

Stability of *Acacia stuhlmannii* (Taub) Extracts as a Botanical Control Option to *Ralstonia solanacearum*

Nyarieko, G. N.^{1*}, Guyo, P. M.² & Mwafaida, J. M.¹

¹Department of Biological Sciences, Pwani University, Kenya

²Department of Chemistry and Biochemistry, Pwani University, Kenya

Abstract: In this study, root bark extracts of *A. stuhlmannii* were subjected to variations in pH, temperature and UV-B (ultra violet-B) then evaluated for bioactivity against *Ralstonia solanacearum*. Crude fractions of ethanol and hexane extracts were obtained separately through single solvent maceration technique. Bioactivity was carried out using disc diffusion method against *R. solanacearum* cultures. Extracts not subjected to variations to the test environmental conditions were used as control(s). Petri-plates were incubated at 35°C for 36 hrs then assessed for their ability to suppress growth of pathogen *in vivo* and the inhibition zone diameters (IZDs) recorded in millimeter. Means from eight replications were analyzed for variance at 5% probability level. Means with $P \leq 0.05$ were considered significantly different using Tukey multiple comparison tool using R studio version R386 3.0.2 statistical package. Both hexane (non-polar) and ethanol (polar) extracts were relatively stable against changes in pH, temperatures and UV-B (280–315 nm) activity. Findings in this study recommend application of *A. stuhlmannii* root extract as stable control of *Ralstonia solanacearum* that cause bacterial wilt disease.

Key words: Stability, *Acacia stuhlmannii*, bioactivity

1. Introduction

Phytochemicals in crude extracts have antibacterial and antioxidant property that accounts for universal antimicrobial application [4]. Many studies have reported use of crude extract from leaves, stem, root, fruits and seeds against plant pathogens [3]. Study by Lalitha *et al.* [6] reported the extraction of metabolites of *Acacia mellifera* (BENTH) using maceration in hexane, ethyl acetate ethanol and methanol. These crude extracts had significant activity when they were tested against gram negative and gram positive bacteria. In a different study minimum inhibition concentration (MIC) of methanol extracts obtained from *Solanum*

sisymbriifolium leaves was used to assess stability in bioactivity at extreme pH and thermal treatment. Further, disc diffusion method was used to evaluate bioactivity and consistence of inhibition zone diameters (IZDs) stated as stability against pH and heat exposure [4].

Like other species in *Acacia* genus, *Acacia stuhlmannii*, produce phytochemicals with *in vitro* bioactivity against *Ralstonia solanacearum*, [8]. Notwithstanding reported bioactivity, stability of *Acacia stuhlmannii* in extreme pH, changes in temperature and UV-B variation is not known. This study therefore was designed to evaluate consistence in bioactivity of *A. stuhlmannii* after exposure to environmental vagaries. Potential stability of *Acacia stuhlmannii* extracts forms a basis for development of effective antimicrobial agent against *R. solanacearum* that cause bacterial wilt in solanaceous crops.

2. Materials and methods

2.1. Determination of minimum inhibition concentration

Minimum inhibition concentration (MIC) assessed using the broth dilution method as described by Wiegand *et al.* [9] was adopted with little modifications.

Broth media preparation; Muller Hinton Broth (M391-500G) was prepared by suspending 21g 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; Minimum inhibition concentration for hexane and ethanol extracts were determined using dilution method. Masses of 100 mg of each extract were reconstituted separately in sterile test tubes using 1ml of 10% v/v Dimethyl sulfoxide (DMSO), to increase solubility. A 1 ml portion of each extract at 100 mgml⁻¹

concentration was added to 9 ml of Muller Hinton Broth to form (1:9) ratio.

The ratio was serially diluted to 10^{-6} dilution factor. Each test tube was inoculated using 100 μ l of *R. solanacearum*, biovar 1, serotype 1 suspension containing 10^7 colon forming unit per milliliter (CFUml⁻¹) then sealed aseptically. The test tubes were incubated at 35°C and agitated for 30 seconds after every 9 hrs. Turbidity was assessed after 36 hrs using 0.5 McFarland standards. The treatments were replicated four times. Concentrations in mgml⁻¹ that showed turbidity were averaged and used as Minimum Inhibition Concentrations (MIC).

2.2. Bioactivity on pH adjusted extracts

Adjustment of pH was done using one molar acid (1M HCl) and base (1 M NaOH) solutions as described by Gupta *et al.* [4] 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 in separate 50 ml beakers were used to prepare MICs of hexane and ethanol extracts of *A. stuhlmannii*. Hexane and ethanol extract with pH of 7.0 (neutral) were considered as control. Only 20 μ l w/v of each extract was infused in sterile commercial paper disc. The loaded discs were placed on 90 mm plates containing Muller Hinton agar seeded with 100 μ l of 10^7 CFUml⁻¹ of *Ralstonia solanacearum*. The plates were sealed using parafilm and incubated at 35°C. Inhibition zone diameter (IZDs) in mm was assessed after 36 hrs of incubation.

2.3. Thermo-stability assessment of extracts

Thermo-stability was evaluated as described in article by Gupta *et al.* [4] with little adjustment. One milliliter of hexane or ethanol extracts were placed in sterilized Eppendorf tubes. The tubes were exposed to varied temperatures then tested for bioactivity using the disc diffusion method. The temperatures considered were; 40, 60, 80, 100 and 120°C. Five clinical Eppendorf tubes for each extract were heated in a water bath. The tubes were then withdrawn in that order of varied temperatures upto a temperature of 120°C using an autoclave. Extracts that were not thermal treated were used as control. Disc diffusion tests were then as described in section 2.2.

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

Filter sterilized (0.2 μ m \varnothing) hexane and ethanol extracts were treated separately using UV-B rays then evaluated for bioactivity. A volume of 1 ml of each extracts at MIC rate was placed in sterilized Eppendorf tubes. These tubes were opened and exposed to UV-B (280–315 nm) rays from the source. Time of exposure was varied as follows; 20,

40, 60 and 80 minutes. Extract in control tubes were not exposed to any UV-B rays. Bioactivity was assessed using disc diffusion sensitivity test as described in section 2.2.

2.5. Data analysis

Inhibition zone diameters (IZDs) were recorded in mm from eight replications of each treatment. The means of each variable of pH, varied temperature, and UV-B treatments were compared for statistical difference at 5% probability level. Means with $P \leq 0.05$ were considered significantly different using Tukey multiple comparison tool using R studio version Ri386 3.0.2 statistical package. Data obtained was presented in simple descriptive format with tables and trend plots where necessary. Trend plots were prepared using MS-excel version 2010. Trends were compared per treatments and gaps within treatments discussed.

3. Results and Discussion

3.1. MICs of *A. stuhlmannii* root extracts

Minimum inhibition concentrations were noted at 18.3 ± 0.3 and 25.7 ± 0.6 mgml⁻¹ w/v, for hexane and ethanol extract, respectively. The MIC values \pm standard error were averages obtained from four replications.

3.2. pH stability of *A. stuhlmannii* extracts

Hexane 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 however maintained significant ($P < 0.05$) bioactivity against *R. solanacearum*. There was no significance ($P > 0.1$) in combined effect of extract by solvent type and pH adjustments ($F_{8, 105} = 0.034$).

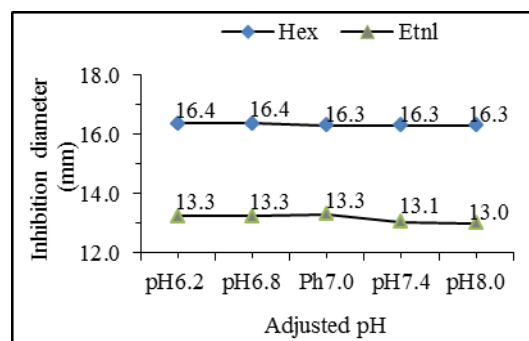


Figure 1. Trend of *invitro* bioactivity crude *A. stuhlmannii* extracts after exposure to varied pH.

Though crude extracts from *A. stuhlmannii* showed significant stability, there is a slight lability associated with increase in pH, see Figure 1. The

trend implies that soils with extreme usage of lime or wood ash may not favor the efficacy of *A. stuhlmannii* as a biocontrol. Findings by other scholars in which trials using methanol extracts of *Cassia alata* (Linn) roots showed optimal antibacterial activity when acidic which diminished when alkalinity is increased [2]. This study is also supported by scholarly findings where root extracts from *Peganum harmala* L. showed stable bioactivity over wide range of pH against Methicillin Resistant *Staphylococcus aureus* [1]. Since non-metal oxide are acidic, *A. stuhlmannii* extracts are potent enough for use with most fertilizers with nitrogen and phosphorous components.

3.3. Thermo stability of *A. stuhlmannii* extracts

Temperature increase had slight effect on bioactivity of hexane and ethanol extracts. Both extracts had a better bioactivity at elevated temperatures up to 80°C. Further, 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 *invitro* bioactivity against *R. solanacearum*. There was a noted difference ($P < 0.05$) in *invitro* bioactivity for combined effect of extracts by solvent type and thermal treatments ($F_{10, 54} = 6.793$).

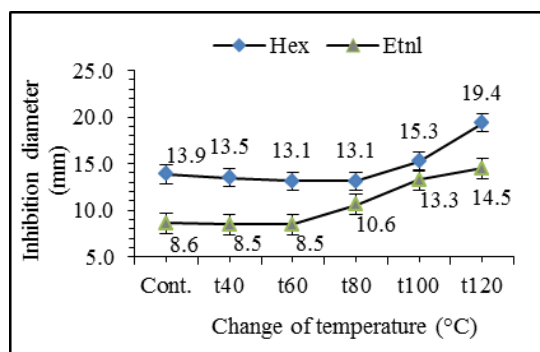


Figure 2. Trend of *invitro* bioactivity crude *A. stuhlmannii* extracts heat treatment.

Work in this research recognized a better performance of hexane 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 [7]. 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* [5]. This potency explains why there was better bioactivity of extracts at temperature above 80°C as described in Figure 2. It further explains that ethanol and hexane extracts begin to volatilize at 60°C and 80°C respectively. *Acacia stuhlmannii* extracts are expected to remain stable and effective control of

bacterial wilt disease under varied environmental conditions. Meaningful suppression is expected in soils media under high tunnels with higher daytime temperature.

3.4. UV-B stability of *A. stuhlmannii* extracts

Fractions of crude extracts obtained using hexane and ethanol extracts showed significant bioactivity against *R. solanacearum* in *invitro* assessments. Bioactivity of ethanol increased slightly but maintained stable bioactivity beyond 20 minutes exposure. Hexane extracts showed consistent bioactivity in all exposure except when exposed for 60 minutes. Activity however recovered when exposed for 80 minutes. Generally, extract by solvent type and UV-B treatment had significant ($P < 0.05$) bioactivity $F_{2, 126} = 76.444$ and $F_{5, 126} = 14.095$, respectively. There was combined significant ($P < 0.05$) interaction between extract by solvent type and length of UV-B exposure, $F_{10, 126} = 2.161$.

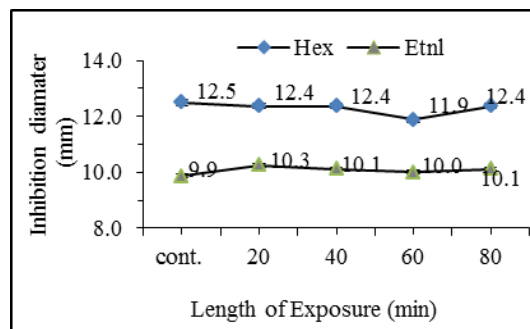


Figure 3. Trend of *invitro* bioactivity of crude *A. stuhlmannii* extracts after UV-B exposure.

The dynamics in bioactivity of crude extracts with UV-B exposure is solely due to two factors namely; changes in reconstituted absorption molecules and partly due to formation of antimicrobial bioactive compounds [10]. According to previous studies, plants respond to active UV-B (280–315 nm) via increase in anti-stress secondary metabolites [10]. This implies daily exposure of plants to UV-B accumulates meaningful phytochemicals with antibacterial property. This study did not record significant increase in bioactivity of *A. stuhlmannii* extracts. Trend in Figure 3 though suggest slight enhancements. The stable nature of hexane and ethanol extracts recommends ability of extracts against lability by UV-B band stress.

4. Conclusion and Recommendation

Findings of this study reveal that extracts maintained reasonable bioactivity even when subjected to changes in pH and UV-B band treatments. Exposure of extract to variation in temperatures seemed to boost bioactivity mainly due

to increased solubility. Based on these findings, root extracts of *A. stuhlmannii* can be recommended as biocontrol of *Ralstonia solanacearum* in a media with dynamic environmental conditions.

5. Acknowledgement

Authors are grateful to the National Commission for Science, Technology and Innovation (NACOSTI) of Kenya for funding. This work was supported by Pwani University laboratory.

6. References

- [1] Darabpour, E., Bavi, A. P., Motamedi, H. and Nejad, S.M.S., "Antibacterial activity of different parts of *Peganum harmala* L. growing in Iran against multi-drug resistant bacteria", *EXCLI J*, 2011, pp. 252–263.
- [2] El-Mahmood, A. M. and Doughari, J. H., "Phytochemical screening and antibacterial evaluation of the leaf and root extracts of *Cassia alata* Linn.", *African Journal of Pharmacy and Pharmacology*, 2008, pp.124–129.
- [3] Gurjar, M.S., Ali, S., Akhtar, M. and Singh, K.S., "Efficacy of plant extracts in plant disease management" *Agricultural Sciences*, 2012, pp. 425.
- [4] Gupta, K., Simlai, A., Tiwari, M., Bhattacharya, K. and Roy, A., "Phytochemical contents, antimicrobial and antioxidative activities of *Solanum sisymbriifolium*", *Journal of Applied Pharmaceutical Science*, 2014, pp. 75–80.
- [5] Hosseinzadeh, S., Masoud, S. and Ebrahim, H., "Effects of sub-bactericidal concentration of plant essential oils on pathogenicity factors of *Ralstonia solanacearum*", *Archives Of Phytopathology And Plant Protection*, 2013, pp. 643–655.
- [6] Lalitha, S., Rajeshwaran, K., Kumar, S., Deepa, K. and Gowthami, K., "In vivo Screening of Antibacterial Activity of *Acacia mellifera* (BENTH) (Leguminosae) on Human Pathogenic Bacteria", *Global Journal of Pharmacology*, 2010, pp. 148–150.
- [7] Li, X., Du, Y., Wu, G., Li, Z., Li, H., and Sui, H., "Solvent extraction for heavy crude oil removal from contaminated soils", *Chemosphere*, 2012, pp. 245–249.
- [8] Nyarieko, N., Mghalu, M., Guyo, P. and Mwandogo C., "Screening for Bioactivity of Phytochemical Extracted from *Acacia stuhlmannii* Against *Ralstonia solanacearum*", *Journal of Biology, Agriculture and Healthcare*, 2016, pp. 65–70.
- [9] Wiegand, I., Hilpert, K. and Hancock, R.E., "Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances", *Nature protocols*, 2008, pp. 163.
- [10] Zhang, J. and Björn, O., "The effect of ultraviolet radiation on the accumulation of medicinal compounds in plants", *Fitoterapia*, 2009, pp. 207–218.