# The potential of papaya leaf extract in controlling *Ganoderma boninense*

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# The potential of papaya leaf extract in controlling *Ganoderma boninense*

**Z H Tay and K P Chong**[1]
Sustainable Palm Oil Research Unit (SPOR), Faculty of Science and Natural Resources.
Universiti Malaysia Sabah, 88400, Jalan UMS, Kota Kinabalu, Sabah, Malaysia

Email: [1]chongkp@ums.edu.my

**Abstract.** Basal Stem Rot (BSR) disease causes significant losses to the oil palm industry. Numerous controls have been applied in managing the disease but no conclusive result was reported. This study investigated the antifungal potential of papaya leaf extracts against *Ganoderma boninense*, the causal pathogen of BSR. Among the five different solvents tested in extraction of compounds from papaya leaf, methanol and acetone gave the highest yield. *In vitro* antifungal activity of the methanol and acetone extracts were evaluated against *G. boninense* using agar dilution at four concentrations: 5 mg $mL^{-1}$, 15 mg $mL^{-1}$, 30 mg $mL^{-1}$and 45 mg $mL^{-1}$. The results indicated a positive correlation between the concentration of leaf extracts and the inhibition of *G. boninense*. ED50 of methanol and acetone crude extracts were determined to be 32.016 mg $mL^{-1}$and 65.268 mg $mL^{-1}$, respectively. The extracts were later semi-purified using solid phase extraction (SPE) and the nine bioactive compounds were identified: decanoic acid, 2-methyl-, Z,Z-10-12-Hexadecadien-1-ol acetate, dinonanoin monocaprylin, 2-chloroethyl oleate, phenol,4-(1-phenylethyl)-, phenol,2,4-bis(1-phenylethyl)-, phenol-2-(1-phenylethyl)-, ethyl iso-allocholate and 1-monolinoleoylglycerol trimethylsilyl ether. The findings suggest that papaya leaf extracts have the ability to inhibit the growth of *G. boninense*, where a higher concentration of the extract exhibits better inhibition effects.

## 1. Introduction

Oil palms in Malaysia are mostly affected by Basal Stem Rot disease (BSR), which is caused by *Ganoderma boninense*. The disease causes a yearly economic loss of up to RM1.5 billion to the oil palm industry [1]. BSR was first discovered in Malaya in 1928 and was reported to be affecting only old oil palm trees but had slowly changed to palms as young as 10-15 years. After 1957, the disease was observed to have spread to younger oil palms even at the nursery stage [2]. Numerous methods have been established in an attempt to control the disease. For instance, cultural control methods such as sanitation, replanting, removing the root masses and digging trenches around the infected plants are adopted to prevent the spread of the disease [3]. Chemical controls such as fungicide are applied but are ineffective. This led to the development of biological control methods using biological agents such as bacteria and fungi to suppress the growth of *G. boninense*. However, no conclusive result has been reported to stop the disease completely, extensive research is required to combat this pathogen.

[1] To whom any correspondence should be addressed.

In the current study, the leaves of papaya were tested for their antifungal potential against *G. boninense*. Papaya leaves have been used in cancer cell line studies for its anti-tumour properties, showing it to be potentially useful in the medical field [5]. Juice made from papaya leaves was shown to increase platelet counts in patients with dengue fever, as compared with the control group [6]. Papaya leaves also exhibit many other antibacterial and antifungal activities. Previous research showed that papaya leaf extract has high inhibition activities towards gram-negative and gram-positive bacteria and fungi *Candida albicans* [7] and *Xanthomonas axonopodis,* which causes Cassava blight disease [8]. Papaya leaf extract was also found to be effective in controlling soft rot disease affecting yam caused by *Mucor circinelloides* and *Rhizopus nigricans* [9]. In this paper we report the antifungal activity of papaya leaf extract against *G. boninense*.

## *2.* Methodology.

### *2.1 Collection of papaya leaves*

Fresh papaya leaves collected from Kota Kinabalu were washed first under running tap water, followed by sterile distilled water. After drying at 40 $^{o}$C for 72 hours [10], the leaves ground to a fine powder using a mechanical blender (Waring ® Commercial Blender). The dry weight was determined, and the powder was stored in an air-tight container at -20 $^{o}$C until further use [11].

### *2.2 Preparation of* Ganoderma boninense *culture*

A stock culture of *G. boninense* was obtained from Genetic Laboratory of Faculty of Science and Natural Resources, University of Malaysia Sabah. The mycelia was transferred onto PDA agar plates and incubated at 25 $^{o}$C. Culture plates fully covered with white mycelia were used within two weeks.

### *2.3 Solvent Extraction of Papaya Leaves*

The compounds of interest in the papaya leaves were extracted using polar and non-polar solvents: methanol, acetone, chloroform, ethyl acetate and n-hexane. One hundred grams of powdered leaves were immersed in conical flasks containing 1000 mL of methanol, acetone, chloroform, ethyl acetate and hexane, respectively. The flasks were then placed in a sonicator (Branson ® 5510) for 10 minutes at 25 $^{o}$C. The extracts were pump filtered through Whatman No. 1 filter paper. The filtrates were evaporated and condensed using Rota Vapor (BUCHI) at 40 $^{o}$C, 150 rpm, to obtain the final volume of 1 mL of extract per 10 g of plant sample. The aliquots were then weighed and kept at -20 $^{o}$C for further use [12]. The extraction yield was determined using the following formula:

$$\text{Yield (\%)} = \frac{\text{Dry weight of extract}}{\text{Dry weight of plant powder}} \times 100$$

### *2.4 In vitro Antimicrobial Assay*

Crude extracts were dissolved in Dimethyl sulfoxide (DMSO), and then a serial concentration of plant solvent extracts at 5, 15, 30, 45 mg mL$^{-1}$ was prepared. Agar with DMSO (no plant extract) was prepared as negative control; agar (no DMSO or extract) was the absolute control.*G. boninense* was taken from the edge of a seven day old culture using a sterile micropipette tip sized 0.8 cm and then placed in the middle of the media. Growth of the pathogen was measured (in cm). Each extract concentration was assayed in triplicates and the mean values were calculated. $ED_{50}$ for both the methanol and acetone crude extracts were determined using SPSS Probit analysis.

### *2.5 Preparation of Solid Phase Extraction (SPE) papaya leaf extract*

The leaf crude extracts were purified using solid phase extraction (SPE) installed with Strata$^{TM}$ C18 cartridges (500 mg/ 6 mL). Absolute methanol (1 mL) was used to activate the sorbent which was equilibrated with sterile deionized distilled water (1 mL) afterwards. Crude extracts were loaded and left in the sorbent matrix for up to a minute, then washed with 1 % methanol (1 mL) to obtain the 'flush fraction'. The 'elute fraction' was obtained by using 5 mL of methanol: acetonitrile (1:1; v:v) [12].

*2.6 Identification of Potential Antifungal Compounds using GC-MS*

Compounds in the SPE extracts were identified using Gas Chromatography (model: Agilent, 7890A) interfaced with Mass Spectrometer (Model: Agilent, 5975C) which was equipped with Agilent J & W GC capillary column with HP-5MS stationary phase (30 m x 0.25 mm x 0.25 µm) and composed of (5%-phenyl)-methylpolysiloxane. An electron ionization system with ionization energy of 70eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 0.8 mL $min^{-1}$and an injection volume of 1 µL (split ratio of 10:1). With the injector temperature at 250 $^{o}$C and ion-source temperature at 280 $^{o}$C, the oven temperature was programmed from 110 $^{o}$C (isothermal for 2 min) with an increase of 10 $^{o}$C $min^{-1}$, to 200 $^{o}$C, then 5 $^{o}$C $min^{-1}$ to 280 $^{o}$C (isothermal for 9 min). Mass spectra were taken at 70 eV (a scan interval of 0.5 secs and fragments from 40 to 550 Da). The total GC running time was 41 min. The relative percentage of each component was calculated by comparing its average peak area to the total area. The software used to handle the mass spectra and chromatograms was GC/MSD ChemStation [2].

*2.7 Data Analysis*

Each treatment was replicated three times and the results were expressed as mean ± standard deviation. The mean values were subjected to Analysis of Variance (one-way ANOVA), and the Post Hoc tests (SPSS statistical package, version 22) was used to determine the significant differences ($p < 0.05$) between treatments.

## 3 Results

*3.1 Extraction yield of papaya leaves*

Powdered papaya leaves were extracted using organic solvents at the ratio of 100 g of leaf samples to 1000 mL of solvents. Different types of solvents resulted in different extraction yields (table 1). Methanol gave the highest yield of extraction (8.24%) while n-hexane gave the lowest (2.06%).

**Table 1.** Extraction yield of papaya leaves using different solvents

| Solvents | Solvent Polarity Index | Yield (%) |
|---|---|---|
| Methanol | 6.6 | 8.24 |
| Acetone | 5.4 | 7.20 |
| Ethyl acetate | 4.3 | 6.87 |
| Chloroform | 4.1 | 4.04 |
| n-Hexane | 0.1 | 2.06 |

*3.2 In Vitro inhibitory effect of papaya leaf crude extract against* G. boninense

The antimicrobial activity of the papaya leaf crude extracts from the two solvents with the highest extraction yield (methanol and acetone) was studied via *in vitro* antimicrobial assay against *G. boninense* (figure *1* and figure *2*). *G. boninense* had grown to the maximum size of the plate in negative control at day 16. There was a significant reduction of growth rates for *G. boninense* in plates with methanol and acetone papaya leaf crude extract with the slowest growth shown in the highest tested concentration of methanol extract (45 mg $mL^{-1}$). An exception was the case of methanol and acetone extracts (5 mg $mL^{-1}$), where the inhibitory effect was lower than the negative control containing DMSO during the first week of observation. However, this enhanced growth did not continue until the end of the observation. Statistical analysis indicated that fungal growth treated with both the methanol and acetone crude extracts were significantly lower ($p< 0.05$) as compared to negative control. *G. boninense* were affected less in acetone extracts which were of similar concentrations as the methanol extracts. All the tested concentrations of methanol and acetone papaya leaf crude extracts failed to totally inhibit growth of the pathogen. From Probit analysis, $ED_{50}$ of

methanol and acetone crude extract was determined to be 32.015 mg $mL^{-1}$ and 65.268 mg $mL^{-1}$ respectively.

The morphology of *G. boninense* was observed for plates incorporated with methanol and acetone extracts. In the control and methanol plates, the fungus grew to form a white mat that appeared cotton-like, which grew outwards from the fungal plug [14]. In the acetone extract plates, the mycelia growth zone was unclear, and the structure was not white and cotton-like. The acetone extract at 45 mg $mL^{-1}$ had white cotton-like mycelia but was malformed and stunted (figure *3*).

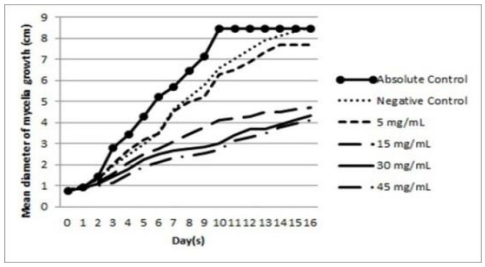


**Figure 1.** *G. boninense* mycelial growth on PDA incorporated with different concentrations of methanol extract of papaya leaf. Each mean obtained from three replicates. Absolute control: agar without addition of DMSO. Negative control: agar with addition of DMSO.

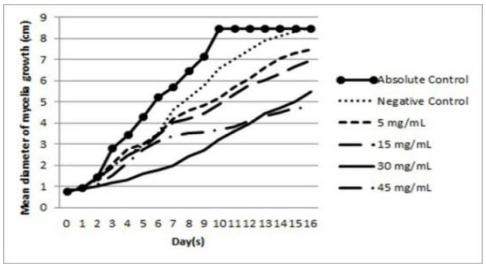


**Figure 2.** *G. boninense* mycelial growth on PDA incorporated with different concentrations of acetone extract of papaya leaf. Each mean obtained from three replicates. Absolute control: agar without addition of DMSO. Negative control: agar with addition of DMSO.

### *3.3 GC-MS analysis of antifungal compounds in papaya leaves*

The methanol and acetone crude extracts of papaya leaves were fractionated using solid phase extraction into elute and flush fractions. The compounds in the fractions were analysed separately using GC-MS and identified by comparing to the National Institute of Standard and Technology (NIST) database. Different compounds were identified from the eluant and flush fractions of the papaya leaf SPE extracts from methanol and acetone.

Five classes of compounds were identified from the methanol and acetone extracts: carboxylic acid, ester, fatty acid ester, phenol and steroid. Two steroid compounds were discovered in the eluted fraction of methanol SPE extracts: 1-monolinoleoylglycerol trimethylsilyl ether (RT= 35.05 min; 77.32%) and ethyl iso-allocholate (RT= 33.40 min; 22.67 %). In the flush fraction of methanol SPE extracts, the amount of fatty acid ester, dinonanoin monocaprylin (RT=32.63min; 71.78%) was much higher than 1-monolinoleoylglycerol trimethylsilylether (RT= 32.38 min; 20.35%) while octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl- (RT= 34.00 min) was only around 4.84 %. Three compounds were found in eluted fraction of acetone SPE extracts: phenol,4-(1-phenylethyl)- (RT= 13.51 min; 0.09 %), phenol,2,4-bis(1-phenylethyl) (RT= 27.23 min; 0.29 %) and octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl- (RT=28.52min; 0.75%). Flush fraction of acetone SPE extracts contained four compounds with Z,Z-10-12-Hexadecadien-1-ol acetate being the most aundant (RT=20.99min; 46.26%), followed by 2-chloroethyl oleate (RT= 21.11 min; 38.24%), phenol-2-(1-phenylethyl)- (RT= 13.52 min; 15.50 %) and decanoic acid,2-methyl- (RT= 17.21 min; 11.15 %).

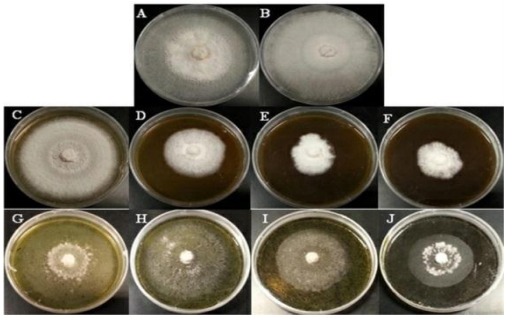


**Figure 3.** Growth of *G. boninense* on incorporated PDA. (A) Absolute control (B) Negative control with

Methanol extract: (C) 5 mg $mL^{-1}$ (D) 15 mg $mL^{-1}$ (E), 30 mg $mL^{-1}$, (F), 45 mg $mL^{-1}$, with Acetone extract: (G) 5 mg $mL^{-1}$ (H) 15 mg $mL^{-1}$ (I) 30 mg $mL^{-1}$, (J) 45 mg $mL^{-1}$ on day 16.

## 4 Discussion

### *4.1 Extraction of papaya leaves using different solvents*

The current study showed methanol to produce the highest yield in extracting compounds from papaya leaves as compared to acetone, ethyl acetate, chloroform and n-hexane, consistent with previous work [15, 16]. Higher extraction yield achieved by methanol could be explained by its ability to dissolve more compounds including phenols and endogenous compounds [17]. Besides the polarity of the solvents, the chemical compositions of the sample also determine the yield of extraction. Polar solvents extract polar compounds while less polar solvents extract non-polar compounds [17]. Polar compounds are also easier to extract compared to non-polar compounds [18]. It is likely that papaya leaves contain more polar than non-polar compounds.

### 4.2 *Inhibitory effects of papaya leaf crude extracts to* G. boninense

Four concentrations of extracts were tested, and the fungicidal effects to *G. boninense* were shown to have a positive correlation, except in the case of methanol and acetone extracts (5 mg $mL^{-1}$). This was also shown in a previous report on *Microsporum canis* where plant extracts at low concentrations enhanced mycelia growth [19]. It is possible that the low concentration of extract meant that the inhibition only started taking effect after a short while. Methanol extracts showed a higher inhibition than acetone extracts, possibly due to its higher extraction yield resulting in higher concentration of bioactive compounds in the extract. It is also possible that the different compound composition resulted in higher inhibition in the methanol extract.

### *4.3 Identity of potential inhibitory compounds in papaya leaf SPE extract*

The identified compounds were classified to five classes based on their possible activities; carboxylic acid, ester, fatty acid ester, phenol and steroid. Each of these compounds has been shown previously to have antifungal activities.

Phenolic compounds were reported to have antifungal activities against *G. boninense* [20], *Candida* species [21], *Alternaria solani*, *Botrytis cinerea* and *Fusarium culmorum* [22], possibly by diffusing through the fungal membrane and interfering with the synthesis of essential components such as ergosterol, glucan, chitin, proteins and glucosamine [23]. Fatty acids or carboxylic acids are one of the essential components in plants with antimicrobial activities. For instance, the saturated fatty acid, decanoic acid,2-methyl- was shown to inhibit hyphae formation of *C. albicans* [24]. Fatty acid esters extracted from *Phyllanthus amarus* was shown to be inhibit growth of several bacteria species [25]. The identified ester, dinonanoin monocaprylin, in this experiment was shown to have the potential to inhibit pathogens such as *Streptococcus* species and *Escherichia coli* [26].The identified steroids, ethyl iso-allocholate and 1-monolinoleoylglycerol trimethylsilyl ether were also shown previously in various reports to have antimicrobial and antibacterial potential [27, 28], the latter being a major compound in leaf and stem extracts of *Sesuvium portulacastrum* [29].

Generally, steroids were detected in the methanol extract while the phenolic compounds and carboxylic acids were from the acetone extracts. Fatty acid esters were detected in both methanol and acetone extracts. The compounds of different categories could have different inhibitory effect shown in *in vitro* antimicrobial assay.

## 5 Conclusion

The inhibitory effect to *G. boninense* was greater at higher concentrations of papaya extracts, but the effects were not complete inhibition. $ED_{50}$ of methanol and acetone crude extracts of papaya leaves were determined to be 32.016 mg $mL^{-1}$ and 65.268 mg $mL^{-1}$, respectively. The eluted and flush

fractions of the papaya leaf extracts were carboxylic acid, ester, fatty acid ester, phenol and steroid. These bioactive compounds had the potential in contributing to the inhibitory effect of papaya leaf extracts towards *G. boninense in vitro*. Future research can be conducted to identify the minimum fungicidal concentration of papaya leaf extract in inhibiting growth of *G. boninense*. The extracts can be further purified and re-tested with the pathogen.

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