

POTENTIAL ANTIMICROBIAL PROPERTIES FROM OIL PALM ROOT AGAINST *Ganoderma boninense*

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OBJECTIVES

The project aimed:

- i) To identify potential antimicrobial properties from oil palm root against *G. boninense*
- ii) To quantify potential antimicrobial properties using High Performance Liquid Chromatography (HPLC)
- iii) To investigate the *in vitro* effect of the antimicrobial properties from oil palm root to the growth of *G. boninense*

MATERIALS AND METHODS

Seed and plant materials

Pre-germinated seeds, varieties of Ekona, Calabar and AVROS were kindly provided by Borneo Samudera Sdn Bhd and grown to one year old in Peat Vriezenveen Substrate, Product of Holland in the field house of UMS.

Ganoderma boninense (GB)

Fruiting bodies of GB were collected from oil palm trees in Borneo Samudera Langkon Estate in Sabah. Internal tissues of fruiting bodies was excised and cultured on *Ganoderma* Selective Medium (GSM). Once the pure culture of GB was isolated, it was transferred and maintained at 25°C on Potato Dextrose Agar (PDA) for normal growth.

Oil Palm Root Elicitation with Chitosan

Stimulation of the defence mechanisms was conducted on seedlings of oil palm aged one year old. A ratio of 5g of chitosan (R & M Chemicals, Essex, UK) was incorporated into 5 kg of Peat Vriezenveen Substrate.

Extraction of phenolic from roots for SPE and HPLC analysis

Oil palm seedlings root were homogenized using 1Ka A11 basic grinder and soaked into methanol for two days before filtered through Whatman® No. 1 filter paper, dried up in a Buchi® rotary evaporator and resuspended into milli-Q® ultra pure water. The aliquot was centrifuged with 13,000 rpm for 15 minutes before proceed to solid phase extraction (SPE).

Solid phase extraction (SPE)

Strata X 33µm Polymeric Reversed Phase (200mg/6ml) by phenomenex® were chosen based

on its reliable sorbent system. Other standard SPE protocols applied. The final concentration was adjusted to 5 g of tissue per 1 ml of milli-Q® ultra pure water.

Confirmation of phenolic acids in oil palm root using injection with standards

The Variable Wavelength Detector (VWD) with 280nm was set for the elution with a flow rate of 1 ml/min. The HPLC conditions as described below:

Acetic acid (C₂H₄O₂) 0.3% (A) and MeOH (B); A/B= 80:20 (0 min)→30:70 (23-24 min)→80:20 (24 min)

Reference compounds used were vanillin, transcinnamic, benzoic, 4-hydroxybenzoic (4-HBA), 3, 4 -dihydroxybenzoic, gallic, syringic, p-coumaric, caffeic, ferulic, and sinapic acids.

Confirmation of phenolic acids in oil palm root using Liquid Chromatography Mass Spectrophotometry/Q-tof (LC-MS/Q-tof)

LC conditions: column, a Waters Acquity UPLC BEH C18 (2.1 mm x 100 mm, particle size 1.7 µm); injection volume, 2 µl; column temperature, 40°C; PDA, 200-500 nm; flow rate, 0.5ml/min; mobile phase, HPLC H₂O (A) and MeOH (B); A/B=100:0 (0 min)→50:50 (2.5-5 min)→ 0:100 (5-5.5 min).

MS/Q-ToF conditions: Accurate mass analysis was conducted with the same Waters LC system described above coupled with a Waters Synapt Q-ToF system equipped with electrospray ionization positive ionization mode (ESI) probe, lock spray inlet system and mass correction was done using injection of leucine enkephalin (556.2771Da). Highly accurate mass acquisition of the ion of interest was performed by chromatography injection. Two microliter injections of the extracts were injected on the C-18 column maintained at a temperature of 15°C using the same eluting condition as before. Mass spectrometric ionization conditions were as follows: desolvation temperature, 350°C; source temperature, 90°C; capillary voltage, 2.3 kV; desolvation gas, 500L/hr, ToF detector voltage, 1700; sampling cone, 45; extraction cone, 4; trap collision energy, 6; transfer, 4.0. For samples analyzed in scan mode the scan range was 100-1500 Da.

HPLC analysis and quantification for phenolics

An efficient gradient of the HPLC system as developed (in confirmation of phenolic acids) was used for HPLC analysis and quantification for phenolics. Serial dilutions of known concentration of standards (from Sigma®) (syringic acid, caffeic acid and 4-HBA) were injected into the HPLC column and ran using the same system. Areas under the peaks were integrated with the known concentrations and standard curves were produced for each of the standard.

***In vitro* bioassays in agar and broth**

A series of 0, 50µg, 70µg, 90µg, and 110 µg/ml of syringic acid, caffeic acid and 4-HBA purchased from Sigma® were incorporated into the 10% Potato Dextrose Agar (PDA), Oil Palm Root Agar (OPRA) and Oil Palm Root Broth (OPRB), which the phenolics were first dissolved in acetone: water (50:50; v/v) before incorporated into the media or broth. Agar or broth without phenolics served as control. The GB was taken from the edge of a seven to eight days old culture using a sterile micropipette tip sized 0.8 cm and introduced to the

middle of the media. The growth of the pathogen was expressed in centimeter of radial growth. For GB incorporation into broth, mycelia first were washed, filtered through Whatman® filter paper No.4, rinsed thoroughly using the milli-Q® ultra pure water and oven-dried overnight at 80°C for approximate eight hours. The dry weight was recorded as initial weight and later introduced into the OPRB with different concentration of phenolics, dry weight were recorded daily and expressed as the growth of the pathogen.

RESULTS

Confirmation of phenolic acids in oil palm root using injection with standards

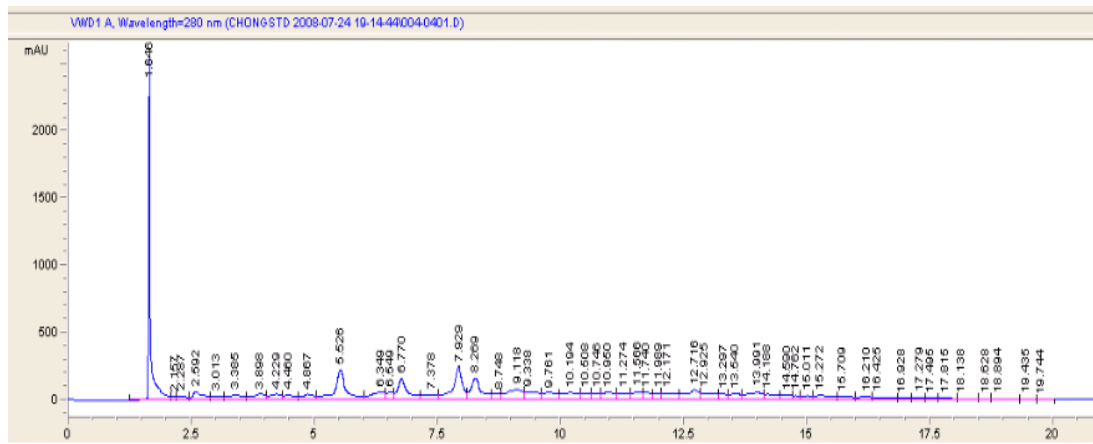


Figure 1: Chromatogram showing the peaks commonly presence in oil palm root extract both in control and elicited roots detected by variable wavelength detector (VWD) after separation by solid phase extraction (SPE) and High Performance Liquid Chromatography (HPLC)

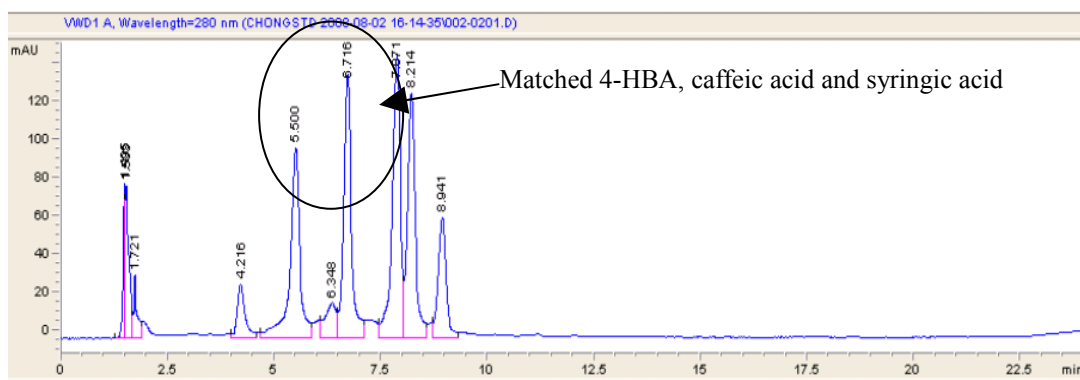


Figure 2: Chromatogram showing injection with sample and the most potential standards that may match the compound of interest. Arrowed: 4-HBA, caffeic acid and syringic acid

Confirmation of phenolic acids in oil palm root using Liquid Chromatography Mass

Spectrophotometry/Q-tof (LC-MS/Q-tof)

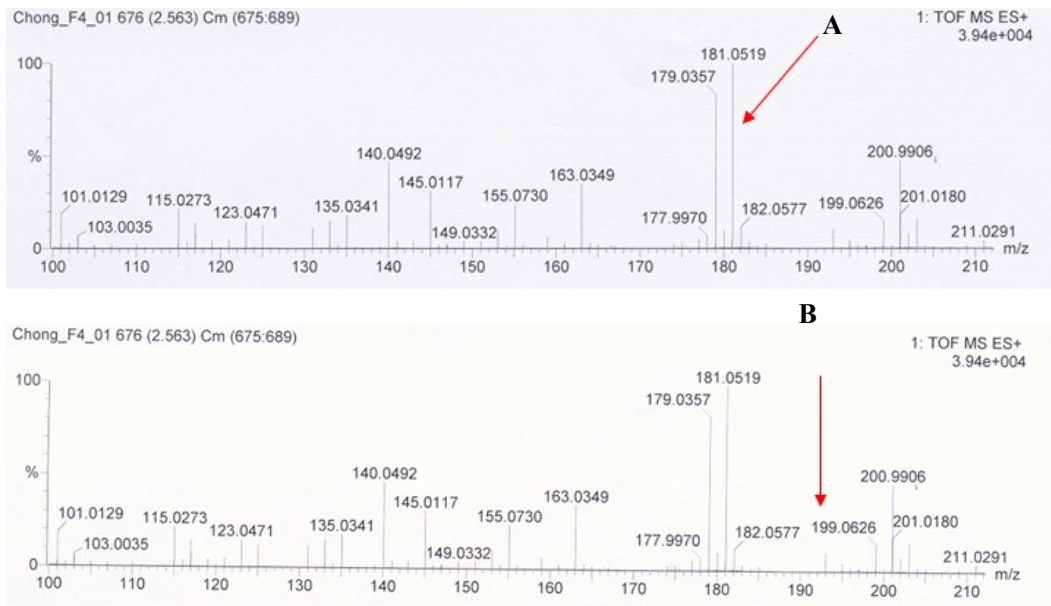


Figure 3: Electro spray mass spectrum of caffeic acid (181.05), and syringic acid (199.06). x-axis shows m/z ratio and y-axis shows the percentage. Arrowed: Caffeic acid (A) and syringic acid (B)

HPLC analysis and quantification for phenolics

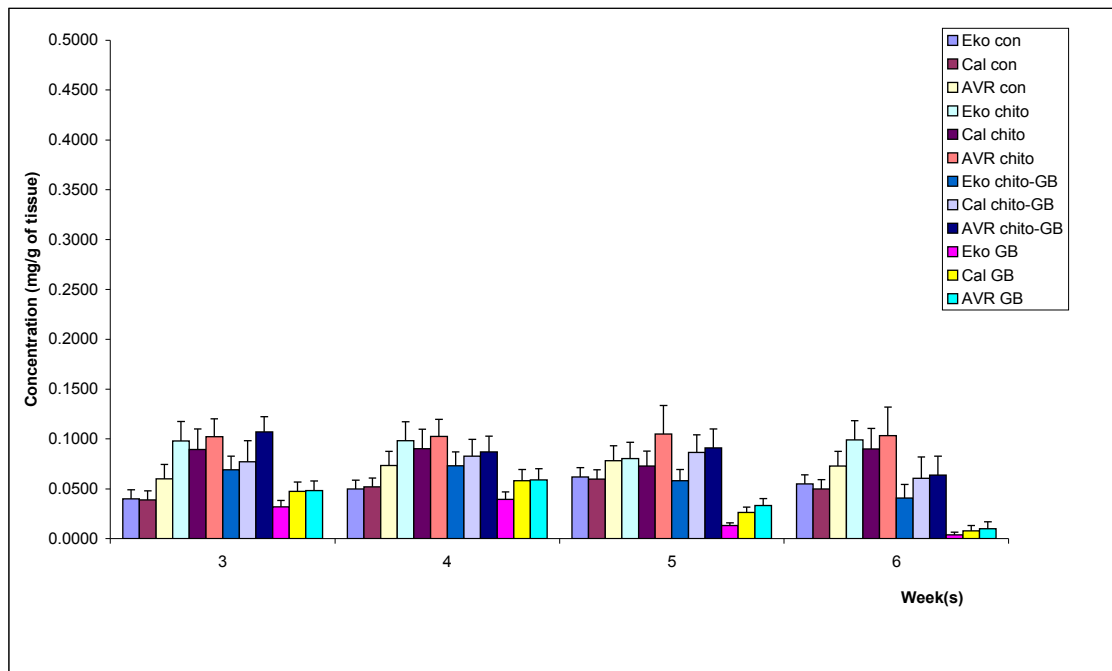


Figure 4: Accumulation of syringic acid in different varieties of oil palm root at different weeks after different treatments

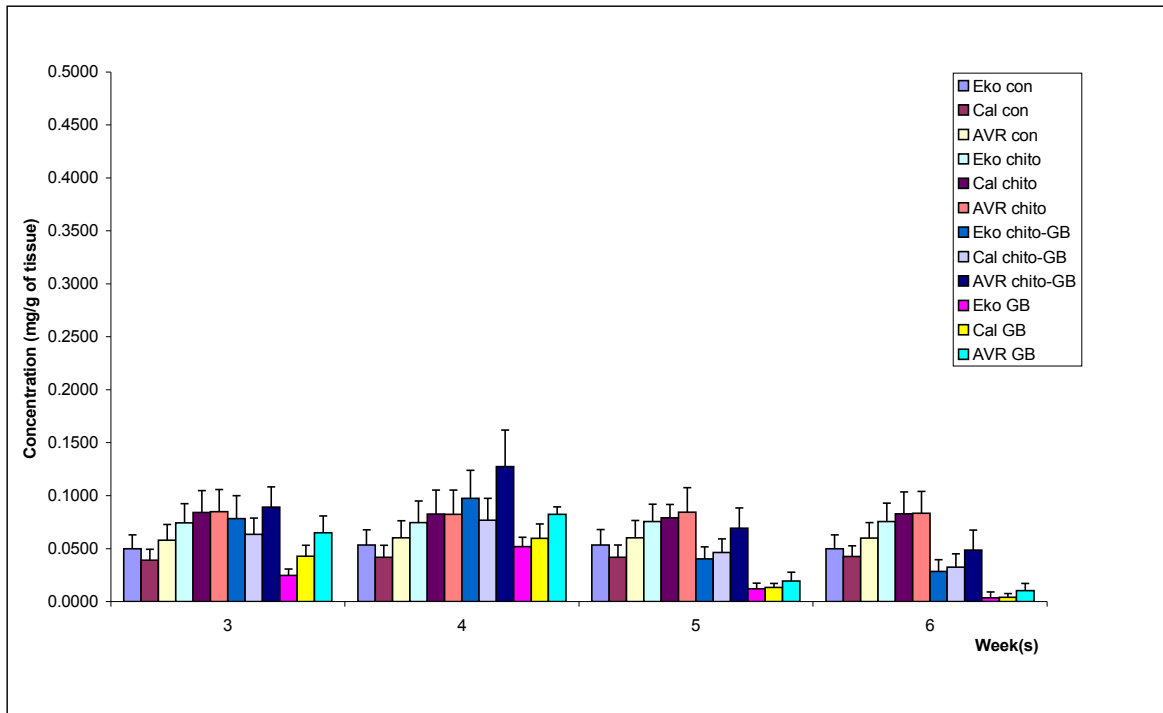


Figure 5: Accumulation of caffeic acid in different varieties of oil palm root at different weeks after different treatments

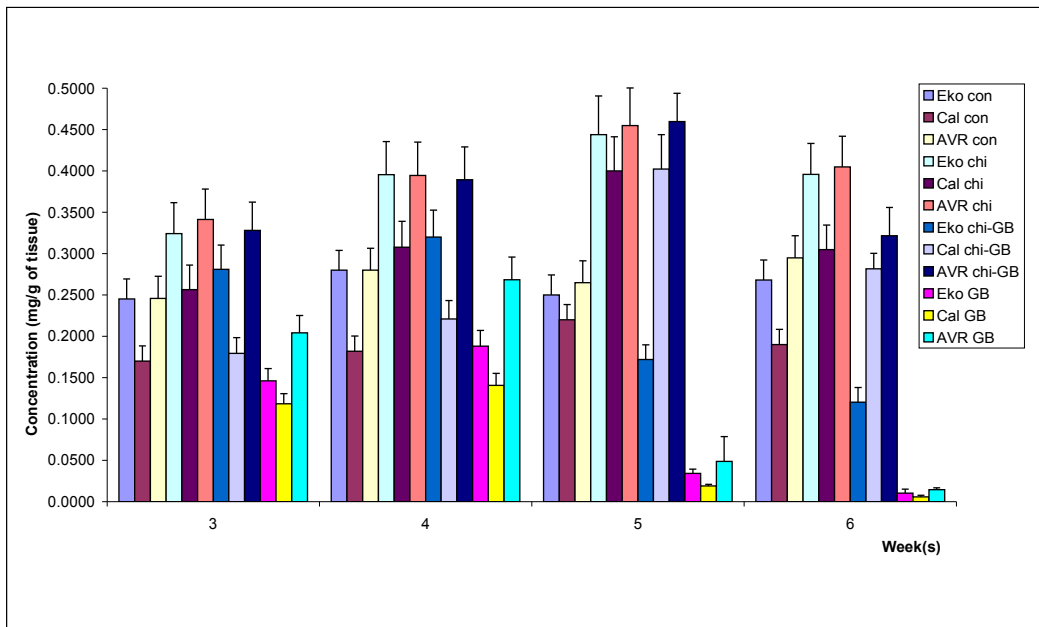


Figure 6: Accumulation of 4-HBA in different varieties of oil palm root at different weeks after different treatments

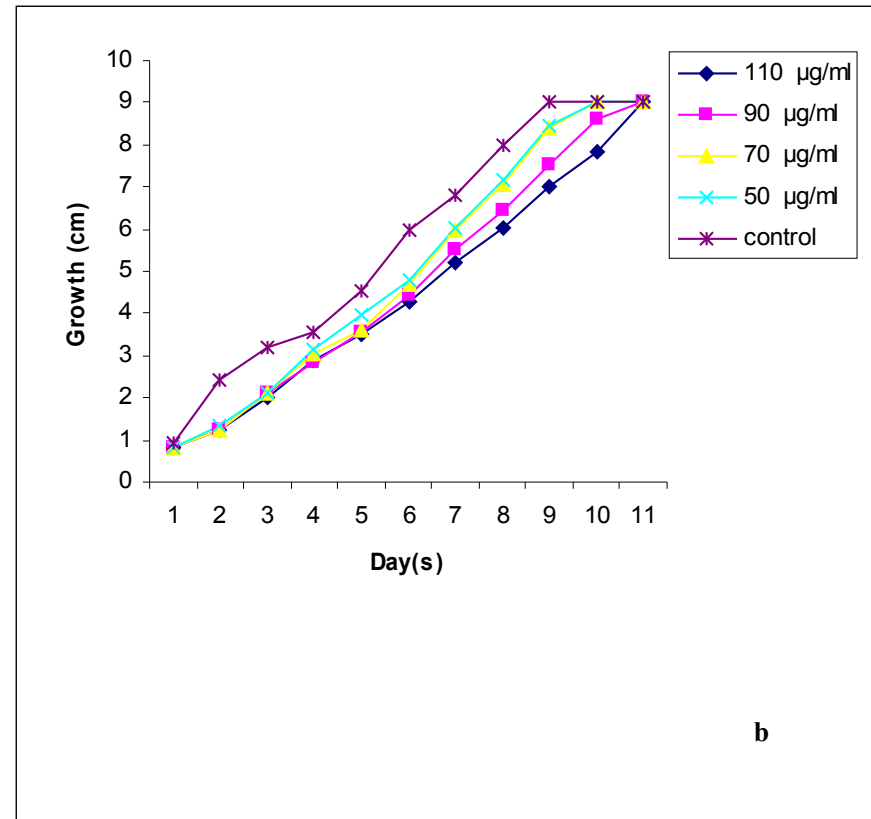
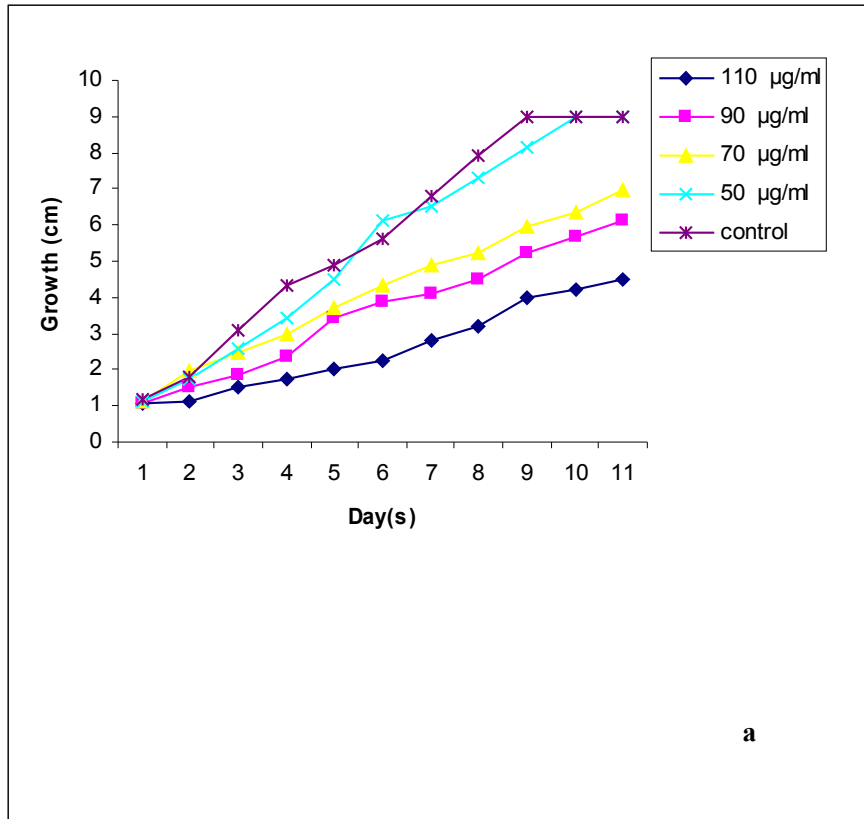


Figure 7: The radial growth of *G. boninense* mycelia on 10% PDA (a) and OP media (b) with different concentrations of syringic acid incorporated into the agar incubated for 11 days

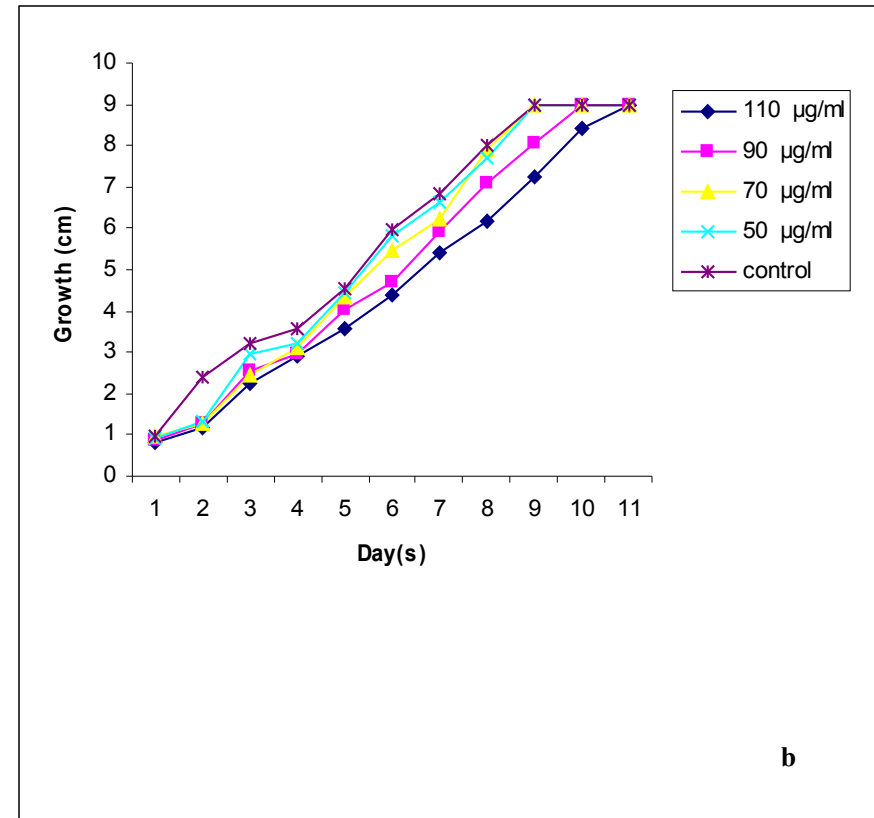
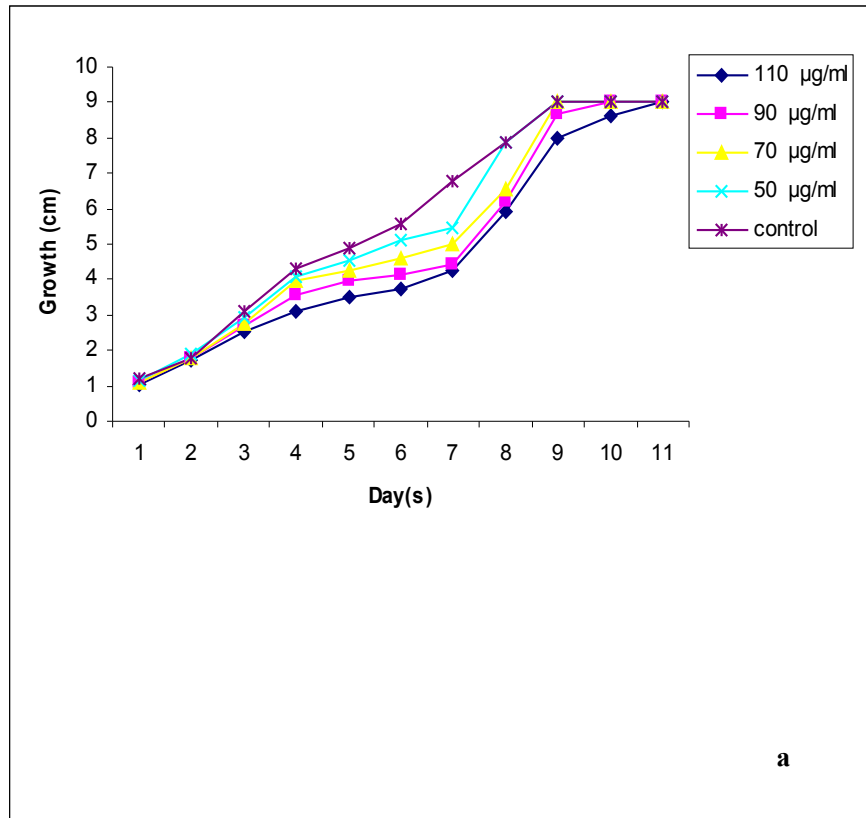


Figure 8: The radial growth of *G. boninense* mycelia on 10% PDA (**a**) and OP media (**b**) with different concentrations of caffeic acid incorporated into the agar incubated for 11 days

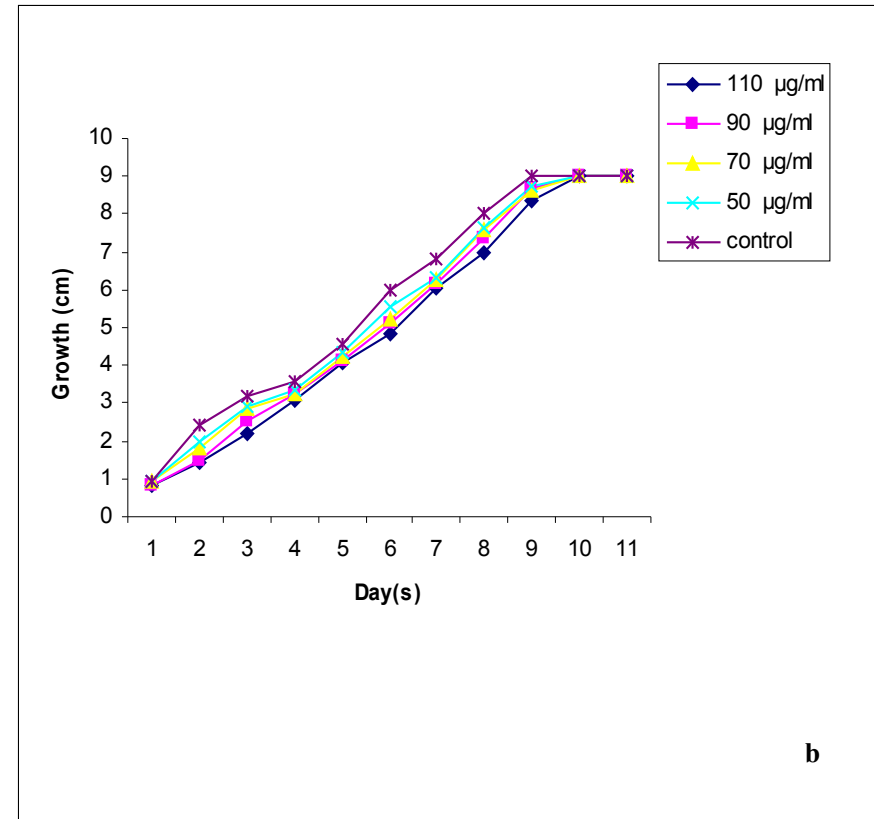
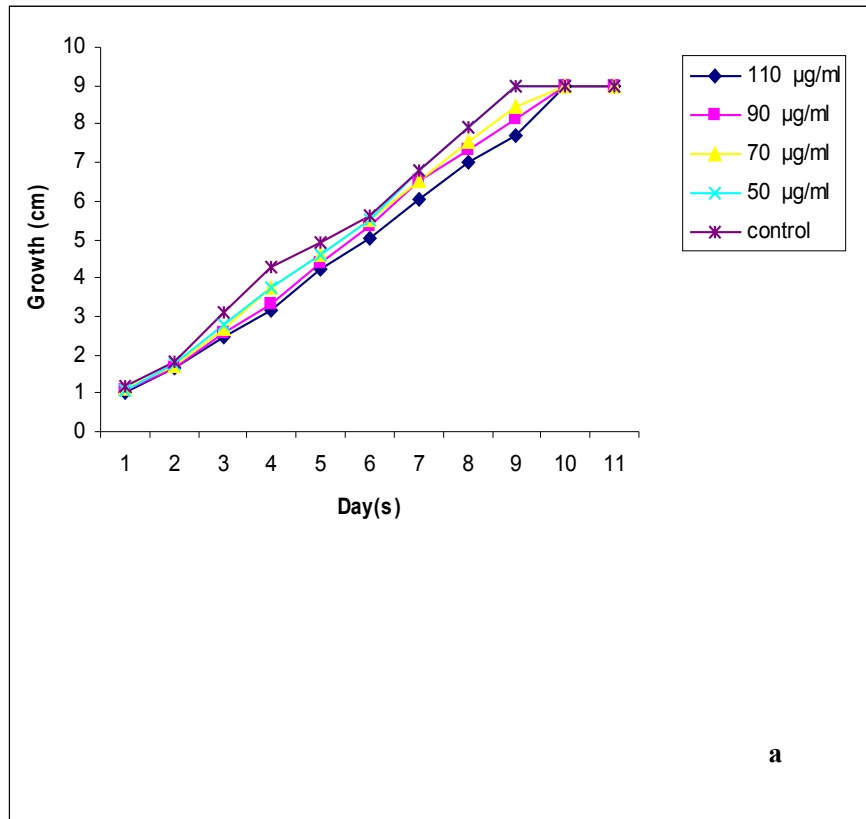


Figure 9: The radial growth of *G. boninense* mycelia on 10% PDA (a) and OP media (b) with different concentrations of 4-HBA incorporated into the agar incubated for 11 days

CONCLUSION

HPLC analysis and LC-MS/Q-tof confirmed the presence of three important phenolic acids; syringic, caffeic and 4-hydroxybenzoic acid in oil palm root defence mechanism. AVROS variety found to have higher concentration of these phenolic acids and less susceptible to *G. boninense* infection compared to Ekona and Calabar. Although *in vitro* studies showed that phenolic acids potentially inhibit the growth of *G. boninense* but low concentration of these phenolics that presence in normal oil palm root failed to stop further invasion of the pathogen.

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