POTENTIAL ANTIFUNGAL ACTIVITY OF *Dioscorea daemona* (Ubi Gadong) TUBER EXTRACT AGAINST *Pyricularia grisea* PATHOGEN OF RICE BLAST DISEASE

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Abstract

Many of plants in Malaysia potentially can be used as bio fungicides. *Dioscorea daemona* (Ubi Gadong) has been used in traditional herbal medicine and as decoction in various areas for decades. Tuber extracts of this plant have also been reported to possess antifungal, antibacterial and antioxidant activities. Tuber crude extracts of *D. daemona* was consecutively separated into hexane, chloroform and methanol and screened for antifungal activities in vitro against *Pyricularia grisea*, pathogen of rice blast disease using well diffusion method on Potato Dextrose Agar. Results of this study showed that the methanol crude extract of *D. daemona* exhibited a very strong inhibitory activity against *P. grisea* with diameter of inhibition zone by 1.5cm (62.5% inhibition) at concentration 150,000ppm. Antifungal activities of most effective extracts were supported by the presence of chemical constituents such as alkaloids, saponin, flavonoids, terpenoids and phenol that were responsible for the antifungal activity. It could be inferred that methanol tuber extracts of *D. daemona* at 150,000ppm concentration can serve as bio-fungicides against the growth of *P. grisea*.

Keywords: Antifungal activity*, Dioscorea daemona,* blast disease, *Pyricularia grisea*

Introduction

Rice (*Oryza sativa* L.) is important staple food and cash crop of the world as well as Malaysia. Unfortunately, rice plants in Malaysia being affected by rice blast disease caused by seed-borne fungus, *Pyricularia grisea* (Cooke.) Sacc. (Formerly known as *Pyricularia oryzae* Cavara.) (Rossman, et al. 1990) Anamorph of *Magnaporthe grisea* (Hebert) Brar (Babu, at al. 2011). *Pyricularia oryzae* (Hubert, et al. 2015) also known as *Magnaporthe grisea* (teleomorph) (Greer and Webster, 2001). Rice blast is the most destructive diseases in rice plantation instead of sheath blight, brown spot disease, bacteria blight and others. Generally, rice blast can cause the losses up to 90% from the rice production depend on the part of plant infection (Shahijahan, et al. 2010). Rice blast is cause by the seed borne fungus *P. grisea* which is the most aggressive and dangerous pathogen in rice world both in terms of its distribution and the damage it causes (Ou, 1980).

P. grisea can affect all part of rice plant such as node, collar, leaf, neck, part of panicle and sometimes leaf sheets. Leaf and panicle are the commonly part that are affected by rice blast fungus. Panicle infection causes the most important economic losses via reduction of yield compares to other part (Roumen, 1992). Rice blast pathogen are easy to spread in low soil

moisture area and prolong period of rain distribution and cool temperature during the daytimes.

The common control method to control this disease is chemical control (Oh, 1980; Anwar, et al. 2002; Gohel, et al. 2008). The regular control of rice blast disease is by using chemical fungicide such as Mencozeb, Carpropamid, Fenoxanil and Tadinilto (Pooja and Katoch, 2014). However, the repeated use of fungicides on crops may cause hazards to human being, develop resistance to fungicide, non-target effect and residual toxicity in plant parts.

On the other hand, Iftikhar *et al.,*(2010) and Babar *et al.,*(2011) proved that some bio-control agents and botanical fungicides were more secure and have no adverse effect on environment. *Trichoderma* spp. and *Bacillus substilis* reported to be effective as biological control agents of plants disease and known as antagonistic fungus are widely used in agriculture as biofungicides (Ali and Nadarajah, 2014). *Trichoderma* spp. inhibited the mycelial growth of rice blast fungus (Ouazzani, et al. 1998). Some studies pointed out that *Trichoderma* and *B. substilis* species are appealing candidates for control rice blast disease. *B. substilis* has being reported can control sheath blight and rice blast disease by individually or combination with other microorganisms such as *Trichoderma* spp. or chemical fungicides (Abeysinghe, 2012; Steindorff, et al. 2012; Ren, et al. 2008; Yang, et al. 2009).

Similarly, plant extracts like those that leaves extract of *Ocimum gratissimum, Chromolaena odorata, Cymbopogon citratus,* and seeds *of Eugenia aromatica, Piper guineense,* and nuts of *Garcinia kola* contain antifungal activity that able to inhibit the growth of *P. grisea* successfully (Olufolaji, et al. 2015). Mycelial growth and spore formation of rice blast fungus was also significantly reduced by acetone, methanol, distilled water and petroleum ether leaf extracts of C*hromolaena odorata* L. (Manjappa, 2013). The purpose of this study was to extract and screening the antifungal activity of *D. daemona* tuber against *P. grisea*.

Materials and Methods

Plant Materials

The tuber of *D. daemona* was collected from Kampung Belantek, Sik, Kedah, Malaysia. The tuber was cut into smaller size and dried at room temperature (20 ºC to 26ºC with an average of 23°C) for 1 month. The tuber was grinded using electric grinder to a powder form and keep in air tight container until required for extraction.

Preparation of Tuber Extracts

The dried fine powdered tuber of *D. daemona* sample about 1 kg was separately and sequentially extracted with hexane, chloroform and methanol at room temperature. The extracts were filtered through Whatman no. 1 filter paper and concentrated by rotary evaporator to give fifteen dark sticky semisolid extracts. The stock solution (200mg/ml) were serially diluted with the mixture dimethyl sulfoxide and methanol (6:4) to obtain desired concentration for test solution of 150, 100, 50, 10 mg/ml.

Preparation of Fungal Culture

The infected paddy plants were collected from the paddy field at IADA Pekan, Pahang. The symptom of rice blast disease can be seen on all part of rice plants including the leaf, neck, panicle, and stem node. *P. grisea* from diseased rice plant parts were inoculated. The plant sample was cut into small pieces (about 2 mm) and surface sterile in 10% Clorox then rinse twice with sterile distills water and blotted dry. The samples were then placed on the Potato Dextrose Agar (PDA) with flamed scalpel and then incubated in controlled environment for 12 hours at room temperature. Fungal was sub-cultured to get a pure culture. Pure culture was

maintained in Potato Dextrose Agar (PDA) until needed.

***In Vitro* Test**

The antifungal activities of *D. daemona* tuber extract were tested by using well diffusion method (Shinwari, et al. 2009). Two holes of 5 mm were then made at 2.5 cm from fungal plug in PDA plate by using sterile core borer. About 10 µl of plant extract was pipette into wells. The plates were then incubated at room temperature for 7 days incubation. The experiment was replicated five times for each treatment and the mean values taken. Fungitoxicity was recorded in terms of percentage colony inhibition and calculated according to the formula of (Pandey, et al.1982).

$$PIRG = \frac{Rc - Rt}{Rc} \times 100\%$$

Where,
PIRG : percentages of radial growth
Rt : radial growth of the fungal pathogen in the treatment
Rc : radial growth of the fungal pathogen in the control

Five concentrations (10 000, 50 000, 100 000, 150 000 and 200 000 ppm) of tuber crude extracts were prepared. Hexane and chloroform crude extracts were dissolved in 5 ml of methanol: dimethyl sulfoxide (DMSO) (4:6) while methanol crude extracts were dissolved in 100% methanol. Methanol: dimethyl sulfoxide (DMSO) (4:6) solvent was used as a negative control for hexane and chloroform crude extracts while methanol (100%) for methanol crude extracts. Mancozeb® fungicides served as the positive control treatment.

Microscopic Analysis

The fungal plug were taken at inhibitory zone and placed on the slides. A drop Lactophenol Cotton Blue (LCB) was placed on the fungal plug then covered with cover strip. The slides were then viewed under light microscope at 40 x magnification.

Phytochemical Analysis of Extract

Phytochemical analysis of the tuber of *D. daemona* extracts were conducted according to the methods by Harborne (1998) with slight modification. The analysis was conducted to screen the presence of active ingredient in the tuber extract.

Test for Alkaloids

Methanol was added to powdered tuber and evaporated to dryness in a boiling water bath. The residue was dissolved in 2 N HCL. The mixtures were filtered and the filtrate was treated with a few drop of Mayer's reagent. The formation of white precipitate showed the present of alkaloids (Harborne, 1998).

Test for Saponins

About 0.2 g of the tuber extracts of *D. daemona* was shaken with 5 ml of distilled water and then heated to boil. Saponins presence was observed there is appearance of creamy miss of small bubbles known as frothing (Egwaikhid and Gimba, 2007).

Test for Flavonoids

About 0.2 g of active tuber extracts of *D. daemona* was dissolve in diluted NaOH and HCL. A

yellow coloration was observed to indicate the present of flavonoids compound (Egwaikhid and Gimba, 2007).

Test for Tannins
Positive test for tannins was represented by the occurrence of the brownish green or a blue-black coloration. This coloration formed by the addition a few drop of $FeCl_3$ into the dried powdered tuber sample which was boiled in a test tube before the mixtures were filtered (Harborne, 1998).

Test for Glycosides
Coarsely powdered stem bark (1gm) was added into two separate beakers. To one of the beakers was added 5 ml of dilute sulphuric acid while 5ml of water was added to the other beaker. The two beakers were heated for 3– 5min and the contents filtered into labeled test tubes. The filtrate was made alkaline with 5%sodium hydroxide and heated with Fehling's solution for 3min. The presence of reddish precipitate in the acid filtrate and the absence of such precipitate in the aqueous filtrate were regarded as positive for glycosides (Harborne, 1998).

Test for Terpenoids
Plant extract was mixed with chloroform and sulfuric acid was slowly added to form a layer. A reddish brown coloration if the interface was formed to show the present of terpenoids (Harborne, 1998).

Test for Phenols
Ferric chloride solution was tested on the extract with 3 to 4 drops. The positive result of phenols was showed by the formation of bluish black color (Harborne, 1998).

Statistical Analysis
The antifungal activity was determined using a completely randomized design in five replicates with *D. daemona* type of extracts and concentration levels as factors. All data were subjected to analysis of variance (ANOVA) where significant ($P<0.001$) differences between means were determined by Turkey's standardized range test (HSD). MINITAB16 software used to perform all analysis.

Result and Discussion

In Vitro* Antifungal Screening of tuber extracts of *Dioscorea daemona* against *Pyricularia grisea
The radial growth inhibition of *P. grisea* pathogens of rice blast were measured and results showed that the methanol tuber crude extract effectively inhibited the growth of *P. grisea* compared to the hexane and chloroform tuber crude extracts (Table 1). Methanol gave 62.5% inhibition; chloroform gave 35.5% inhibition and hexane 35.0% inhibition at 150000 ppm. Result found that methanol extract showed greater inhibitory activity against tested pathogen meanwhile, hexane and chloroform extract showed slightly inhibitory activity toward the tested fungi. However, compare to positive control, methanol extract showed better inhibition where positive control gave 61.5% inhibition. Napisah, et al., (2011) reported that the ethanol tuber extract of *D. daemona* were effective at all concentration against *Staphylococcus aureus, Bacillus cereus, Pseudomonas aeruginosa* and *Candida albicans*. Result also indicated that, the antifungal activity of *D. daemona* tuber extract via stunted the growth of sporangia and hyphae.

Table 1 showed that hexane and chloroform tuber extract of *D. daemona* gave a slightly inhibitory activity toward the pathogen of rice blast, *P grisea*. Result found that *D daemona* tuber chloroform and hexane extracts not infected by *P grisea*. There is no significant different between all the treatments with different concentration either tuber chloroform and hexane extraction with negative control except tuber chloroform extract of *D daemona* at 10000 ppm. For methanol tuber extract of *D. daemona* showed inhibition in the range of 55 to 62 % for all concentrations. The results are not significantly different form positive control (61.5%) but is significant different from with negative control (17.5 %)l. *D daemona* tuber methanol extractions gave the best result as inhibit the radial growth of *P grisea*. Result found that the highest percentage of inhibitory activity is 62.5% at 150000 ppm while the lowest percentage of inhibitory activity is 55.5% at 50000 ppm. The minimal inhibitory concentration of methanol tuber crude extract was at 150,000 ppm. The result also showed that only methanol tuber extract of *D. daemona* had the ability to inhibit the growth of radial align with the study by Arifullah *et al.* (2014)

Table 1. The percentage of inhibition of *D. daemona* tuber extracts against *P. oryzae*

Concentration (ppm)	Hexane	Chloroform	Methanol
10 000	20.5% a	45.5% b	59.0% b
50 000	45.0% bc	32.5% a	55.5% b
100 000	32.0% ab	34.0% ab	57.0% b
150 000	35.0% ab	35.5% ab	62.5% b
200 000	40.5% abc	35.0% ab	59.5% b
Positive control	61.5% c	61.5% c	61.5% b
Negative control	17.5% ab	17.5% a	17.5% a

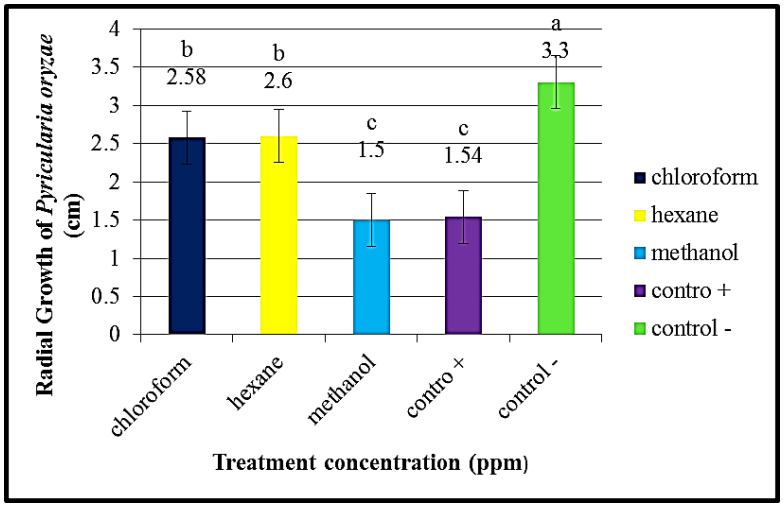

Figure 1 Comparison of solvent extractions of same level of concentration with different type of solvent extraction of hexane, chloroform, methanol negative control and positive extract

Microscopic observation of antifungal activity

By using Digital Light Microscope, the effect of *D. daemona* tuber extract against *P. grisea*

was observed. Visual observation of antifungal activity was showed the formation of inhibition zone, which limits the growth of fungi. Results showed the difference between the treated and untreated mycelia (control). Comparison between treated *P. grisea* and control negative were observed. Result found some alteration on the hyphae and sporangia of *P. grisea*. Antifungal activities of active tuber extracts of *D. daemona* tuber against *P. grisea* were via hyphal retardation, lysis, burst and disruption (Figure 2).

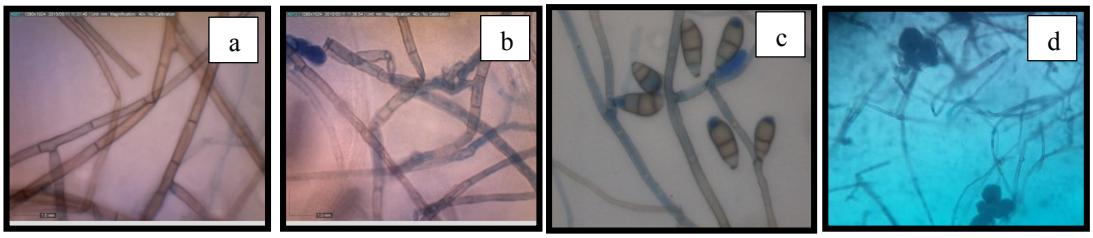

Figure 2 Microscopic observation of antifungal activity of *Dioscorea daemona* tuber extracts against rice blast disease under 40 x magnifications. a) Healthy hyphae of *P grisea* b) Stunted hyphae of *P grisea* c) Healthy sporangia of *P grisea* d) Stunted sporangia of *P grisea*

Figure 2 showed that the mycelia treated with the crude extracts (b and d) had an altered morphology that was shorter, retarded, lysed and ruptured compared to control (a and c). The mycelia in the treatments also grew much slower than those of control. The comparison between control and treated (methanol tuber extracts of *D. daemona*) hyphae showed smooth walls and straight-shape in *P grisea* normal hyphae in microscope observation. However, hyphae which were treated with methanol tuber extracts of *D. daemona* were found with rough, abnormal, irregular shape and granular like surface. Semagun, (2006) reported that several mode of action may have resulted from antifungal substances from plants including responses such as degradation of cell wall, alteration of cell permeability, inhibition of enzymatic activities in the fungal cells that is turned off the cell permeability. The surface tension of cell membrane is reduced, which resulted in cell lysis. As a consequence, the growth of a fungus is suppressed (Rachmawai and Karlina, 2009).

Phytochemical analysis on active crude extracts

Phytochemical analysis of the tuber extracts was conducted according to the methods by Harborne (1998). The phytochemicals analyzed the presence of alkaloids, saponin, flavonoids, tannins, glycosides, triterpenoids and phenol. Phytochemical analysis, found that the methanol tuber crude extracts of *D. daemona* were contained alkaloid, saponin and some flavonoid, phenol and terpenoids that were able to inhibit the growth of *P. oryzae* that contributed to antifungal activity. Previous studies by Liang, (2015) stated that *D. daemona* contained saponin glycoside and diosgenin as their major active compund.

Table 2. *Phytochemical analysis test of Dioscorea daemona tubers extract*

	Hexane	**Chloroform**	**Methanol**
Alkaloids	-	-	+
Saponin	-	-	+
Flavonoids	-	-	+
Tannins	-	-	-

Glycosides	-	-	-
Terpenoids	-	-	+
Phenol	-	-	+

Key:
- Absent
+ Present

Conclusion

Result from the study indicated that methanol extract of *D. daemona* was found to have an active antifungal activity to control the growth of *P. oryzae*. The methanol extract gave 62.5% radial growth inhibition meanwhile hexane and chloroform gave 0%. Result also indicated that, the antifungal activity of *D. daemona* tuber extract via stunted the growth of sporangia and hyphae. The phytochemical analysis, result found that the *D. daemona* tuber extract contain of alkaloid, saponin and little of flavonoid, phenol and terpenoids those are able to give minimal inhibition growth of *P. oryzae* that contributed to antifungal activity.

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Conflict of interest

This research has not been submitted for publication nor has it been published in whole or in part elsewhere. We attest to the fact that all Authors listed on the title page have contributed significantly to the work, have read the manuscript, attest to the validity and legitimacy of the data and its interpretation, and agree to its submission to the Journal Gading.

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