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Screening for Bioactivity of Phytochemical Extracted from Acacia stuhlmannii Against Ralstonia solanacearum

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Abstract
Ralstonia solanacearum causes bacterial wilt in tomato and other important crops with significant losses in the Agriculture sector. Unfortunately this disease has no known cure to date. This study intended to screen crude extracts from *Acacia stuhlmannii* against this soil pathogen using disc diffusion techniques. Crude extracts were obtained from about 1 kg of powder by single solvent maceration with polar (ethanol) moderately polar (ethyl acetate) and non-polar (hexane) solvents that yielded 7.94, 4.90 and 3.27%, respectively. Disc diffusion tests were used to evaluate bioactivity of the phytochemical against *Ralstonia solanacearum*. Growth inhibition zones were measured in mm to reflect the bioactivity of extracts. Hexane extracts were the most potent fraction with bioactivity range of 13.1–23.8 mm compared to 11.4–19.1 mm and 10.3–10.8 for ethyl acetate and ethanol extracts, respectively. These findings therefore suggest that crude extracts from *A. stuhlmannii* root barks are a potential biocontrol agent in the management of *Ralstonia solanacearum.*
Keywords: Bioactivity, *Acacia stuhlmannii, Ralstonia solanacearum*

1. Introduction

Acacia stuhlmannii **Taub** is a flora that belongs to the family Leguminosae-mimosoideae, found in Kenya, Tanzania, Ethiopia, Zimbabwe, Botswana, Transvaal (Thulin *et al.,* 2008). Research elsewhere showed *Acacia stuhlmannii* root powder promotes animal health and is repellant to snakes (Minja, 1994). In Kenya, *A. stuhlmannii* commonly known as Msaro among the Giriama community of the Kenyan Coast is valuable in treatment of pneumonia, malaria, syphilis, sterility and stomach aches. The perceived antibiotic component in the plant extracts of *A. stuhlmannii* may therefore be beneficial against *R. solanacearum* bacterium.

Biological management of *R. solanacearum* bacteria is the most current strategy applied (Yuliar *et al.,* 2015). Study elsewhere reported nonpolar, moderately polar and polar plant compounds of *Agave sisalana* leaves are extracted using hexane, ethyl acetate and methanol respectively (Chigodi *et al.,* 2013). Polar compounds have reported significant antibacterial activity against *R. solanacearum* as described by Alemu *et al.* (2013). Asran *et al,* 2009 extracted bioactive compounds against *R. solanacearum* using cold and hot water (Abo-Elyousr and Asran, 2009). However, extracts obtained using water have higher impurity (Bandar *et al.,* 2013). Ethanol was preferred in this study because it has higher heating efficiency and better solubility for polar phytochemicals (Wu *et al.,* 2014).

The present study was designed to explore the bioactivity of extracts from the root barks of *A. stuhlmannii* against *R. solanacearum* pathogen with further intent of developing sustainable control strategy against these important bacteria in agronomy.

2. Materials and methods

2.1. Collection, extraction and screening of bioactive compounds from A. stuhlmannii root barks

2.1.1. Collection and drying of A. stuhlmannii root barks

Barks of *Acacia stuhlmannii* were collected from Danicha area within Ganze Sub-county in Kilifi County. The area centered around 39° 46´E latitude, 03° 28´S longitude and 3 m altitude. Root barks were washed in tap water, and then rinsed in sterile distilled water (SDW). Plant materials were cut into small pieces, dried in a shade before milled into a fine powder using an electrical miller. The powder was weighed into batches of 1 kg each, packed in the Ziploc/freezer bags and stored at -4°C until further use.

2.1.2. 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 (Figure 1). About 1 kg of the powdered barks were each soaked in five (5) liters of ethanol, hexane and ethyl acetate 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).

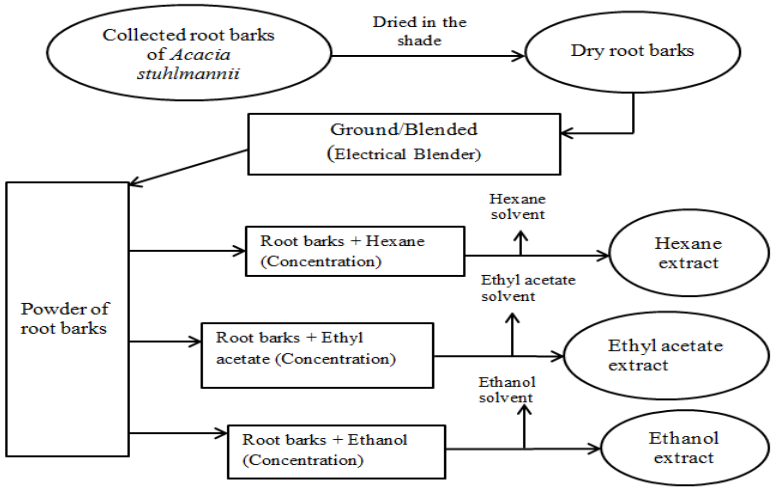

Figure 1: Illustration showing single solvent maceration method.

2.1.3. 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 H_2O 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% $FeCl_3$ 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 by Chugh *et al.,* 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 Chugh *et al.,* 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 $FeCl_3$ 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 Chugh *et al.,* 2012.

Test for steroids (Salkowski test): Extracts were tested for steroids. Two milliliter of concentrated sulphuric acid (H_2SO_4) was carefully added to a test tube containing 5 ml concoctions. Red brown ring at the junction was a characteristic indicator of steroids (Yadav *et al.,* 2014).

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% H_2SO_4, 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).

2.2. Evaluation of suppressive effect of A. stuhlmannii extracts against R. solanacearum invitro trials

2.2.1. Preparation of R. solanacearum (PU-Rs-01) inoculum

Isolate PU-*Rs*-01 of ***R. solanacearum,*** biovar 1, phylotype I was obtained from Kenyatta University. The isolate was bulked in casamino peptone glucose agar (CPGA). Colonies were scrubbed from CPGA into 50 ml SDW and resultant aliquots adjusted 10^7CFU/ml) before use.

2.2.2. 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.

2.2.2.1. Preparation of culture media

Muller Hinton Agar (38 g) 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 (470 ml) was collected in 65 ml 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).

2.2.2.2. Reconstitution of extracts and bioassay

Concentrates of extract, 50, 100, 150, 200 and 250 mg of hexane, ethyl acetate and ethanol extracts were reconstituted separately using 1 ml 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 cotrimozazole octo-disc as a positive control and 10% v/v DMSO disc as a negative control. The plates were sealed and incubated at 35°C. Inhibition zone diameter (IZDs) in mm was evaluated after 36 hrs of incubation.

2.3. 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 percentage (w/w) of product and ground materials used. Phytochemical screening was a qualitative test indicating the presence or absence of the test compounds in the extract. Presence was scored as +ve and absence as –ve in the data collected. Antimicrobial activity was an average of inhibition zone diameters (mm) recorded from four (4) plates. Mean diameter score were analyzed as a function of the extract concentration and solvent type.

3. Results

3.1. Extraction of bioactive compounds

3.1.1. Extraction yield

Powdered root barks yield 7.94%, 4.90% and 3.27% for ethanol, hexane and ethyl acetate respectively (Table 1) from the single solvent extraction method.

Table 1: Weight of powdered root bark and corresponding yield of crude extract

		Root powder	Extract yield	
Solvent	solvent type	grams (g)	grams (g)	percentage (%)
Ethanol	Polar	995.02	79.05	7.94
Hexane	Non-polar	994.54	48.65	4.90
Ethyl acetate	Moderately polar	995.08	32.55	3.27

3.1.2. Phytochemical compounds screened in hexane, ethyl acetate and ethanol extracts

Alkaloids and tannins were absent in hexane extract while nonpolar saponins tested positive. Phlobatannins were completely absent. Flavonoids, steroids, coumarins and cardiac glycosides were extracted in all solvents hexane, ethyl acetate and ethanol solvents (Table 2).

Table 2: Results of tested compounds in *Acacia stuhlmannii* root bark extracts

		Solvent partitions		
Phytochemical content		Hexane	Ethyl acetate	Ethanol
1.	Alkaloids	–	+	+
2.	Tannins	–	+	+
3.	Saponins	+	–	–
4.	Flavonoids	+	+	+
5.	Phlobatannins	–	–	–
6.	Steroids	+	+	+
7.	Coumarins	+	+	+
8.	Cardiac Glycosides	+	+	+

(Key: + = present, – = absent)

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

Screened antimicrobial activity revealed inhibitory activity by hexane, ethyl acetate and ethanol extracts against *R. solanacearum* with values ranging from 13.1–23.8 mm for hexane, 11.4–19.1 mm for ethyl acetate and 10.3–10.8mm for Ethanol extracts. Both hexane and ethyl acetate extracts had a consistent increase in activity with increases in concentrations (Plate 1). Lower concentration of ethanol extract had better antimicrobial activity at high concentrations (Table 3). Generally, both the solvent and concentration of extract had significant effect on mean IZDs at $F_{4,\ 75} = 2153.52$, $P<0.05$ 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.

Table 3: Antimicrobial activity of *Acacia stuhlmannii* root bark extracts against *R. solanacearum* by disc diffusion method

	Inhibition diameter (mm)			
Concentration (mg/ml)	Hexane	Ethyl acetate	Ethanol	COT
50	13.1±0.6d	11.4±0.8c	10.8±0.4a	25.8±0.7a
100	16.6±0.3c	15.3±0.3b	10.5±0.2a	25.9±0.4a
150	18.9±0.4bc	14.5±0.4b	10.3±0.3a	26.3±0.3a
200	20.0±0.6a	17.0±0.6ab	10.4±0.1a	25.5±0.6a
250	23.8±0.9a	19.1±0.6a	10.3±0.4a	25.4±0.6a

Values are mean of triplicates inhibition diameter ± standard error; Means followed by same letter(s) in each column are not significantly different at 5% probability level according to Tukey multiple comparisons test.

(a) (b) (c) (d) (e)

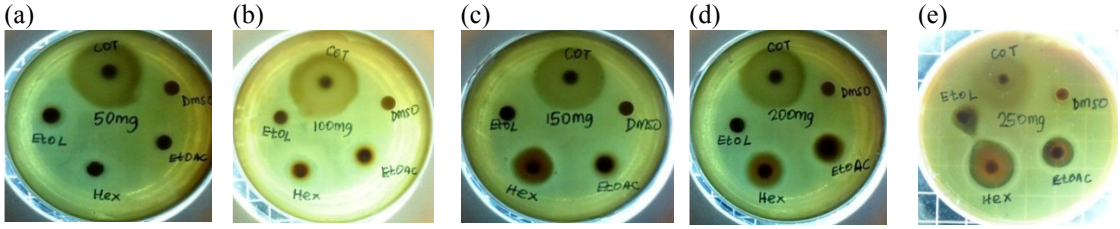

Plate 1: Inhibition area evaluated on extract loaded discs against PU-*Rs*-01 strain incubated at 35°C. Discs sets were loaded with *A. stuhlmannii* extract separately with 20µl of 50, 100, 150, 200 and 250 mg of Hex=hexane, EtOL=ethanol, EtOAc=ethyl acetate, COT=Co-Trimoxazole commercial antibiotic disc, DMSO=10% dimethyl sulfoxide.

4. Discussion

4.1. Screened phytochemical contents of Acacia stuhlmannii

4.1.1. Polar extracts by ethanol solvent

Ethanol yielded (7.94%) quantitatively higher amount of extract than hexane (4.90%) and ethyl acetate (3.27%). Consistent yield was reported for screening of *Acacia mellifera* (BENTH) which is in the same genus (Lalitha *et al.,* 2010) with *A. stuhulmanii*. Other studies support comparatively higher amount of polar compounds yields than the nonpolar reported in the Lebanese stinging nettle *(Urtica dioica* L.) using maceration method (Bandar *et al*., 2013). Presence of Tannins which facilitate healing of wound and flavonoids with antioxidative and free radical scavenging property suggest extract potent, against cancer activity (Doss *et al.* 2009). Other metabolites cardiac glycosides, steroids and coumarins were confirmed present in ethanol extracts (Table 2). Confirmed alkaloids and tannins suggest bioactive principle against bacterial infections (Doss *et al.* 2009).

4.1.2. Moderately polar extracts by ethyl acetate solvent

Screened phytochemicals in ethyl acetate alkaloids, tannins, cardiac glycosides, flavonoids, steroids and coumarins in ethanol extracts also tested positive in ethyl acetate (Table 2). Lalitha *et al.* (2010) screened alkaloids, reducing sugars, flavonoids and steroids compounds in *Acacia mellifera* using ethyl acetate solvent. Although ethanol and ethyl acetate revealed the presence of similar content, difference in yield can be explained by greater ability of ethanol to dissolve the bioactive compounds and higher heating stability which enable retention of volatile compounds (Wu *et al.,* 2014). Tannins extracted were hydrolysable tannins with similar property as condensed tannins soluble in nonpolar solvents (Paulsen 2010). Ethanol and ethyl acetate solvent can solubilize hydrolysable tannins in extract According to research only hydrolysable tannins were present in *A. stuhlmannii* root bark (Table 2).

4.1.3. Non-polar extracts by hexane solvent

Hexane extracts were positive for cardiac glycosides, saponins, flavonoids, steroids and tannins and coumarins. The difference in macerated amount in hexane solvent was due its outstanding ability to extract non-polar metabolites compared to moderately polar (ethyl acetate) solvents (Paret *et al.,* 2010).

4.2. Bioactivity of crude extracts in invitro assays

4.2.1. Antibacterial inhibition of extracts

Acacia stuhlmannii root bark extract showed antimicrobial activity range of 10 - 24 mm. Quantitative assessment of inhibitory diameter shows highest activity (23.8±0.9 mm) by hexane extract at 250 $mgml^{-1}$. Ethyl acetate extract had antibacterial activity of (19.1±0.6 mm) while ethanol performed least (10.3±0.4 mm) at similar concentration. Findings therefore indicated that hexane is the most potent solvent compared to ethyl acetate and ethanol for extraction purpose. However, this is not consistent with the reported antibacterial activity of methanol (polar) that was better than hexane extracts (Saini *et al.* 2008).

4.2.2. Antibacterial potent of hexane extract

The potent activity of extracts by hexane solvent also remains qualitatively active even at very minute amounts (Table 3 and Plate 1). Bioactivity is attributed to compounds that constitute essential oils and saponins classified as non-water soluble (condensed) tannins. Condensed tannins have restricted solubility in water which decreases with the increase in molecular mass (Paulsen, 2010).

Preliminary research work shows presence of saponins in hexane (Table 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 its derivatives. This implies that hexane extracts have compound(s) that better target the virulent factors in *R. solanacearum* hence inhibit its growth in media (Hosseinzadeh *et al.,* 2013).

5. Conclusion

The *Acacia stuhlmannii* crude extracts provides a potential bioagent as a strategy to control *Ralstonia solanacearum.* Extracts obtained by single solvent maceration had significant ($P<0.05$) bioactivity against *Ralstonia solanacearum.* Hexane extract was the most potent fraction at all concentrations with bioactivity range of 13.1–23.8 mm compared to 11.4–19.1 mm and 10.3–10.8 for ethyl acetate and ethanol extracts, respectively. Further, more work is recommended 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 also require further investigations.

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