Evaluation of selected herbs for biocontrol of Rice Blast Disease

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Abstract. Rice blast disease caused by Pyricularia oryzae is the most destructive disease of rice worldwide. It kills seedlings or plants up to the tillering stage. The most common practice controlling method is using chemical fungicide, however there are several negative impact of using synthetic chemicals to the human consumption as well as to the environment. In order to solve this problem, this study was conducted to find other alternative methods to control blast disease by using plant extracts from family Zingiberaceae which are Alpinia galanga, Curcuma longa and Zingiber officinale. Screening for antifungal activities of these plant extracts were done in vitro on Potato Dextrose Agar (PDA). After 7 days of treatment, result showed that crude extracts of Alpinia galanga hexane crude extract exhibited strong inhibitory against Pyricularia oryzae with the highest percentage of inhibition 52.9%, followed by Curcuma longa hexane crude extract with 49.1% and Zingiber officinale methanol crude extract with 43.5% inhibition. The antifungal activities may be due to the presence of some chemical compounds such as alkaloids, saponins, tannins, phenols, glycosides, flavonoids and terpenes that been proved thru phytochemical test. However, further trial such as field trial is necessary to be done to evaluate the effectiveness of these extracts to control rice blast disease under field condition.

1. Introduction
Rice are most important staple food for half of the world's population, especially in Asia. But, the production of rice begins to decrease due to the occurrence of rice blast disease which caused by Pyricularia oryzae. Once infected, the disease can cause either leaf blast or neck blast. This type of fungus can infect rice at different growth stage, including grain [1]. In Malaysia alone, the estimated yield loss due to rice blast disease is about 90 000 tan per season which equal to RM 72 million [2]. The most common controlling measures to this disease via chemical control. However, there are some negative impact faced such as chemical residues, air pollution, non-target effect and development of pathogen resistance. In order to reduce the dependency on synthetic chemicals, researchers conducted various experiment to find alternative controlling methods such as using natural products derived from plant. It is because application of naturally derived products is more environmentally friendly and cost effective. The botanical fungicides are safer because it contained various active compounds such as alkaloids, saponin, flavonoids, phenols, tannins and glycosides that are biodegradable and no residue in
the environment [3]. Several researchers have successfully reported the effective control of rice diseases using plant extracts [4][5][6]. Wilart, et al., [7] reported that hexane, ethyl acetate, acetone or methanol extract of the rhizome of *Alpinia galanga* shows the anti- Phytophthora capsici activities. Moreover, Yulia et al., [8] stated that water and ethanol extracts from galangal (*Alpinia galanga* L. Willd.) rhizomes are more efficient in inhibiting spore germination of *Colletotrichum gloeosporioides* isolated from black pepper. Uda, et al [9] reported that the extracts from *Aloe vera, Citrus hystrix, Sabah Snake Grass and Zingiber officinale* potentially to inhibit the growth of *Pyricularia oryzae in vitro*. Hence, this study was conducted to evaluate the antifungal activity of *Alpinia galanga, Curcuma longa* and *Zingiber officinale* crude extracts against *Pyricularia oryzae*, pathogen of rice blast disease.

2. Material and methods

2.1. Extraction of plant samples

Plant materials (rhizomes) were collected and inspected for any disease infections before cleaned thoroughly using tap water to remove all the dirt and sliced thinly. They were dried at room temperature for one week before finely ground into powder using an electric blender. Methods of extraction were followed by method highlighted [10]. The sequential extraction started with 200 g of dried sample powder soaked in hexane for 48 hours and filtered through Whatman No. 1 filter paper and the filtrate were dried using rotary evaporator (HANH VAPOR) in the 70°C water bath for 30 minutes to yield the pure hexane extract. The residue was then soaked again with chloroform and methanol sequentially and followed the same procedure.

2.2. Isolation of *Pyricularia oryzae*

Infected paddy was collected from rice field at Pahang Tua, Pekan, Pahang and brought back to the laboratory for isolation according to method highlighted [11]. The infected tissue was cut into small pieces and soaked in 10% sodium hypochlorite for surface sterile for 1-3 minutes. Then, rinse 3 times with sterile distilled water and blotted dry. The plant samples were then transferred into PDA plate incubated for 48 hours at 27°C. The fungal pathogen was sub-culture until pure culture were obtained and undergo Koch’s postulates before been observed under light microscope for identification and characterization of the pathogen.

2.3. Phytochemical screening

Phytochemical screening was conducted on each of plant extracts to determine the presence of flavonoids, alkaloids, saponin, tannins, phenolics, glycosides, triterpenoids, steroids according procedure that been highlighted [12][13].

2.3.1. Flavonoids

About 2 ml of crude extracts was treated with 1 ml of methanol solution. Then, the solution was warmed using double boiled and metal magnesium was added. Then 5-6 drops of concentrated hydrochloric acid (HCl) were added. Color changes of orange were observed for the presence of flavonoid

2.3.2. Alkaloids

About 2 ml of crude extracts was boiled with 2 ml of 2% HCl on a steam bath. The mixture was filtered and treated with 3-4 drops of Dragendorff’s reagent. Orange to red precipitate indicated the presence of alkaloids.

2.3.3. Saponin

About 0.4 g of crude extracts was dissolved in 5 ml of distilled water and shaken vigorously till a stable persistent froth was obtained. The froth was mixed with 3 drops of olive oil and shaken vigorously and then observed for emulsion.

2.3.4. Tannin
A few drops of 10% of lead acetate were added in 2 ml of crude extracts. White precipitate indicated the presence of tannins.

2.3.5. Phenol
About 1 ml of crude extracts was treated with 5 % of ferric chloride. Formation of deep blue or black color precipitation indicated the presence of phenols in the compound.

2.3.6. Steroid and Triterpenoids
About 5 ml of chloroform was added in 1 ml of plant extract followed by the addition of 0.5 ml of acetic anhydride and 1 ml of concentrated Sulphuric Acid. The present of blue green color at the junction indicated the presence of steroids while appearance of red, pink or violet color at the junction indicated the presence of triterpenoid.

2.3.7. Glycosides
About 1 ml of crude extracts was treated with a few drops of glacial acetic acid and ferric chloride. Then, 3-4 drops of concentrated Sulphuric Acid were added along the sides of the test tube. Presence of glycosides was indicated by formation of blue-green color.

2.4. In vitro antifungal activity screening of plant extracts against Pyricularia oryzae
The antifungal activity of plant extracts was determined by well diffusion method [14]. A plug of mycelia from 14-day-old pure cultures of P. oryzae was made using 5 mm diameter of sterile cork borer and placed at the center of PDA plate. Then, 8 mm diameter wells were made 2.5 cm away from the center of the plate with sterile cork borer. Each of the well were filled with 10 µl of each of plant crude extracts. For negative controls, the well was filled with solvents used for extraction meanwhile for positive control, the well was filled with fungicide. All the plates were incubated at 28°C for 72 hours and diameter growth colony was measured daily for 7 days incubation. The percentage of growth inhibition of P. oryzae were calculated [15].

2.5. Determination of effective inhibitory concentration (EIC) of plant extracts
The effective inhibitory concentration of A. galangal, C. longa and Z. officinale crude extracts that exhibited antifungal activity was determined by using well diffusion method using Potato Dextrose Agar (PDA). Two holes of 5 mm were made 2.5 cm away from fungal culture using sterile cork borer. About 10µl of plant extracts at different concentration of 50,000 ppm, 100,000 ppm, 150,000 ppm, 200,000 ppm and 250,000 ppm were pipette into the wells. The radial growth of fungal pathogen measured daily for 7 days incubation and percentages of radial growth inhibition were calculated [15].

2.6. Microscopic observation of antifungal activity of active plant extracts
The mechanism of antifungal activity of A. galangal, C. longa and Z. officinale crude extracts against P. oryzae was evaluated microscopically. The effects of each crude extracts against hyphae and spores were observed under light microscope. The slides were viewed under 40 x magnification and compared to the healthy (control) hyphae and conidia for comparison of changes and effects of the crude extracts.

3. Results and Discussion
3.1. Isolation of Pyricularia oryzae
The study was able to isolate P. oryzae, the fungal pathogen of rice blast disease. The identification of P. oryzae was made based on the morphological characteristics. The culture was matured after 14 days of incubation and grow fully in PDA after 7 days. After 21 days incubation, the perithecia was appeared. All the pure culture obtained shows good mycelial growth with smooth colony margin [16]. P. oryzae were identified based on three-celled conidia which are pale brown to hyaline and pyriform (pear-like) in shape. The shape of conidia is pyriform with base rounded, apex narrowed, 2-4 celled, 2-3 septate and middle cells were broader than others (Figure 1b and 1c) [16]. The perithecia have long neck and
cylindrical ascis with aligned [17]. The colony color of \textit{P. oryzae} are varies from white, light gray or dark gray and will turn grayish to black depending on media used (Figure 1a). [18] [19]. Under the microscope, the conidia are brown in color and transparent.

![Figure 1.](image)

**Figure 1.** (a) Cultured \textit{P. oryzae} on PDA, (b) \textit{P. oryzae} colony under microscope with 10x magnification (c) \textit{P. oryzae} conidia under microscope with 40x magnification.

3.2. **Phytochemical screening**

Phytochemical screening revealed that \textit{A. galangal}, \textit{C. longa} and \textit{Z. officinale} crude extracts showed the presence of alkaloids, tannins, steroid, triterpenoids, saponins, glycosides, phenols and flavonoids as shown in Table 1.

| Phytochemical test | \textit{Alpinia galangal} | \textit{Curcuma longa} | \textit{Zingiber officinale} |
|--------------------|---------------------------|------------------------|-------------------------------|
| Flavonoids         | +                         | +                      | +                             |
| Alkaloids          | +                         | +                      | +                             |
| Saponins           | +                         | +                      | +                             |
| Tannins            | +                         | +                      | +                             |
| Phenols            | +                         | +                      | +                             |
| Steroids           | +                         | +                      | +                             |
| Triterpenoids      | +                         | +                      | +                             |
| Glycosides         | +                         | +                      | +                             |

(+) Present (−) Absent

The presence of these active compounds indicated that these plants potentially can be used as source of phytochemical for antifungal activity. Result from Table 1 showed that \textit{A. galangal} crude extract contained alkaloids and tannins. Alkaloids and tannin are widely known for their pharmacological properties and possessed antibacterial and antiviral properties [20]. Presence of phenols and Saponin in \textit{C. longa} crude extracts contributed to its antifungal activities [3]. Saponin act as an important precursor for steroidal substances which have been proved to have a wide range of pharmacological activities [20]. Furthermore, inhibition growth response of \textit{P. oryzae} to \textit{Z. officinale} crude extract may be contributed by flavonoids. Flavonoids work by inhibiting the synthesis of nucleic acids of fungi and caused the cell membranes became unstable due to the changes of cell membrane permeability that may cause the exchange of fluid in the cell [20]. \textit{Z. officinale} crude extract also showed to contained saponins and tannins. Saponin works as an antifungal agent by interfering with the permeability of the fungal cell wall. Moreover, saponins also naturally occurring glycosides that showed cytotoxic activity [22].

3.3. **In vitro antifungal screening of plant extracts against Pyricularia oryzae**

Result from the study (Figure 2) showed that, the highest percentage inhibition of fungal pathogen radial growth was showed by \textit{A. galangal} hexane crude extract with 52.9% inhibition followed by \textit{C. longa}
hexane crude extract (49.2% inhibition), Z. officinale methanol crude extract (43.5% inhibition) and Z. officinale hexane extract (42.1% inhibition). Meanwhile, A. galangal chloroform crude extract, C. longa methanol and chloroform crude extract, Z. officinale chloroform crude extract and A. galangal methanol crude extract gave 38.1%, 32.8%, 27.6%, 27.1% and 23.3% growth inhibition respectively. Comparison the treatments with positive control (fungicide) showed that fungicide act better antifungal activity in inhibiting the radial growth of P. oryzae with 54.6% growth inhibition.

![Figure 2](image)

**Figure 2.** Antifungal activity screening of Alpinia galangal, Curcuma longa and Zingiber officinale crude extracts against Pyricularia oryzae.

Various researches have been done to evaluate the antifungal activities of A. galangal, C. longa and Z. officinale crude extracts against various fungi and bacteria. Many reports showed that A. galangal has numerous biological activities. Result from the previous research stated that hexane, ethyl acetate, acetone and methanol extract of A. galangal shows antifungal activity against Phytophthora capsici [23]. Moreover, application of A. galangal extracts on fungal pathogen can inhibit the spore formation and the growth of hyphae of P. oryzae [24]. In addition, ethanolic extract of A. galangal was also exhibited promising antifungal activity against phytopathogen that infected fruits and vegetables such as Rhizopus stolonifer, Colletotrichum musae, Fusarium oxysporum and Aspergillus niger which can potentially be used as fungicides in agriculture [25]. The result of this study also showed that C. longa hexane extract had the ability to inhibit the radial growth of P. oryzae where it been reported that ethanol and hexane extracts of C. longa showed significant antifungal activities against ten different pathogenic fungi including Botrytis cinerea, Chaetomium olivaceum, Fusarium graminearum, and Magnaporthe grisea by interfering with the development of mycelia [26]. The antifungal activity of Z. officinale hexane extract was slightly inhibiting the radial growth of P. oryzae. However, some studies reported that Z. officinale crude extract was proven effective against some phytopathogenic fungi such as Pythophthora infestans, Fusarium solani and Pyricularia oryzae [27].

### 3.4. Determination of effective inhibitory concentration (EIC) of plant extracts

Among the all the crude extracts of A. galangal, C. longa and Z. officinale tested against P. oryzae, the A. galangal hexane crude extract expressing the highest reduction in mycelial growth of pathogen by 52.9% growth inhibition. However, the EIC values for all the active crude extracts (A. galangal and C. longa hexane crude extract, Z. officinale methanol crude extract) were at 250,000 ppm. Effective inhibitory concentration is the effective concentration of the plant extracts that able to inhibit the growth of the fungal pathogen at 50% inhibition. Kim et al [28] reported that 1000 mg/L of ethyl acetate extract of C. longa able to inhibit the growth of R. solani, P. infestans, Puccinia recondita, and Botrytis cinerea. Meanwhile, Chowdhury, et. al. [29] revealed that C. longa extract and curcumin oil showed antifungal activities against Fusarium solani and Helminthosporium oryzae at 19.73 g/ml and 12.7 g/ml,
respectively.

**Table 2. Effective Inhibitory Concentration (EIC) of A. galangal, C. longa and Z. officinale crude extracts against *P. oryzae***

| Plants  | Type of solvent extracts | Percentage of radial growth inhibition (%) | Concentration (ppm) |
|---------|--------------------------|-------------------------------------------|---------------------|
|         |                          |                                           | 50,000   | 100,000 | 150,000 | 200,000 | 250,000 |
| Galangal| Hexane                   |                                           | 32.43    | 33.30   | 33.30   | 42.05   | **52.95** |
|         | Chloroform               |                                           | 16.20    | 21.77   | 31.02   | 20.15   | 36.61   |
|         | Methanol                 |                                           | 11.32    | 10.12   | 6.39    | 19.96   | 23.20   |
|         | Hexane                   |                                           | 36.62    | 39.54   | 38.71   | 44.97   | **49.11** |
| Turmeric| Chloroform               |                                           | 13.01    | 11.92   | 36.43   | 31.41   | 27.21   |
|         | Methanol                 |                                           | 31.56    | 30.81   | 30.01   | 36.80   | 32.55   |
|         | Hexane                   |                                           | 34.96    | 36.64   | 31.68   | 34.12   | 42.04   |
| Ginger  | Chloroform               |                                           | 35.78    | 15.66   | 37.83   | 35.47   | 25.08   |
|         | Methanol                 |                                           | 29.15    | 22.72   | 28.76   | 30.85   | **43.14** |

*The bold number is the value of highest percentage of radial growth inhibition.

Overall, difference of antifungal activities of each extract may be due to the higher concentration or activity from the active compound in the crude extracts. If the amount of active compound in the crude extract very high, and highly active, it will show high activity even at very low concentration. But if the active compound in crude extract very low and highly active, it will show positive activity at an increasing concentration. As the concentration increased, the inhibition of fungal pathogen also increased gradually. In this study, increasing concentration of *A. galangal, C. longa* and *Z. officinale* crude extracts, the higher the inhibition of radial growth of *P. oryzae*.

**3.5. Microscopic observation of antifungal activity of active plant extracts**

The effects of *A. galangal, C. longa* and *Z. officinale* active crude extracts against the growth of *P. oryzae* hyphae and conidia were observed using Digital Light Microscope. The mycelium was taken from the inhibition zone area. From the result, it showed clearly the differences between treated and untreated mycelia. The treated mycelia have abnormalities, rough, irregular shape and granular like surface. It also being retarded (slower growth), lysis and ruptured compared to untreated mycelia (Figure 3c) where, they have smooth like surface and straight shape (Figure 3a). Furthermore, the treated spores showed stunted growth, abnormalities, irregular shape and ruptured (Figure 3d) compared to untreated spores which have clear pyriform shape and 2-3 septate (Figure 3b). The lysis of hyphae and conidia of *P. oryzae* caused by active substances in *A. galangal* hexane crude extract which showed the best inhibition of radial growth of *P. oryzae* with 52.9% inhibition.
From Figure 3, slow growth of mycelial of *P. oryzae* after being treated may be attributed by active compound such as alkaloids, glycosides, tannins and phenols where the compound inhibits the mycelium growth of *P. oryzae*. Taiga and Friday [21] reported that alkaloid and flavonoids obtained from *Aloe vera, Azadirachta indica* and *Nicotiana tabacum* inhibited mycelial growth, spore germination and germ tube elongation of *P. oryzae*. It also been reported that crude extract of *Epicoccum* sp. could destroyed the hyphae and spores of *Magnaporthe oryzae* (*P. oryzae*) [34]. In addition, ruptured and lysis of *P. oryzae* hyphae may be due to presence of phenols in the crude extracts that able to suppress growth of hyphae by mechanism such as the alteration of cell permeability, degradation of cell wall, inhibition of enzymatic activities in the fungal cells [3]. Present study showed that crude extracts of *A. galangal, C. longa* and *Z. officinale* showed a remarkable antifungal activity against *P. oryzae* through lysis of mycelial cells of *P. oryzae* suggested that this extracts potentially can be used as bio-fungicides to control rice blast disease.

4. Conclusion

*Alpinia galangal, Curcuma longa* and *Zingiber officinale* have been widely used to treat many diseases due to the presence of the bioactive compounds that can act as antifungal activity. In this study, all plant extracts showed a positive antifungal activity against *Pyricularia oryzae*, pathogen of Rice Blast disease under *in vitro* condition on PDA medium. However, the best treatment was showed by *A. galangal* hexane crude extract at 250,000 ppm concentration that gave 52.9% of growth inhibition and the extract significantly inhibited the growth of *P. oryzae*. The extract also able to caused lysis, ruptured and stunted growth of both hyphae and spores of *P. oryzae*. The antifungal activities may be due to the presence of various bioactive compounds in the plant itself. However, it is important to conduct field research in order to evaluate the effectiveness of the extracts to control rice blast disease in field condition and to do chemical analysis using Gas Chromatography-Mass Spectrometry (GC-MS) to confirm the exact compound that present in each of plant crude extracts that act as antifungal agent.

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