Saponins form froth and emulsification when the froth settles.

Test for cardiac glycosides: Tested according to procedure by Journal (2012) and Chigodi et al. (2013) with few adjustments. Concentrates of crude extracts (0.2 g) were mixed with 5 ml of distilled water and boiled in a water bath (65°C) followed by filtration. Two milliliter of glacial acetic acid with 1 drop of FeCl₃ was added to the mixture in the test tube. Brown ring appeared suspended on a violet-gradual green complex ring an indication of deoxysugar (cardenolides) in the sample.

Test for phlobatannins: Each sample of crude extract (5 ml) in different test tube was boiled with 1 % HCl in a water bath. A deposition of a red precipitate indicates presence of phlobatannins as described in Journal (2012).

Test for steroids (Salkowski test): Extracts were tested for steroids according to the method by Yadav et al. (2014). Two milliliter of concentrated sulphuric acid (H₂SO₄) was carefully added to a test tube containing 5 ml concoctions. Red brown ring at the junction was a characteristic indicator of steroids.

Test for flavonoids: Flavonoids were tested using protocol described by Doss et al. (2009) with modifications. Two (2 g) of extract concentrate were added to 3 ml of 1% ammonia solution in test tube, 1ml of 1.0M HCl. Yellow colour which is non-persistent indicate presence of flavonoids.

Test for Alkaloids: Alkaloids were tested according to Chigodi et al. (2013) with modification. About 2 ml of each extract was warmed with 3 ml 2% H₂SO₄, for two
minutes then filtered. Three (3) drops of Dragendorff reagent was added and observed for an orange red precipitate which indicated presence of alkaloids constituents.

**Test for Coumarins:** Two milliliter of extract was added to 3 ml of sodium hydroxide (10%). Coumarins are confirmed present by a visual yellow coloration as described in Yadav *et al.* (2014).

### 3.3. Evaluation of suppressive effect of *A. stuhlmannii* extracts against *R. solanacearum* invitro trials

#### 3.3.1. Preparation of *R. solanacearum* (PU-Rs-01) inoculum

Isolate PU-Rs-01 of *R. solanacearum*, biovar 1, phylotype I was obtained from Kenyatta University originally characterized by Dr. George Kariuki’s group. The isolate was stored in heat sterilized potted soil in a shaded area and also in 15% glycerol. *Ralstonia solanacearum* maintained in soil was re-isolated using protocol described by EU, (1998) with few adjustments. Soil sample (30 g) was mixed with 100 ml of sterile distilled water (SDW) and agitated for 30 minutes. Ten milliliter of the mixture was adjusted serially to $10^{-5}$, $10^{-6}$, $10^{-7}$ and $10^{-8}$ dilution factor. Each dilution factor was evaluated for colony forming units (CFUml⁻¹) on 0.5% w/v of 2, 3, 5-Triphenyl Tetrazolium Chloride (TTC) selective media. About 100µl was plated on TTC media and smeared using sterile glass rod. Plates were then incubated at 30°C for 48 hrs. Colonies were counted manually using a colony counter and pen. Colony forming unit was computed by the standard formula described in American society for microbiology (ASFM, 2013).

$$\text{Colony forming unit (CFU/ml)} = \frac{\text{Mean number of colony}}{\text{Volume plate (ml)} \times \text{Total dilution used}}$$
Table 3.1: Colony Forming Units (CFU ml\(^{-1}\)) of \textit{R. solanacearum} evaluated on TTC media petri plates incubated at 30°C after 48 hrs

<table>
<thead>
<tr>
<th>Dilution Factor (DF)</th>
<th>Amount of suspension plated of 90 mm petri plates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 µl</td>
</tr>
<tr>
<td>10(^{-5})</td>
<td>17</td>
</tr>
<tr>
<td>10(^{-6})</td>
<td>9</td>
</tr>
<tr>
<td>10(^{-7})</td>
<td>4</td>
</tr>
<tr>
<td>10(^{-8})</td>
<td>2</td>
</tr>
</tbody>
</table>

### 3.3.2. Maintenance of virulent PU-Rs-01 strain for bioassays

Cream colored, opaque, fluidal and spread colonies with a pink centre were considered virulent (Chaudhry and Rashid, 2011). A single colony was streak twice in succession on 0.5% w/v of 2, 3, 5-Triphenyl Tetrazolium Chloride (TTC) filter sterilized (0.2 µm Ø) stock solution added to 0.125% casamino acids, 1.25% peptone, 0.3125% dextrose, 1.875% agar all w/v. petri plates were incubated at 30°C for 48 hrs. Throughout the study, pure cultures of PU-Rs-01 strain were grown in casamino acid peptone glucose agar (CPGA) prepared according to Alemu \textit{et al.} (2013) with few adjustments, 0.2 g casamino acid, 2 g peptone, 1.0 g glucose, 3.4 g agar in 200 ml distilled water. The growth media was adjusted to pH of 7.0 using 3% w/v sodium hydroxide and sterilized at 121°C for 15 minutes. The isolates were maintained at a temperature of 4°C in sterilized 15% glycerol.

### 3.3.3. Morphological and biochemical tests to confirm \textit{R. solanacearum} (PU-Rs-01) sample

Biochemical tests were done to further confirmation of \textit{R. solanacearum} strain that causes bacterial wilt in tomato.
**Gram staining:** A loopful of bacterium was smeared onto a glass and fixed in low flame, 15% of aqueous crystal violet solution was spread over the smear for 50 seconds then rinsed in running tap water for one minute. Gram stain iodine was swabbed over the slide for 50 seconds, washed in tap water and decolorized with 95% v/v ethanol according to Chaudhry and Rashid (2011). This was followed by flooding with safrin counter-stain for about ten (10) seconds.

**Catalase test:** A loop full of fresh culture was transferred from CPGA solid medium onto a glass slide. About one milliliter of 5% v/v solution of Hydrogen Peroxide (H$_2$O$_2$) was added onto the glass slide, and observed for production of bubbles.

**Potassium hydroxide (KOH) test:** KOH solubility test was performed using 12-18 hrs culture. Three drops of 5% w/v aqueous KOH were put onto a glass slide. A single colony of test strain was touched with sterile tooth pick and stirred into the solution and observed for slime mucoid string after approximately ten (10) seconds.

**Kovac’s oxidase test:** The test was done as described by Chaudhry and Rashid (2011) with minor adjustments. A filter paper was placed within glass the petri plate. Two drops of 1% Kovac’s oxidase reagent (1% tetra-methyl-p-phenyl diamine dihydrochloride) was transferred onto the filter paper. Loopful amount of test strain was gently rubbed on loaded oxidase reagent. The filter paper was observed for purple colour, characteristic of the bacteria strain.

### 3.3.4. Evaluation of virulence level of PU-Rs-01 in tomato seedlings- Koch’s rule

Further, virulence of PU-Rs-01 to cause bacteria wilt was evaluated by Koch postulate as described by Alemu *et al.* (2013) with some adjustments. About 50 tomato seeds of the susceptible tomato cultivar Cal J were raised in 500 ml plastic pots at the rate of ten (10) plants per pot in four (4) sets each. The pots were filled with soil sterilized done at 80°C for two hours in an oven. Seeds were sown and allowed to grow for two (2)
weeks. The seedlings were inoculated with 20 ml $10^{-8}$, $10^{-7}$, $10^{-6}$ and $10^{-5}$ CFUml$^{-1}$ R. solanacearum suspension around the root zone. Pricking with a sterile sharp pin was done to enhance infection (Adebayo, 2011). The set-ups were incubated at 25°C and 70% humidity with regular watering. Diseases incidence was then evaluated four times at an interval of three (3) days. No Treatments using A. stuhlmannii plant extracts were done at this stage. Seedlings were irrigated regularly to ensure the disease was well spread in the soil (AVRDC, 2005).

3.3.5. **In vitro bioassays using A. stuhlmannii extract against PU-Rs-01**

3.3.5.1. **Re-isolation of PU-Rs-01**

Samples of diseased tomato plants were collected randomly from the infected pots in 3.4.3 and the diseases confirmed using ‘bacteria streaming ooze test’ (Champoiseau *et al.*, 2009). The stems of the diseased plants were sliced into small pieces and gently agitated in 3 ml SDW in Eppendorf tube. A creamy streaming suspension was observed. The isolate in creamy solution was isolated twice on 0.5% w/v TTC media and bulked in casamino peptone glucose agar (CPGA). Colonies were scrubbed from CPGA into 50 ml SDW and resultant aliquots adjusted to an optical density (OD) of 0.401 at 660 nm (approx. $10^7$CFU/ml) before use.

Plate 3.1: Appearance of PU-Rs-01 colonies at 0.401 OD.
3.3.5.2. Disc diffusion sensitivity test

Level of bioactivity of hexane, ethyl acetate and ethanol extracts of *A. stuhlmannii* against PU-Rs-01 strain was determined by susceptibility test method as described by Abo-Elyousr and Asran (2009) with little adjustments. Sterile Petri dishes of 90 mm diameter were filled to a depth about 2 mm with Muller Hinton Agar (M173-500G) susceptibility test media, 38 g of Muller Hinton Agar was suspended in 1000 ml of distilled water brought to boil to dissolve, followed by sterilization at 121°C for 15 minutes in an autoclave. The media was allowed to cool to about 37°C before it was enriched with 5% de-fibrinated sheep.

Sheep Blood was aseptically using sterile glass bottle containing glass beads that were centrifuged and dispensed in citrated Baxter Healthcare donor bags. One pint of blood (470ml) was collected in 65ml of citrate phosphate dextrose anticoagulant. The blood in collection bag was sterilized under chilled condition for seven (7) days before use (Russell *et al.*, 2006).

Concentrates of extract, 50, 100, 150, 200 and 250 mg of hexane, ethyl acetate and ethanol extracts were reconstituted separately using 1ml of 10% v/v DMSO. Antibiotic discs were prepared separately in sets using reconstituted hexane, ethyl acetate and ethanol extracts. Each disc was infused with 20 µl of (w/v) extracts. Susceptibility test of PU-Rs-01 was done in four (4) replications using Co-Trimoxazole octo-disc as a positive control and 10% v/v DMSO disc as a negative control. The plates were sealed and incubated at 28°C for 36 hrs. Inhibition Zone Diameters (IZDs) were measured accurately to the nearest millimeter (mm). Sets of experiments were repeated using the same concentrations at incubation temperature of 35°C and inhibition zone diameter (IZDs) in mm after 36 hrs of incubation were taken. Each trial was replicated four times.
3.3.6. Determination of minimum inhibition concentration (MIC)

Minimum inhibition concentration was done by broth dilution method as described by Andrews (2006) with little modifications.

**Broth media preparation;** Muller Hinton Broth (M391-500G) was prepared by suspending 21 g in 1000 ml of distilled water and brought to heat while stirring gently to dissolve. The broth was sterilized at 121°C for 15 minutes then allowed to cool to room temperature before use.

**Reconstitution of plant Extract;** MIC was determined separately using 50, 100, 150, 200 and 250 mg masses of filter sterilized (0.2 µm Ø) hexane, ethyl acetate and ethanol extract. Masses of sterilized extracts were reconstituted in sterilized test tubes using 1 ml of 10% v/v DMSO to form corresponding concentrations. One milliliter of each concentration was added to 9 ml of Muller Hinton Broth to dilution factor of 10⁻⁶.

Each test tube was inoculated using 100 µl of PU-Rs-01 suspension (10⁷ CFUml⁻¹) then sealed aseptically. The test tubes were incubated at 35°C and agitated for 30 seconds at every 9 hrs. Turbidity was assessed after 36 hrs using 0.5 McFarland standards.

3.4. Stability evaluation of bioactive compounds from *A. stuhlmannii* root barks

The extracts were subject to varied temperature, pH and UV-B exposure to assess their stability when exposed to stress. The extracts were subjected to temperature range of 40–120°C, pH range from 6-8 and UV-B exposure for 30–100 minutes before testing for bioactivity in *invitro* tests.

3.4.1. Exposure of extract to UV-B rays at varied time

Hexane, ethyl acetate and ethanol extracts at corresponding MIC (18.3, 19.3 and 25.7 mgml⁻¹ respectively) were exposed to UV-B rays for 20, 40, 60 and 80 and 100 minutes. Products were evaluated using disc diffusion sensitivity test.
3.4.2. Thermo-stability evaluation on MIC of extracts

Extracts from hexane, ethyl acetate and ethanol (1 ml each) at corresponding MIC were heated in separate test tubes and in sets on a water bath. Sets were withdrawn at temperatures of 40, 60, 80, 100 and 120°C in that order then assessed for the level of bioactivity using disc diffusion test.

3.4.3. Bioactivity on pH adjusted extracts

Adjustment of pH was done using 1 M HCl and 1 M NaOH as described by Gupta et al. (2014), with few modifications. Aliquots of 10% v/v DMSO was adjusted to pH 6.2, 7.0, 6.8, 7.8 and 8.2. Adjusted aliquots were used to prepare MIC of hexane, ethyl acetate and ethanol extracts of A. stuhlmannii. Disc diffusion sensitivity test was then used to access changes in bioactivity.

3.4.4. Disc diffusion evaluation

Minimum Inhibition Concentrations (1ml) of hexane (18.3 mgml⁻¹), ethyl acetate (19.3 mgml⁻¹) and ethanol (25.7 mgml⁻¹) extract of A. stuhlmannii root bark were evaluated for stability against UV-B rays, temperature and pH. Invitro bioassays were done by loading 20 µl of extract onto sterile paper discs against 100 µl of 10⁷ CFUml⁻¹ PU-Rs-01 suspensions. The inoculated petri-plates were incubated at 35°C for 36 hrs and observed.

3.5. Invivo efficacy trials of A. stuhlmannii extracts against bacterial wilt disease

3.5.1. Invivo screening using potted seedlings

Application of extracts was done using soil drenching method as described by Sangoyomi et al., (2011) with few modifications. Ten tomato seedlings were raised (for two weeks) in each 500 ml plastic pot filled with sterile soil in black shade net condition. After two weeks A. stuhlmannii plant extracts (20 ml) plant extract applied as
described in Alemu et al. (2013) two (2) days before inoculation, at inoculation time and two (2) days after inoculation. Each pot was inoculated with 15 ml of $10^7$ CFUml$^{-1}$ of *R. solanacearum* standard suspension (EU, 1998). Seedlings were pricked using a sharp sterile pin at two (2) opposite points on the root region. The potted seedling were saturated to 70% humidity by daily watering and exposed to 12/12 hrs light regime. Disease severity index (DSI) of bacteria wilt disease was assessed every three (3) days until twelve (12) days after inoculation (DAI). Disease severity index values were rated using six point scale (0 to 5) adopted from Winstead and Kelman, (1952) and compiled using the formula by Cooke, (2006) with little adjustment.

$$\text{DSI} = \sum \left( \frac{(d \times n)}{(N \times m)} \right)$$

Where:  
- $d =$ disease rating on each plant
- $m =$ maximum disease rating possible
- $n =$ number of plants in each scored pot
- $N =$ total number of plants examined in each replicate

Percent Reduction (PR) of DSI was calculated using the formulae described by Aliye et al. (2008)

$$\text{PR} = \sum \left( \frac{(\text{PC} - \text{PT})}{\text{PC}} \right) \times 100\%$$

Where:  
- PR = percent reduction,
- PC = percentage value of the control and
- PT = percentage value of the treatment group.

### 3.5.2. *In vivo* efficacy evaluation of hexane extracts against BW disease on tomato seedlings

Treatment using hexane extracts was done using MIC rate. Concentrate of crude hexane extracts were constituted into concentrations of 36.52, 18.26, 9.13 gml$^{-1}$ which respectively corresponds to double, full and half application rates of the minimum inhibition concentration. Application of extract was done simultaneously with $10^7$ CFUml$^{-1}$ using soil drench method. Seedlings were pricked as described in 3.5.1.
Seedlings were irrigated regularly to allow spread and entry of *R. solanacearum*. Disease severity index (DSI) of bacteria wilt disease was assessed as described in 3.5.1. Diseases severity index values were scored using six point scale of 0 to 5 as described by Winstead and Kelman (1952). Further, the most effective fraction was assessed for efficacy at half MIC, single MIC, and doubled MIC application rate. This was done for comparative evaluation of efficacy when large or small doses are applied.

3.5.3. *Invivo* efficacy of *A. stuhlmannii* plant extracts on BW disease using potted tomato plants under screen house conditions

Application of minimum inhibition concentration of extracts was done using soil drench method as described by Sangoyomi *et al.* (2011) with few modifications. Minimum inhibition concentration (MIC) was determined by dilution method. Masses of hexane, ethyl acetate and ethanol concentrates were separately reconstituted at 18.3, 19.3, and 25.7 mg ml\(^{-1}\) w/v, respectively. Healthy tomato seedlings were raised in the sterile soil on a tray for three (3) weeks. The seedlings were then transplanted into sterile soil in three (3) liters black paper pots and allowed to establish for one week. Pots were inoculated as described by Alemu *et al.* (2013) with little adjustment where 30 ml of \(10^7\) CFU/ml of PU-Rs-01 suspension was introduced around the root zone of the tomato seedlings. The roots of the seedling were pricked as described in 3.5.1 to enhance infectivity. Application of *A. stuhlmannii* plant extracts at 40 ml per pot was done using three (3) methods namely: a) two (3) days before planting, b) at planting time and c) three (3) days after planting. Pots were arranged on 1 m raised beds in a randomized complete block design (RCBD) in black screen house. Seedlings were saturated at 70% humidity (by daily watering and kept at 12/12 hrs light regime. Other agronomical practices applied were; weeding, trellising, pruning and application NPK fertilizer (1 tea
spoon per pot after every three weeks). Insect and fungal disease controls were done by regular sprays with Bestox® 100EC (Alphacypermethrin) and Power 76 WP® (cymoxanil and propineb/dithiocarbamate) respectively at recommended rates.

Bacteria wilt disease was assessed weekly for seventy five (75) days. Disease Severity Index (DSI) for development of bacterial wilt symptoms was evaluated based on a six point rating scale (0 to 5) where: 0 = no wilt symptoms, 1 = 25% leaves wilted, 2 = 50% leaves wilted, 3 = all leaves except the tip wilted, 4 = whole plant wilted and 5 = death (collapse) of the whole plant. Disease Severity Index (DSI) was calculated using the formulae adopted from Cooke, (2006). Production data (weight of fruits/plant) was also assessed and compared across the treatments with control set. Fruits per plant, per plot were harvested and recorded. Yield data was compared at 75 days from transplanting based on cumulative production at the end of the experiment. The DSI values were computed based on the following formula by Cooke, (2006).

\[
DSI = \left( \frac{(n0 \times 0) + (n1 \times 1)(n2 \times 2)(n3 \times 3)(n4 \times 4)(n5 \times 5)}{N} \right) \times 100\%
\]

Where:
- \(n0\) = no of seedlings with no wilt symptoms
- \(n1\) = no of seedlings with 25% wilt symptoms
- \(n2\) = no of seedlings with 50% wilt symptoms
- \(n3\) = no of seedlings with all leaves wilted except the tip
- \(n4\) = no of seedlings completely wilted
- \(n4\) = no of seedlings dead and collapsed
- \(N\) = 10 (total number of seedling evaluated)

**Key**: no=Number

### 3.6. Data collection and analysis

Percentage yield of extract was considered as production for each solvent used in the extraction process. All data was converted to % (w/w) of product and ground materials used. Phytochemical screening was a qualitative test indicating the presence or absence
of the test compounds present in the bioactive products. Presence was scored as +ve and absence as –ve in the data collected. Mean of Inhibition zone diameters (mm) was recorded from four plates. Diameter for disc diffusion sensitivity experiments were analyzed as a function of the extract concentration and solvent type. Diameters of inhibition zone (mm) for pH, temperature, and UV-B stability treatments were evaluated as function of treatments (variable). Means of diameter were compared for statistical difference at 5% probability level. Diseases severity indices (DSI) on tomato seedlings were evaluated as factor of both extract by solvent type and time of application. Averages sets of diseases severity indices (quadruplicates) for hexane, ethyl acetate and ethanol extracts every three days up to day 12 were compared to application time. Means with a p<0.05 were regarded significantly different. The DSI was converted to percentage disease incidence (PDI) as described by Cooke in 2006 in 3.5.1 above. Further, percentage of disease incidence scores for hexane extracts for three application times were compared weekly and means were evaluated as a function of time. Means of disease incidence with a probability of at least 5% were considered statistically different. Significantly different score were compared to yield fruit per treatment. Disease suppression was expressed as a percentage reduction (PR) of disease as described in by Aliye et al (2008) in 3.5.1 above.

All data was subjected to analysis of variance (ANOVA) and the calculated means compared at 5% significance level. Means were separated using Tukey multiple comparison tool using R studio version Ri386 3.0.2 statistical package. Data obtained was presented in simple descriptive format with bar graph and trend plots where necessary. Bars and trend plots were prepared using MS-excel version 2010. Trends were compared per treatments and gaps within treatments discussed.
CHAPTER FOUR

RESULTS

4.1. Extraction of bioactive compounds

4.1.1. Extraction yield

Powdered root barks yield 7.94%, 4.90% and 3.27% for ethanol, hexane and ethyl acetate respectively (Table 4.1) from the single solvent extraction method.

Table 4.1: Weight of powdered root bark and corresponding yield of crude extract

<table>
<thead>
<tr>
<th>Solvent used</th>
<th>Solvent type</th>
<th>Root powder (grams)</th>
<th>Extract yield (grams)</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>Polar</td>
<td>995.02</td>
<td>79.05</td>
<td>7.94</td>
</tr>
<tr>
<td>Hexane</td>
<td>Non-polar</td>
<td>994.54</td>
<td>48.65</td>
<td>4.90</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>Moderately polar</td>
<td>995.08</td>
<td>32.55</td>
<td>3.27</td>
</tr>
</tbody>
</table>

4.1.2. Phytochemical compounds in hexane, ethyl acetate and ethanol extracts

Table 4.2: Results of tested compounds in Acacia stuhlmannii root bark extracts

<table>
<thead>
<tr>
<th>Phyto-constituents</th>
<th>Solvent partitions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hexane</td>
</tr>
<tr>
<td>1. Alkaloids</td>
<td>–</td>
</tr>
<tr>
<td>2. Tannins</td>
<td>–</td>
</tr>
<tr>
<td>3. Cardiac Glycosides</td>
<td>+</td>
</tr>
<tr>
<td>4. Saponins</td>
<td>+</td>
</tr>
<tr>
<td>5. Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>6. Phlobatanins</td>
<td>–</td>
</tr>
<tr>
<td>7. Steroids</td>
<td>+</td>
</tr>
<tr>
<td>8. Coumarins</td>
<td>+</td>
</tr>
</tbody>
</table>

(Key: + = present, – = absent)
4.2. Bioassay results

4.2.1. Colony morphology of *R. solanacearum*

A virulent colony with pink centre appeared red in a streak done on 0.5% w/v 2, 3, 5-Triphenyl Tetrazolium Chloride (TTC) selective media. Successful streak appeared cream, and fluidal.

Plate 4.1: PU-Rs-01 strain streaked on TTC selective media (A) colonies of *Rs* mated in soil, (B) streak of a cream colony with a pink center, (C) streak of a loopful obtained from plate (B).

4.2.2. Morphological and biochemical characteristics of *R. solanacearum*

A collection of biochemical tests had a positively confirmation of the re-isolated strain as *R. solanacearum* (Table 4.3). The results were consistent to description by Chaudhry and Rashid, (2011).

Table 4.3: Summary of tests for confirmation of PU-Rs-01 as a strain of *R. solanacearum*

<table>
<thead>
<tr>
<th>Test</th>
<th>Morphological test</th>
<th>Biochemical test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Colony colour</td>
<td>Appearance</td>
</tr>
<tr>
<td>PU-Rs-01</td>
<td>Cream with pink centre</td>
<td>Fluorescent</td>
</tr>
</tbody>
</table>
4.2.3. Virulence of PU-Rs -01 on tomatoes var. Cal J

There was a gradual disease progression of bacteria wilt disease symptoms marked by observed epinasty, and chlorosis (Plate 5.1). There was significant difference (P<0.05) in the virulence of the isolate within the incubation period (F3, 48= 167.227). Research findings also established significant difference (P<0.05) in different dilution factors of PU-Rs-01 strain (F3, 48 = 7.784). There was also a strong interaction (P=0.191078) between days after inoculation and dilution factor at 5% probability level (F9, 48= 1.458).

![Figure 4.1: Bacteria wilt severity caused by PU-Rs-01 on tomatoes var. Cal J evaluated every 3 days until 12 days. DSI values are averages of four (4) replicates each with four (4) pots. Means followed by same letter(s) for each day (3, 6, 9, and 12) are not significantly different at 5% probability level according to Tukey multiple comparisons test.](image)

A dilution of 10^-5 is potent enough to cause a disease, 10^-8 was equally good. Treatments in subsequent experiments settled on 10^-8 which gives 10^7 CFUml^-1 standard inocula for R. solanacearum (EU, 1998). Virulent R. solanacearum bacteria (PU-Rs-01) were maintained in tomato plant and re-isolated to maintain viability and virulence (Thomas and Upreti, 2014).
4.2.4. *In vitro* bioassays using disc sensitivity test

4.2.4.1. Inhibition zone diameter recorded at incubation temperature of 28°C

Mean IZDs recorded for hexane at 28°C was relatively higher compared to ethyl acetate and ethanol (Fig. 4.2 and Plate 4.2a–e). Although, Hexane extract had highest mean diameter zone (18.3 mm) it was significantly different (P<0.05) from bioactivity of co-Trimoxazole (approx. 24 mm) control. Ethyl acetate bioactivity results at a concentration of 50 mgml⁻¹ was significantly different (P<0.05) from both hexane and ethanol extracts of similar concentrations. The bioactivity of ethanol extracts differs with hexane extracts at concentrations of 100 mgml⁻¹ and above but there was no significant difference to that of ethyl acetate extracts, (Fig. 4.2). Both concentrations and solvent had significant effect on mean IZDs (F₄, ₇₅ = 85.11, P<0.05 and F₄, ₇₅ = 2793.70, P<0.05), respectively. There was also solvent to concentration interaction effect (F₁₆, ₇₅ = 13.63, P<0.05).

![Figure 4.2: Mean inhibition zone diameter (IZDs) in mm by different concentrations of extracts against PU-Rs-01 on 5% MHSBA at (a) 28°C after 36 hrs incubation. Hex=hexane, Etac=ethyl acetate, Etnl=ethanol extracts. COT=Co-Trimoxazole commercial antibiotic disc. Bars consist of means IZDs, error bars are corresponding standard error (SE). Means followed by same letter(s) for each extract by solvent are not significantly different at 5% probability level according to Tukey multiple comparisons test.](image-url)
Plate 4.2: Inhibition area evaluated on extract loaded discs against PU-Rs-01 strain incubated at 28°C. Discs sets were loaded with A. stuhlmannii extract separately with 20µl of 50, 100, 150, 200 and 250 mg of Hex=hexane, EtOL=ethanol, EtOAc=ethyl acetate, COT=Co-Trimoxazole commercial antibiotic disc, DMSO=10% dimethyl sulfoxide.

4.2.4.2. Inhibition zone diameter recorded at incubation temperature of 35°C

Then average growth inhibition of *R. solanacearum* by hexane extracts at incubation temperature of 35°C ranged from 13.1 to 23.8 mm and by ethyl acetate extracts stood at 11.4 to 19.1 mm. Both extracts had a general and consistent increase in inhibition with increases in concentrations (Plate 4.3a–e). Ethanol crude extract however, had decreased growth inhibiting with increased concentration, (Fig. 4.3). Bioactivity of ethyl acetate extract is significantly different from that of hexane at 100 mgml⁻¹ but not different statistically for concentration of 150 mgml⁻¹. Bioactivity of ethyl acetate extracts was highest at 250 mgml⁻¹ although not significant from hexane extracts at 200 mgml⁻¹ rates. Hexane extracts had the highest activity at a concentration of 250 mgml⁻¹, which is significantly different (P>0.05) to bioactivity recorded at 150 mgml⁻¹ and below. Generally, both the solvent and concentration of extract had significant effect on mean IZDs (*F*₄,₇₅ = 2153.52, *P*<0.05 and *F*₄,₇₅ = 38.41, *P*<0.05), respectively. A solvent to concentration interaction effect (*F*₁₆,₇₅ = 18.55, *P*<0.05) was also observed.
Figure 4.3: Mean inhibition zone diameter (IZDs) in mm by different concentrations of extracts against PU-Rs-01 on 5% MHSBA at 35°C after 36 hrs incubation. Hex=hexane, Etac=ethyl acetate, Etnl=ethanol extracts. COT=Co-Trimoxazole commercial antibiotic disc. Bars consist of means Inhibition Zone Diameters (IZDs), error bars are corresponding standard error (SE). Means followed by same letter(s) in each solvent extract are not significantly different at 5% probability level according to Tukey multiple comparisons test.

Plate 4.3: Inhibition area evaluated on extract loaded discs against PU-Rs-01 strain incubated at 35°C. Discs sets were loaded with A. stuhlmannii extract separately with 20µl of 50, 100, 150, 200 and 250 mg of Hex=hexane, EtOL=ethanol, EtOAc=ethyl acetate, COT=Co-Tirmoxazole commercial antibiotic disc, DMSO=10% dimethyl sulfoxide.

4.2.5. Minimum inhibition concentration of A. stuhlmannii extracts on PU-Rs-01 strain

Minimum inhibition concentration of A. stuhlmannii extracts had differences in bioactivity against PU-Rs-01 marked for absence of true turbidity in MHB incubates.
Hexane showed the lowest MIC while highest MIC was assessed in extracts done by ethanol solvent. The difference in MIC of hexane and ethyl acetate extracts is relatively small compared to hexane and ethanol extracts for all evaluated concentrations (Fig. 4.4). Data consist of mean of four (4) trial replications each with four (4) sets.

Bioactivity of hexane, ethyl acetate and ethanol extracts of *A. stuhlmannii* against *R. solanacearum* differed significantly (P<0.05). However, there was no significant difference (P>0.05) in bioactivity of extract MIC established for 50, 100, 150 and 200 mgml⁻¹ concentrations. This implies that bioactivity of minimum concentrations of extract by solvent type is not statistically different (P>0.05) at concentrations levels 50, 100, 150 and 200 mgml⁻¹ (Fig. 4.4). Therefore, any set of minimum inhibition concentration can be used for further evaluation. For consistency and accurate results in subsequent experiments a fixed MIC of 18.3, 19.3 and 25.7 mgml⁻¹ for hexane, ethyl acetate and ethanol crude extracts respectively were used.

![Figure 4.4: Minimum inhibition concentration (MIC) for hexane, ethyl acetate, and ethanol extracts of *A. stuhlmannii* at 50, 100, 150, and 200 mgml⁻¹ recorded after 36 hrs incubation at 35°C. Hex=hexane, Etac=ethyl acetate, Etnl=ethanol extracts. MHB=Muller Hinton Broth. Bars are means (n=4) of inhibition concentration. Means followed by same letter(s) for each extract by solvent type are not significantly different at 5% probability level according to Tukey multiple comparisons test.](image)
4.3. Stability evaluation of *A. stuhlmannii* root bark extracts

4.3.1. Stability after UV-B rays exposure

Comparison of means of eight (8) replications for UV-B exposed MIC of hexane, ethyl acetate and ethanol extracts showed significant effects on extract bioactivity. Although there was reduced bioactivity of hexane extract by UV-B exposure for 80 minutes a general stability was observed. Exposure ethyl acetate and ethanol extracts to UV-B enhanced consistent bioactivity. Generally, extract by solvent type and UV-B treatment had significant bioactivity ($F_{2, 126} = 76.444$, $P<0.05$) and ($F_{5, 126} = 14.095$, $P<0.05$) respectively. Extract by solvent type and UV-B exposure had significant ($P<0.05$) interaction effect ($F_{10, 126} = 2.161$). Generally a stable trend of bioactivity was observed for hexane, ethyl acetate and ethanol extracts between 20–80 minutes.

![Figure 4.5: Trend of *invitro* bioactivity of minimum inhibition concentration (MIC) of *A. stuhlmannii* extracts after exposure to active UV-B band. Minimum inhibition concentration of hexane extract=18.26 mgml$^{-1}$, ethyl acetate extract=19.26 mgml$^{-1}$ and ethanol extract=25.69 mgml$^{-1}$ Cont.=control concentration were not exposed to UV-B band. Bars represent standard error of the corresponding mean of eight replicates.](image-url)
4.3.2. Thermo stability of A. stuhlmannii extracts

Temperature increase had slight effect on bioactivity of hexane, ethyl acetate and ethanol extracts. All extracts had a better bioactivity at elevated temperature up to 80°C that was tested. Extracts by solvent type ($F_{2, 54} = 148.932, P<0.05$) and extract at varied temperature ($F_{5, 54} = 66.143, P<0.05$) had significant bioactivity against PU-Rs-01 strain *invitro*. Solvent and extract interaction effect also had significant bioactivity ($F_{10, 54} = 6.793, P<0.05$).

Figure 4.6: *Invitro* bioassay done using thermo treated minimum concentrations of A. stuhlmannii extracts. Inhibition zone diameter was assessed after 36 hrs of incubation at 35°C. Control temp=room temperature, MIC of hexane extract=18.26 mgml$^{-1}$, ethyl acetate extract=19.26 mgml$^{-1}$ and ethanol extract=25.69 mgml$^{-1}$. Bars represent standard error of the corresponding mean of eight replicates.

4.3.3. pH stability of bioactive extracts

Hexane, ethyl acetate and ethanol extracts at corresponding minimum inhibition concentrations showed no significant difference ($P>0.05$) in bioactivity when pH was varied. Extract MIC for each solvent used had significant suppression ($P<0.05$) against virulent PU-Rs-01 strain. There was no significance in combined effect ($P>0.1$) of extract by solvent type and pH adjustment ($F_{8, 105} = 0.034$).
Invivo results of *A. stuhlmannii* extracts against bacteria wilt in potted tomato seedlings

4.4.1. Soil drench with extract two days before inoculation

Comparison of means (n=4) on treatments done two (2) days before inoculation using MICs of hexane, ethyl acetate and ethanol extracts showed significant effect in bioactivity. It was observed that disease severity increased from day three (3) to day twelve (12) of incubation Fig. 4.8(a). Treatment by solvent type had significant (P<0.05) bioactivity on severity of bacterial wilt diseases ($F_{3, 48} = 651.12$). The difference in time of treatment also had a significant effect (P<0.05) on diseases severity ($F_{3,48} = 206.17$). Interaction by solvent type and treatment days had significant effect at P<0.01 on bacterial wilt development ($F_{9, 48} = 13.35$).

Relationship between bioactivity of hexane extracts showed highest ability to manage bacterial wilt diseases than ethyl acetate and ethanol extracts Fig. 4.8(b). A low
reduction in potency of ethyl acetate extracts was noted but generally a stable bioactivity in reference to the time of incubation was observed. Extracts by ethanol applied by this method became less effective between days 6–12, Fig. 4.8(b). The trend of reduction in bioactivity of hexane extracts was relatively higher than ethanol and ethyl acetate extracts. All extracts showed highest bioactivity on day 6 after inoculation which later stabilized Fig. 4.8(b).

Figure 4.8: Effect of A. stuhlmannii root bark extracts against bacterial wilt disease rate; (a) extracts were applied two days before inoculation, (b) reduction trend of extract, bioassays were done on two weeks old seedlings of tomato var. Cal J. dai=days after inoculation, Hex=hexane, Etac=ethyl acetate, Etnl=ethanol extracts, SDW=Sterile Distilled Water (Control). T2dbi=treatment done two days before inoculation. Experiment was done in black shade net condition. Data are means of four replications; means followed with same letter(s) for each extract type are not significantly different at the probability of 5% level according to Tukey HSD test. Error bars are standard error of means.
4.4.2. Simultaneous application of extract and *Rs* inoculation

Disease severity for treatments done simultaneously at inoculation time was significantly reduced by minimum concentration rates for hexane and ethyl acetate, Fig. 4.9(a). Ethanol was relatively effective upto six (6) days of incubation, beyond which typical signs of bacterial wilt were observed in all the replicates (n=4) for ethanol and ethyl acetate. Hexane however remained effective up to day twelve (12) of incubation under black shade net conditions. Treatments by solvent type had significant (P<0.05) bioactivity on bacterial wilt diseases severity (F$_{3, 48} = 141.075$). The difference in time of treatment also had a significant effect (P<0.05) on diseases severity (F$_{3, 48} = 34.262$). Interaction by solvent type and treatment days had significant effect at P<0.05 on bacterial wilt development (F$_{9, 48} = 5.449$). There was generally a stable bioactivity in reduction of bacterial wilt incidence by hexane extracts, Fig. 4.9(b). Trend of reduction observed on ethyl acetate extract was also stable upto 9 days but decreased between 9-12 days. Bioactivity by ethanol extracts was relatively short and marked by a sharp decrease beyond sixth day after treatment and inoculation. Simultaneously applying hexane extract with the pathogen managed the level of bacterial wilt disease better compared to similar treatments using ethyl acetate and ethanol extracts. Bioactivity and trends for ethyl acetate and ethanol applied two days before and at inoculation time were also lower compared to similar treatments using hexane extract, Fig. 4.8(b) and 4.9(b).
Treatment two days after inoculation had slightly different suppression for all extracts. Bioactivity of ethanol and ethyl acetate extracts was not considerably different within the period of twelve (12) days Fig. 4.10(a). Hexane extracts were suppressive but not as
good as when applied before and at inoculation time. General increased disease severity was noted for all extracts. Diseases severity for hexane treated sets remained relatively lower compared to sets treated with ethanol and ethyl acetate extracts. Treatments by solvent type and the difference in time of treatment had a significant (P<0.05) bioactivity on bacterial wilt diseases severity ($F_{3, 48} = 53.434$ and $F_{3, 48} = 63.017$), respectively. Interaction by solvent type and treatment days was not significant (P>0.05) on bacterial wilt development ($F_{9, 48} = 0.668$ and corresponding probability of 0.733).

The potency of hexane, ethyl acetate and ethanol extracts shows reduced bioactivity trends especially from day 6, Fig. 4.10(b). This shows that application of extract using soil drench method after inoculation is not effective to manage the level of bacterial wilt disease. Hexane extracts applied by this method still have a high disease suppression ability compared to treatments using ethyl acetate and ethanol extracts, Fig. 4.10(b).

Figure 4.10a: Effect of *A. stuhlmannii* root bark extracts against bacterial wilt disease rate; extracts applied two days after inoculation, bioassays were done on two weeks old seedlings of tomato var. Cal J. dai=days after inoculation, Hex=hexane extract, Etac=ethyl acetate extract, Etnl=ethanol extract, SDW= Sterile Distilled Water (Control). T2dai=treatment done two days after inoculation. Data are means of four replications; means followed with same letter(s) for each extract type are not significantly different at the probability of 5% according to Tukey HSD test. Error bars are standard error of means.
4.4.4. Disease suppression within using potted seedlings

Inoculation and application by *A. stuhlmannii* extracts, on two weeks old tomato seedlings showed suppression effects of *R. solanacearum* inoculation compared to SDW (control). There was generally lower disease scores compared to the control treatment. Percentage reduction computed as described in 3.5.1 formulae by Aliye *et al.* (2008) show that application of extract two (2) days after *R. solanacearum* inoculation has a suppression of 55.7% for hexane extract at day three (3) and 43.6% at day twelve (12). Ethyl acetate on the other hand had a percent suppression of 34.1% and 22.6% for day three (3) and twelve (12), respectively. Ethanol suppressed bacterial wilt disease by 23.5% at day twelve, relatively lower than 30.7% in the first three days (Fig. 4.11).

Hexane extracts had the highest ability to manage bacterial wilt disease in tomato seedlings than ethyl acetate and ethanol extracts. Further, the percentage reduction by treatment using hexane extracts remained high compared to treatments with ethyl acetate and ethanol extracts at day twelve (12) of incubation. Preliminary results
indicated that the hexane extracts performed best against *R. solanacearum* of tomato. Hexane extracts were therefore, further evaluated for efficacy using different MIC application rates.

![Graph](image)

**Figure 4.12**: Disease suppression compared after day 3–12 of incubation on two-week old Cal J tomato seedlings treated against bacterial wilt inoculum. Means are reduction potency of crude extracts at the start and end of day 12. Formula in section 3.6.1 excludes experiment control. Error bars are corresponding standard error (SE) Means followed by same letter(s) in each treatment are not significantly different at 5% probability level according to Tukey HSD test.

### 4.4.5. Evaluation of hexane extract using varied MICs on tomato seedlings

Findings indicated that MIC of hexane extracts, 18.3 mg ml$^{-1}$ was the best application rate. Doubling the MIC rates for hexane extract did not have any statistically difference in bioactivity (Fig. 4.12). Varied rate of MIC had significant effect (P<0.05) on the incidence of bacterial wilt, $F_{2,36} = 8.859$. Symptoms of bacterial wilt diseases increased significantly (P<0.05) within incubation period, $F_{3, 36} = 28.559$. Interaction by MIC treatment rate within incubation period was not significantly (P>0.05) different, $F_{2,36} = 0.157$. 


4.6 Efficacy of *A. stuhlmannii* crude extract against BW in potted plants

Minimum concentration of *A. stuhlmannii* root barks was suppressive enough to keep bacterial wilt disease severity at minimum level at the week 6 of the season (Fig. 4.14). The findings in reduction were assessed in three treatments (40 ml of MIC extract)
infected with 30 ml of $10^7$ CFU ml$^{-1}$ PU-Rs-01 strain. Extract of *A. stuhlmannii* by hexane applied two (2) days before and simultaneously with inoculation had no significant difference ($P>0.05$) in potency (Fig. 4.14). Hexane extracts applied two (2) days after inoculation had statistically different bioactivity. Suppression by extract in application; two days, simultaneous and two days after inoculation were not significantly different after week two (Fig. 4.14). Generally time had significant difference ($P<0.05$) in bioactivity on bacterial wilt diseases severity ($F_{3, 40} = 23.45$). Evaluation for efficacy shows significant variation ($P<0.05$) in trend of bioactivity up to maturity ($F_{1, 40} = 207.71$). Treatment levels (concentrations) and efficiency (timing) had significant ($P<0.05$) interaction with probability of 0.0113, ($F_{3, 40} = 4.20$).

![Figure 4.15: Suppression of bacterial wilt disease incidence by MIC of hexane extracts obtained from *A. stuhlmannii* root bark on susceptible tomato var. Cal J. T2DBP=Treatment 2 Days before Planting, T2DAP=Treatment 2 Days after Planting, TAP=Treatment at Planting, WAP=Weeks after Planting. Experiment done in black shield house condition. Means followed by same letter(s) for each treatment are not significantly different at a probability of 5% according to Tukey HSD test.](image)

### 4.4.7. **Fruit yield after treatment with bioactive *A. stuhlmannii* extracts**

There was no significant difference between fruit yield from treatments done two (2) days before inoculation and the simultaneous treatments with *R. solanacearum*
inoculation (Fig. 4.15). Treatment applied two (2) days after inoculation had the lowest fruit yield. Tomato yield from sets treated with hexane extract two (2) days after inoculation was lowest in the three application strategies. This yield was however statistically different (P<0.05) from the control set. Findings by our research therefore consider pre-treatment and simultaneous treatment better method of application compared to post-planting treatment.

Figure 4.16: Comparative effect of hexane extract applied at different time on tomato yielded at maturity. T2DBP=Treatment 2 Days before Planting, TAP=Treatment at planting time, T2DAP=Treatment 2 Days after Planting, hex=hexane, Cont.=Control (sterile distilled water) treatment. Means followed by same letter(s) are not different significantly at 5% probability level according to TukeyHSD test.
CHAPTER FIVE
DISCUSSION

5.1. Collection and yield of Acacia stuhlmannii plant metabolites

Due to temperature stress plants synthesize higher amount of essential oils (Razmjoo et al., 2008) and metabolites important in dealing with environmental (biosphere) related stress (Ramakrishna and Ravishankar, 2011). Samples in this study were collected in the month of January, 2014 during hot and dry climate of the coastal belt (Muthaura et al., 2007). Bioactive compounds from plants are produced as secondary metabolites. Production of secondary bioactive compounds is not exceptional since they play specific role in plant function (Paulsen, 2010).

Research before reported that distribution of A. stuhlmannii within altitude range of 100–750 m (Thulin et al., 2008). The present study reveals a collection of root bark samples in low altitudes of up to 3 m above the sea level (3.2.1.). Acacia stuhlmannii is well adapted to water stressed and saline soil in East Africa and Somalia areas (Thulin et al. (2008). The plant therefore produces more metabolites to deal with biotic and abiotic stresses (Ramakrishna and Ravishankar, 2011).

5.1.1. Polar extracts by ethanol solvent

Higher yield of ethanol extract (7.94%) compared to hexane (4.90%) and ethyl acetate (3.27%). This is consistent with research done by (Lalitha et al., 2010) on screening of Acacia mellifera which yielded comparatively lower non-polar extracts than polar compounds. This is also in line with studies done on Lebanese stinging nettle (Urtica dioica L.) that confirmed higher yields of phenolic compounds in polar compared to non-polar extractions done by maceration (Bandar et al., 2013). Alkaloids, tannins, cardiac glycosides, flavonoids, steroids and coumarins were confirmed present in ethanol extracts. Saponins and phlobatannins were however, absent (Table 4.2).
5.1.2. Moderately polar extracts by ethyl acetate solvent

Ethyl acetate solvent has ability to extract moderately polar compounds. According to Lalitha et al. (2010) alkaloids, reducing sugars flavonoids and steroids are moderately polar and easily extracted in ethyl acetate solvent. Alkaloids, tannins, cardiac glycosides, flavonoids, steroids and coumarins in ethanol extracts also tested positive in ethyl acetate (Table 4.2). This explains why bioactivity of ethanol and ethyl acetate was near similar in most experiments. However, the yield difference of ethyl acetate (3.27%) and ethanol extract (7.94%) can be explained by greater ability of ethanol to dissolve the bioactive compounds and a higher heating stability which enable retention of volatile compounds (Wu et al., 2014). Other study by Paret et al. (2010) indicated that non-polar metabolites are better extracted in polar solvent than moderately polar solvents. This also explains why there was a higher yield of ethanol (7.94%) than ethyl acetate (3.27%) extracts.

5.1.3. Non-polar extracts by hexane solvent

Hexane screened positive for cardiac glycosides, saponins, flavonoids, steroids and tannins and coumarins. The difference in bioactivity of hexane is due to saponins non-water soluble tannins and compounds that constitute essential oils. Condensed tannins have restricted solubility in water which decreases with the increase in molecular mass (Paulsen, 2010). It explains the difference in yield mass of ethyl acetate (3.2%) and hexane (4.90%) extracts (Table 4.1). According to research by Paulsen (2010) most properties of condensed tannins are similar to hydrolysable tannins, present in ethanol and ethyl acetate. This affirms that only hydrolysable tannins were present in A. stuhlmannii root bark (Table 4.1).
5.2. Bioactivity of crude extracts in *invitro* assays

*Invitro* assays using *A. stuhlmannii* root bark extract exhibited mild to moderate bioactivity against PU-Rs-01 strain that cause tomato wilt. The potent inhibitory activity of extracts by hexane solvent also remains qualitatively active even at very minute amounts (Fig. 4.2 and 4.3). Findings in this study also indicate a general decrease in *invitro* bioactivity with increase in extract/solvent polarity. This explains better why hexane extracts had highest bioactivity than ethyl acetate. However, Saini *et al.* (2008) reported that antibacterial activity of polar extracts by methanol is more outstanding than hexane extracts. Preliminary research work shows presence of saponins in hexane (Table 4.2). Although, the physiological role of saponins in plants is not yet fully understood they have been grouped as protective molecules (phytoanticipins or phytoprotectants) which include glycosylated steroids, triterpenoids, and steroid alkaloids (Saxena *et al.*, 2013). This resarch therefore attribute bioactivity of hexane as a factor of saponins and derivatives. Hexane extracts showed significant activity against PU-Rs-01 at concentration 50, 100, 150, 200 and 250 mgml⁻¹ at 5% (P<0.05). *Invitro* experiments show significant bioactivity of hexane extract at minimum concentration of 18.26 mgml⁻¹. This implies that hexane extracts have compound(s) that better target the virulent factors in *R. solanacearum* (Hosseinzadeh *et al.*, 2013).

5.3. Stability of bioactive extracts in extreme environmental conditions

5.3.1. Stability of extract against UV-B

Crude extract of ethanol exposed to UV-B had slight increase of *invitro* activity. Bioactivity however, was maintained in long exposure observed beyond 40 minutes. This implies that more exposure of ethanol extracts has no additional effect in bioactivity. Bioactivity of extracts obtained using ethyl acetate and hexane solvent reduced slightly followed by in stability in exposures after 20 minutes (Fig. 4.5).
was significant (P=0.0243) interaction of solvent and UV-B treatment at 5% probability level. Bioactivity of UV-B treated extract was solely a factor of compounds present in the extract and UV-B exposure. Change in bioactivity is due reconstituted UV-B absorption molecules and partly due to formation of antimicrobial bioactive compounds (Zhang and Björn, 2009).

5.3.2. Temperature stability

This research established a better performance of hexane and ethyl acetate extracts at higher temperatures than in lower temperatures. This is attributed partly to increased solubility of aliquots and partly to liquid-gas phase ability of non-polar compounds at higher temperatures (Li et al., 2012). This implies that at high temperature, volatile compounds (essential oils) in extracts form liquid-gas state which has suppressive effect on *R. solanacearum* by limiting biofilm formation in *invitro* (Hosseinzadeh et al., 2013). This potency explains why there was better bioactivity of extracts MIC at temperature above 80°C (Fig. 4.6). It further explains that ethyl acetate has compounds that were easily (40°C) volatized validating the increase in bioactivity. Ethanol and hexane extracts begin to volatize at 60°C and 80°C respectively. *Acacia stuhlmannii* extract of are expected to remain stable and effective biocontrol of bacterial wilt disease in soil with higher temperature like greenhouse.

5.3.3. pH stability

It was observed that variation in pH has no effect on the bioactivity of crude extracts from *A. stuhlmannii* (Fig. 4.7). Research elsewhere reported that the bioactivity of pH stable methanoic (polar) extracts from *Solanum sisymbriifolium* leaves against bacterial coagulants is not different to that of moderately polar chloroform and hexane extracts against gram-negative *Bacillus* bacteria (Gupta et al., 2014). Bioactivity of extract at MIC for each extract however, were different (P<0.05) statistically. Preliminary
research work has shown a difference in bioactivity at 35°C of hexane, ethyl acetate and ethanol extract against *R. solanacearum* (Fig. 4.3). Other research where polar solvent (ethanol), ethyl acetate and hexane extracts used against *Bacillus* gram-negative *R. solanacearum* reported significant effect of pH on bioactivity of extract (Tans-kersten et al., 2001). Finding in this research work were different which implies that *A. stuhlmannii* extracts are stable against pH vagaries.

5.4. Efficacy of *A. stuhlmannii* extracts against *Rs* in potted seedlings

5.4.1. Hexane vs. ethyl acetate and ethanol extracts

Extracts obtained using hexane had compounds that showed significant (P<0.05) different bioactivity against PU-Rs-011 at MIC application rate of 18.36 mgml⁻¹. Ethyl acetate (19.3 mgml⁻¹) and ethanol (25.7 mgml⁻¹) MICs had significantly different efficacy in extracts applied two days before and simultaneous with *R solanacearum* inoculation. This finding is consistent with reported difference in bioactivity of plant extract depending on the polarity of the constituent compounds (Lalitha et al., 2010). Hexane extract had consistent efficacy compared to ethyl acetate and ethanol in treatments done two days before, simultaneous and two days after inoculation. Hexane extracts had the highest ability to manage bacterial wilt disease (56–84%), ethyl acetate (23–53%) and ethanol (23–59%) in tomato seedlings (Fig. 4.12). Preliminary results indicated that the hexane extracts performed best against *R. solanacearum* of tomato. Bioactivity of hexane extracts is solely due to compound(s) that better target the virulent factors in *R. solanacearum* both in *invitro* and *invivo* conditions (Hosseinzadeh et al., 2013).

5.4.2. *Invivo* bioactivity of hexane at varied MICs

Preliminary work indicated that MIC of hexane extracts, 18.3 mgml⁻¹ was the best application rate. There was no significance difference when MIC was doubled (Fig.
Although half MIC treatment is low application it was effective to suppress *R. solanacearum* bacteria in inoculated soil. Studies elsewhere have indicated that even small doses of non-polar compounds have ability to disable complex motility important in *R. solanacearum* virulence (Khan et al., 2009; Hosseinzadeh et al., 2013). This explains better why there was no significance difference in bioactivity of half and single and even double MIC treatments (Fig. 4.13).

### 5.4.3. Efficacy of extracts hexane *A. stuhlmannii* against *Rs* in potted seedlings

Hexane extracts are non-polar that consist small chain saturated fatty acids (Desbois and Smith, 2010). Non-polar compounds are easily volatized into liquid-gas phase which has effective penetration (Li et al., 2012). This better explain why extract by hexane solvent effectively managed (at day 12) bacterial wilt in tomato seedlings by 75.4% and 84.2% for applications done before and simultaneous (Fig. 4.14). Efficacy was attributed to penetration ability of volatized compounds (Li et al., 2012).

*Ralstonia solanacearum* forms biofilms aggregation outside host as a strategy to avoid host defenses (Tans-Kersten et al., 2001). Research elsewhere showed that *Ralstonia solanacearum* exhibit important motility like twitching, swarming and flagella aided swimming to optimal infection sites (Hosseinzadeh et al., 2013). Due to liquid gas phase ability hexane extracts penetrated complex synergistic aggregations that immobilized *R. solanacearum* complex motility (Khan et al., 2009). Moreover, research elsewhere reported that bacteria wilt severity in host crops become formidable at temperature between 30-35°C (Momol et al., 2000). *Invivo* results were consistent to preliminary *invitro* work that showed higher bioactivity observed in plates incubated at 35°C compared to 28°C (Fig. 4.2).

Other possible bioactivity by hexane extracts is the disruption of penetration by adhesively binding to cell wall of host plant inhibiting activity of bacterial enzymes
responsible for virulence (Gurjar et al., 2012). Research elsewhere by Hayek et al. (2013) is in supported that essential non-phenolic oil mainly disrupt bacterial cell wall or membrane inhibiting its infectivity. The disruption of bacterial membrane, extract-plant cell wall complex, reduction of motility and bacterial biofilm penetration by the volatized compounds in hexane extracts are the possible bioactivity strategies that successfully controlled bacterial wilt diseases in this work.

5.5. Efficacy of hexane extracts against Rs in potted tomato plants

According this study it was found that, crude extracts of A. stuhlmannii were potent enough to suppress bacterial wilt disease. Findings further, established a variation in bacterial wilt incidence based on the time of R. solanacearum inoculation. Soil drenching two days before and simultaneous with inoculation was more effective in suppressing BW disease before week six. Studies by Alemu et al. (2013) partly support findings simultaneous application is the most effective method of applying crude botanical extracts for disease control. This study however established that efficacy of hexane extracts applied before, simultaneous and after inoculation was not statistically different (P>0.05) in management of bacteria wilt. This is supported by Paret et al. (2010) who reported that plant essential oils have antagonistic affect against bacterial wilt pathogen in both invitro and invivo conditions. This may partly explain why hexane and ethyl acetate extracts were generally more effective than ethanol.

The findings of this study report a variation in bacterial wilt incidence up 75 days. Studies reported that R. solanacearum is effectively non-motile in host xylem vessels which suggest important and complex motility is involved in spread within the host (Tans-kersten et al., 2001). Microscopic structure of R. solanacearum shows ability to form a biofilm aggregation in xylem vessels (Hosseinzadeh et al., 2013). Aggregation ability is important for bacteria survival in latent infection and even saprophytic life
Enhanced ability of root inoculation by pricking may have increased the entry of *R. solanacearum* hence lower BW incidence in treated tomato plants compared to control sets (Fig. 4.15).

5.6. **Fruit yield in tomato crop after treatment with bioactive *A. stuhlmannii* extracts**

Lower yield was observed in control compared to sets which hexane extract was applied (Fig. 4.12). The significant difference indicated that yield was potentially compromised by the effect of bacterial wilt diseases. This is consistent with many reports indicating high losses in tomato farming attributed to bacterial wilt disease (Denny, 2006; Plantpath, 2008). Fruit yields collected from treatments done using hexane extracts before and simultaneous were not significantly different in weight (kg). This confirms the competitive ability of the two methods of application. However, this does not exclude methods not evaluated in this research. Fruit yield in setups that were post-treated with hexane extracts after inoculation scored lower yields and were significantly different (P<0.05) to treatments before and simultaneously with inoculation. The yields correlate with the disease incidence and dying of infected plants (Plate 5.1), even when saturated with moisture (Monther and Kamaruzaman, 2010).

Plate 5.1: Tomato showing symptoms of bacterial wilt disease. Healthy leaves (A), leaves with wilt symptoms (B) and dead plant (C).
CHAPTER SIX
CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

The *Acacia stuhlmannii* crude extracts provides a potential bioagent as a strategy to control *Ralstonia solanacearum* in the field and in greenhouse farming. Extraction methods were single solvent by maceration, concentration and solvent removal by hot air oven which was efficient enough to obtain potent concentrates. Extracts by hexane, ethyl acetate and ethanol at concentration 18.3, 19.3 and 25.7 mgml\(^{-1}\) respectively have suppressive effect against *Ralstonia solanacearum* in tomato. Further, ethyl acetate and ethanol extracts of *A. stuhlmannii* had relatively low stability compared to bioactivity of hexane extracts against *R. solanacearum*. Extracts obtained through hexane solvent was considered the most effective fraction of *Acacia stuhlmannii* against *Ralstonia solanacearum* of tomato. Hexane extract was the best fraction with an average reduction potency of 71%, ethyl acetate-42% and ethanol-40%. Assessment using minimum inhibition concentration (18.3 mgml\(^{-1}\)) of hexane extracts displayed consistent biocontrol of bacterial wilt. Doubling the minimum inhibition concentration to 36.6 mgml\(^{-1}\) or reducing the rate to half (9.13 mgml\(^{-1}\)) does not have any added advantage when applied. Application of large doses is therefore wasteful. This work concludes that; Application of crude extracts before transplanting remains the best application method for tested crude extracts. Further, it was concluded that weight of fruits harvested from treatment done before and simultaneous with inoculation were not significantly different. *Acacia stuhlmannii* extracts are potential to provide a biological control agent against *Ralstonia solanacearum* both *in vitro* and *in vivo* bioassays.
6.2. Recommendation

Extracts from *Acacia stuhlmannii* crude extracts may be used as a biocontrol agent in the suppression of *Ralstonia solanacearum* in infected soils as soil drench before transplanting seedlings. Due to stability of *A. stuhlmannii* extracts, its application is recommended for trials in arable soils with varying pH range within 6.0 through 8.0. The extracts can also be applied in soils with varying temperatures such as in open fields and or in greenhouse conditions and remain effective with stable results.

As this technology picks pace, it is recommended that the vegetation of *Acacia stuhlmannii* in the region need to be improved through deliberate afforestation strategies. The Plant does well in arid and semi-arid lands (ASAL) which are in dire need for afforestation. There may be need to protect the plant through legislature for the purpose of controlling its exploitation. This shall focus on sustainable exploitation of *Acacia stuhlmannii* once the end users discover the medicinal and pesticidal potentials of this plant.

Further, more work is required to determine the formulation, application rates and packaging of hexane extracts from *Acacia stuhlmannii* as an agrochemical product for use in agriculture. The active compounds which are suppressive to *Ralstonia solanacearum*; and the suppression mechanisms in tomato-pathogen require further investigation.
REFERENCES


Paulsen, S. (2010). Highlights through the history of plant medicine, Bioactive compounds in plants - benefits and risks for man and animals. 18–29.


USDA. (2010). Recovery Plan for Ralstonia solanacearum Race 3 Biovar 2 Causing Brown Rot of Potato, Bacterial wilt of Tomato, and Southern wilt of


