



Antifungal properties of *Mimusops elengi* seeds against paddy seed-borne fungi and selected pathogenic fungi

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ABSTRACT

Aims: The ever-revolving fungi strains and environmental and health concerns due to current practice of synthetic pesticide in agricultural fields have encourages more ventures into bio-pesticides research. *Mimusops elengi*, a widely available endogenous plant in tropical countries and most parts of this plant have been proven to possess medicinal and antimicrobial potential. In this study, *M. elengi* seeds crude extracts are tested for their antifungal activities on paddy seed-borne and pathogenic fungi.

Methodology and results: The dried and grinded *M. elengi* seeds are macerated separately using water, methanol, ethyl acetate, dichloromethane and petroleum ether as extraction medium. Crude extract of each solvent was used on paddy seed surface treatment to determine their antifungal inhibition potential on seed-borne fungi and paddy grain germination. Synthetic fungicide mancozeb and thiram are also tested as comparisons to the performance of plant extracts. Water and methanol extracts exerted the best fungal inhibiting and grain germination results from the five crude extracts tested and qualitative phytochemical screening reveals both extracts contained the most number of phytoconstituents including saponin, flavonoids, alkaloids, tannins and phenolic. Water extract, methanol extracts and synthetic fungicides are then subjected to in-vitro bioassay to observe their effect on mycelial growth of several fungi strains pathogenic to paddy namely, *Fusarium fujikuroi*, *Curvularia aerea*, *C. lunata* and *C. eragrostidis*. Water and methanol extracts showed a very similar trend of inhibition on all four fungi strains tested with best percentage of inhibition on mycelia growth of *C. eragrostidis* followed by *C. aerea*, *C. lunata* and least effective on *F. fujikuroi*. Further separation of crude extract need to be done to isolate the specific acting compounds contributing to fungal growth inhibition.

Conclusion, significance and impact of study: Both water and methanol extracts of the seeds contain promising antifungal properties on seed borne fungi which is as good as the synthetic fungicides compared in this study. A broad range of active phytochemical properties it possesses may be the contributing factor for the fungal growth inhibition. This preliminary screening could narrow down the potential of this seed extracts as natural antifungal agents and the acting active compounds.

Keywords: *Mimusops elengi*, antifungal, bio-pesticide, paddy fungi

INTRODUCTION

Rice (*Oryzae sativa*) is a staple food hence extensively cultivated in the Asian region. In Malaysia alone paddy fields covers about 690,000 hectares and produces 1.8 million tonnes of rice annually (Wahab, 2016). Paddy like other cultivated plants faces the same challenges when comes to diseases and infection caused by pest, fungi, bacteria, virus and mycoplasma pathogens (Gnanamanickam, 2009). According to (Agrawal, 1999), more than 50 pathogenic seed borne fungi species from paddy have been reported and some of the most common species found in paddy across the globe includes *Fusarium*, *Aspergillus*, *Cercospora*, *Pyricularia*, *Alternaria*, *Curvularia*, *Rhizoctonia* and *Trichoderma* causing harvest

infection, seed rot, seed necrosis, loss of germination capability, loss of grain nutritional value and seedling damage (Janardhana *et al.*, 1998).

Treatment directly on seeds is the most effective and economical approach in controlling seed-borne fungal diseases and maintaining quality of grains (Chandler, 2005). Synthetic pesticides which are largely used by paddy farmers in Malaysia include mancozeb, maneb, mathalaxyl, thiram and azoxystrobin. Evolution and development of resistance of microorganism towards certain drug over time forces farmers to increase dosage, alternate to more prevalent pesticides and uses concoction of pesticides to protect their crops. Even though synthetic pesticides could efficiently control fungal growth on grains but they should not be applied on grains

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for they might cause pesticides toxicity directly to consumers and active continuous use of chemical fungicides could cause serious damage to the environment and other non-targeted organisms (Margni *et al.*, 2002). Hence, to overcome those issues, continuous search for new and safer antimicrobial drugs or biopesticides should be done to ensure eco-friendly pest control practices.

A wide range of plants and their derivatives have been used for their medicinal, pharmaceutical and therapeutic benefits. Plant metabolites as fungicides could be an alternative practice compared to common synthetic fungicide used. Extracts from plants such as *Syzygium aromaticum*, *Emblica officinalis* Gaertn., *Allium sativum* L. (Sehajpal *et al.*, 2009), *Azadirachta indica* (neem), *Persea americana* (pear) (Jagessar *et al.*, 2015), *H. anthelminthicus*, *X. lanceolatum* and *C. sappan* (Jantasorn *et al.*, 2016) have been reported to exhibit antifungal effect in laboratory studies against paddy and other grains. *Mimusops elengi* plant is an evergreen tree usually cultivated as decorative or shade tree. Its seeds have been traditionally used as remedy for piles, headache, constipation and spermicidal (Gopalkrishnan and Shimpi, 2010). Barks and leaves of this plant have also been proven to exhibit significant antimicrobial characteristics towards several species of bacteria and fungi (Ali *et al.*, 2008).

Therefore, in this study, antimicrobial potential of the extracts of *M. elengi* seeds is screened *in vitro* on its ability to inhibit seed-borne fungal growth on paddy grains and its effects on the paddy grains germination. Phytochemicals of the plants are qualitatively screened to preliminarily ascertain phytoconstituents from *M. elengi* seeds which are responsible in antifungal activities. Extracts with higher potential of antifungal properties are further tested on growth of fungi strains pathogenic to paddy namely, *Fusarium fujikuroi*, *Curvularia aeria*, *C. lunata* and *C. eragrostidis*.

MATERIALS AND METHODS

Collection of paddy grains samples

Paddy grains samples were collected from paddy fields during harvest season and rice mills between 9th to 12th January of year 2015 and 2016 from region of Pulau Pinang and Kedah, Malaysia. Collected samples were bagged in sterile plastic container and kept in 4 °C refrigeration.

Preparation and extraction of *Mimusops elengi* seeds

Ripe fresh fruits of *M. elengi* (Sapotaceae) were collected from its trees growing in Esplanade Bay, Pulau Pinang. Seeds were separated from flesh, washed thoroughly with distilled water, dried in dryer at 40 °C for a week, grinded and readied for solvent extraction process. The grinded seeds are macerated separately using water, methanol, ethyl acetate, dichloromethane and petroleum ether as extraction medium at 1:20 seed to solvent ratio for three

days. The extracts were filtered, concentrated of their solvent and preserved at 4°C in air tight dark bottle till further use.

Germination test

In vitro antifungal activity assay were performed on water, methanol, ethyl acetate, dichloromethane and petroleum ether extract of *M. elengi* seeds against fungal growth on paddy grains using seed surface treatment and direct plating method. The concentrated extracts were dissolved into different vials to 2500 ppm concentration using respective solvents for antifungal test. Conventional pesticide thiram and mancozeb were prepared in aqueous according to its recommended concentration 2000 ppm and 2500 ppm respectively to be used as comparisons with the botanical extracts. Collected paddy grains were surface disinfected with 2% sodium hypochlorite solution for two min and rinsed twice with distilled water to eliminate surface contamination during collection and transference. Surface disinfected grains were then soaked with *M. elengi* water extract for 2 min and then removed and solvents were allowed to dry then kept in plastic container to prevent infestation during observation of the effect of treatment on the paddy grains for 90days. Seeds were then tested for their germination using blotting paper method (Tournas *et al.*, 2001). A total of 25 seeds from each treatment were placed onto a Petri dish containing water soaked blotting paper with four replicates for each treatment. Treatment are repeated with methanol, ethyl acetate, dichloromethane and petroleum ether botanical extracts as well as synthetic fungicide thiram and mancozeb. Percentage of germination of the grains were determined after 10 days.

Phytochemical screening of *Mimusops elengi* seeds

Standard laboratory method was used for qualitative phytochemical screening on each of the *M. elengi* seeds extracts. Phytochemical properties screened includes carbohydrate, reducing sugar, saponin, flavonoids, alkaloids, terpenes, tannins, phenol and glycosides. Presence of carbohydrates and reducing sugar were tested using Molisch's test and Benedict's test respectively. Saponin were tested using Frothing test while flavonoids with Shinoda Test. Dragendorff's test were used to confirm presence of alkaloids while Liebermann-Burchard test for triterpenoids. Both tannins and phenol were tested for presence using ferric chlorides test whereas Kellar-Kiliani test confirm presence of cardiac glycosides. These methods allow phytochemical properties contained in the extracts to be known qualitatively through observation of colour changes that occurred to the extract solutions during tests (Tiwari *et al.*, 2011).

Antifungal bioassay

Potato dextrose agar (PDA) medium with 50 ppm, 150 ppm, 200 ppm and 500 ppm of methanol and water

extracts of *M. elengi* seeds and synthetic fungicide Thiram and Mancozeb were prepared. PDA medium infused with only methanol served as control since methanol were used as the main solvent to dilute the extracts to their intended concentrations. Five mm disc containing single spore of 48 h culture of test fungi were placed at the center of the petri dish, sealed and incubated at 25±2 °C. Pathogenic fungi strains tested includes *F. fujikuroi* (obtained from the School of Biological Sciences, Universiti Sains Malaysia), *C. aerea*, *C. lunata* and *C. eragrostidis* (obtained from the Faculty of Science, Universiti Putra Malaysia). Three replications were done for each set of treatment. Diameter of fungi were measured after 7 days of incubation. The fungi toxicity of the extracts was represented as percentage of inhibition of the mycelia growth, calculated using equation:

$$\text{Percentage inhibition, \%} = \left(\frac{Dc - Dt}{Dc} \right) \times 100\%$$

Where Dc= Average diameter in control, Dt= Average diameter in treatment.

Solvents extracts were prepared by dissolving 0.02 g, 0.06 g, 0.10 g and 0.20 g of dried methanol and water extracts were dissolved in 10mL of methanol. 500 µL of each solvent extract were amended with 20 mL of PDA medium before solidification to produce extracts-amended

PDA with concentration of 50 ppm, 150 ppm, 200 ppm and 500 ppm.

RESULTS AND DISCUSSION

Results of different solvent extracts of *Mimusops elengi* seeds and synthetic fungicide mancozeb and thiram treatment on germination and fungal inhibition on paddy grains are presented in Table 1. These tests revealed that germination percentage and fungal inhibition on paddy grains differed with different solvents extracts and fungicide treatment. Water extract showed highest percentage of total germination at 90% followed by methanol extract (74%), ethyl acetate extract (66%), petroleum ether extract (66%) and lastly dichloromethane (58%). Highest inhibition of fungal growth on paddy grains were observed for methanol and ethyl acetate extracts at 70%. Highest healthy percentage of healthy seedlings where grains germinated and were not infected were methanol treated grains (54%) with longest shoots and roots average length. This were followed by water extract (52%), ethyl acetate extract (46%) and dichloromethane and petroleum ether extracts (30%). Grains treated with synthetic fungicide mancozeb showed 88% of total germination and produced 56% of healthy seedlings while thiram had similar total germination as methanol extract (74%) however only produced 38% of healthy seedlings and had the shortest shoots and roots average length (Figure 1).

Table 1: Effect of different solvents extracts of *Mimusops elengi* seeds on germination and fungi inhibition of paddy grain.

Treatment	Germinated					Non-Germinated			Total Germination (%)	Total Inhibition (%)
	Inhibited (%)	Shoot length (cm)	Root length (cm)	Infected (%)	Shoot length (cm)	Root length (cm)	Inhibited (%)	Infected (%)		
Water extract	52	6.0	7.2	38	4.7	6.7	4	6	90	56
Methanol extract	54	6.1	7.4	20	5.8	6.8	16	10	74	70
Ethyl-acetate extract	46	5.6	5.7	20	6.0	5.2	24	10	66	70
Dichloro-methane extract	30	5.7	6.0	28	4.2	4.8	26	16	58	56
Petroleum ether extract	30	5.7	6.7	36	4.7	4.9	28	6	66	58
Fungicide mancozeb	56	5.9	6.3	32	5.4	6.1	6	6	88	62
Fungicide thiram	38	4.8	4.5	36	4.1	3.9	12	14	74	50

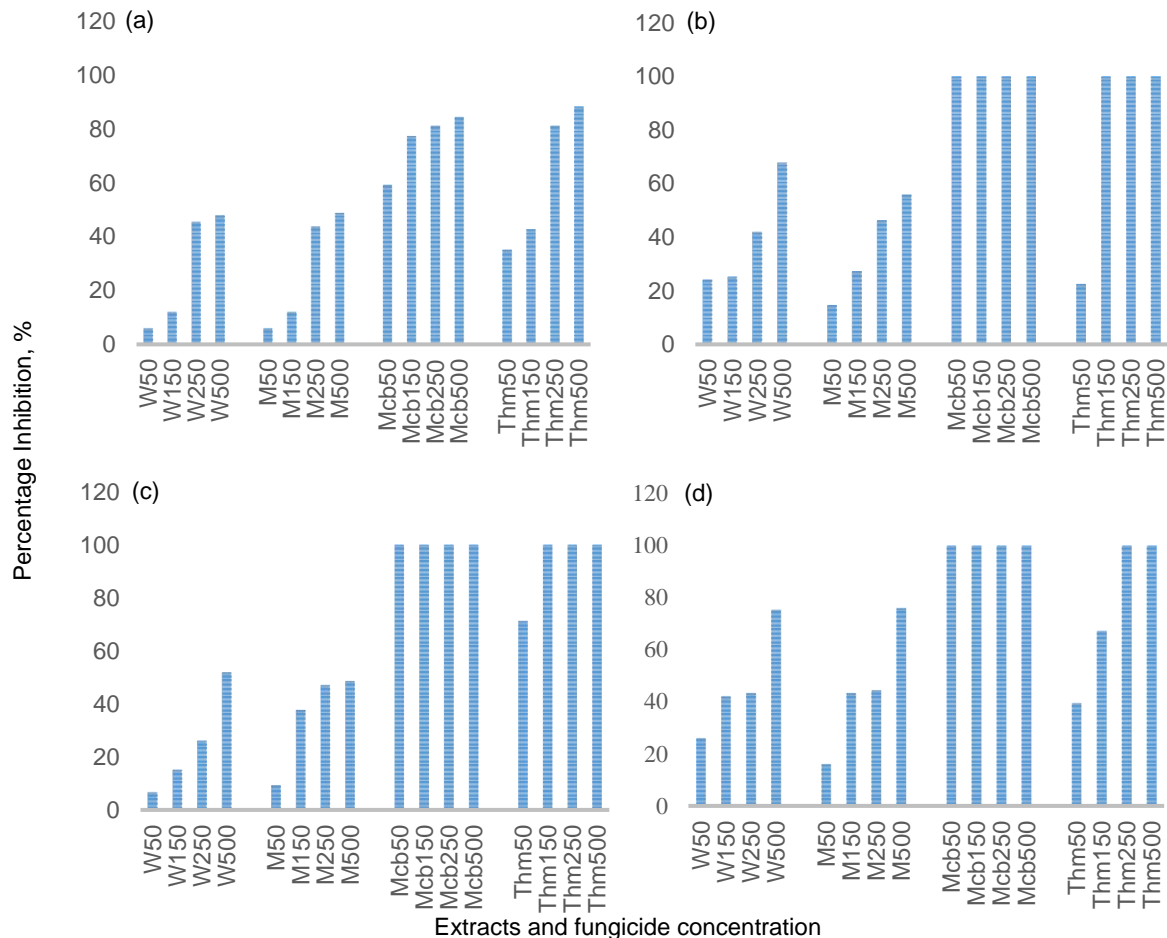


Figure 1: Effect of water and methanol extracts and synthetic fungicide mancozeb and thiram at different concentration on mycelia growth inhibition of (a) *Fusarium fujikuroi*, (b) *Curvularia aerea*, (c) *Curvularia lunata*, (d) *Curvularia eragrostidis*. W50 -50 ppm water extract, W150 -150 ppm water extract, W250 -250 ppm water extract, W500 -500 ppm water extract, M50 -50 ppm methanol extract, M150 -150 ppm methanol extract, M250 -250 ppm methanol extract, M500 -500 ppm methanol extract, Mcb50 -50 ppm mancozeb, Mcb150 -150 ppm mancozeb, Mcb250 -250 ppm mancozeb, Mcb500 -500 ppm mancozeb, Thm50 -50 ppm thiram, Thm150 -150 ppm thiram, Thm250 -250 ppm thiram and Thm500 -500 ppm thiram.

Results from germination test indicated that the paddy grains were able to germinate after being surface treated with the solvent extracts and synthetic pesticides after 90 days storage. This inferred that all the grains samples were not made infertile by the treatments applied. Percentage germination of healthy seedling and length and coloration of shoots and roots of seedlings are important indicators to determine the most efficient extracts as bio-fungicide. Study by (Tomkins and Grant, 1972) showed that application of certain pesticides on grains could cause cytotoxic effect on grains and affect germination, hence to study the effect of extracts on paddy grains are important to determine their suitability to be applied on stored grains. All of the five solvent extracts

showed a certain degree of inhibition of fungal growth on paddy grains when compared to the surface disinfected paddy grains as control. These phenomena indicated the fungal inhibitory effect which the extracts possess. Similar extracts from barks, leaves and fruits crude extracts has been tested to exhibit antimicrobial properties by (Ali *et al.*, 2008). These suggest that some derivatives from *M. elengi* plant are able to act as fungal growth suppressant. The results also indicated that water and methanol extracts were more desirable as antifungal agent as they both exhibited properties which were equivalent to fungal inhibition of both synthetic fungicides such as mancozeb and thiram.

Table 2: Phytochemical screening of different solvent extracts of *Mimusops elengi* seeds.

Phytoconstituents	Observation				
	Water	Methanol	Ethyl Acetate	Dichloromethane	Petroleum Ether
Carbohydrates	+	+	+	+	+
Reducing sugar	+	+	-	-	-
Saponin	+	+	-	-	-
Flavonoids	+	+	-	-	-
Alkaloids	+	+	-	-	-
Triterpenoids	+	+	+	+	+
Tannins	+	+	-	-	-
Phenolic compounds	+	+	-	-	-
Cardiac glycosides	-	-	+	+	-

Different solvent extracts of the seeds were qualitatively screened to determine the phytoconstituents in each solvent extract. The presence of phytochemicals in respective solvent extracts may play a significant role in the fungal inhibition of each extracts. Results of the test are shown in Table 2. Carbohydrates were detected in all solvents extracts but only water and methanol extracts contained reducing sugars. Saponin, flavonoids, alkaloids, tannins and phenolic compounds are found to be present in both water and methanol extracts. Triterpenoids were detected in all five types of solvent extracts while cardiac glycosides were only tested positive in ethyl acetate and dichloromethane extracts. Many of the phytochemicals compound were found in both strongly polar solvent extracts; water and methanol. The presence of majority of phytoconstituents may be a contributing factor to both water and methanol crude extracts having a significant fungal inhibitory effect. Results also ascertained the antifungal potential of both crude extracts are comparable to the inhibitory performance shown by the synthetic fungicides tested.

Qualitative phytochemical screening provides an understanding of possible active constituents which are antifungal. Having most of the phytochemical compounds present in the water and methanol extracts may explains the performance of both extracts having high fungal inhibitory effect. Saponin, a complex sugar distributed in plants and are associated to triterpene or steroid through a hydroxyl group or a carboxyl group well known for surfactant properties (Sahu *et al.*, 2001) and have been known for its medicinal new sweetener and flavor enhancer, also exhibited inhibitory effects against fungi and yeast (Tanaka *et al.*, 1996; Barile *et al.*, 2007). Phytochemical such as flavonoid, alkaloids, tannins, triterpenoids and phenols are phytochemicals which have been actively investigated and proven to a certain extend in recent years for their cytotoxicity, medicinal, antibacterial and antifungal properties. Published work

(Djoukeng *et al.*, 2005; Hazra *at al.*, 2007; Buzzini *et al.*, 2008; Manaharan *et al.*, 2012; Hu *et al.*, 2014; Zhang *et al.*, 2014) have all shown derivatives of these phytochemicals plays crucial roles in the bioactivities of the compound towards pathogens. Further separation and identification work on the crude seed extracts could help to derive active compounds which are useful against pathogenic fungi.

Water and methanol extracts were further tested on mycelia growth of several pathogenic fungi strains. Four strains of fungi pathogenic to paddy, *F. fujikuroi*, *C. aeria*, *C. lunata* and *C. eragrostidis* were tested and results were shown in Figure 1(a)-(d). Treatment using seed extracts and fungicide had all revealed that inhibition percentage increases with increasing concentration of substrate. Percentage of effectiveness of substrate on *F. fujikuroi* are shown in Figure 1(a). Results show water and methanol extract exhibited very similar trend of inhibition at all four-tested concentration with highest percentage of inhibition at 48.1% for water extract and 48.9% for methanol at 500 ppm. Synthetic fungicide mancozeb showed 59.4% of inhibition at lowest concentration and 84.5% at 500 ppm while fungicide thiram achieved 88.4% inhibition at 500 ppm. Figure 1(b) represent the results of treatment on mycelia growth of *C. aeria*. Water and methanol extracts also showed a very high similarity in trend of inhibition with best result of inhibition at 67.8% and 55.9% respectively at 500 ppm. Fungicide mancozeb were able to inhibit *C. aeria* 100% at 50 ppm while thiram needs 150 ppm. Percentage of inhibition *C. lunata* mycelia growth were presented in Figure 1(c) where methanol extract exerted higher inhibition compared to water extract at concentrations of 50 ppm, 150 ppm and 250 ppm. Mancozeb and thiram inhibition on *C. lunata* were similar to that of *C. aeria*. Effect of extracts and fungicides on *C. eragrostidis* were as displayed in Figure 1(d). Water and methanol extracts also showed a highly similar growth

inhibition pattern. *C. eragrostidis* were also fully inhibited by mancozeb at 50 ppm while thiram at 250 ppm.

Water and methanol extracts showed greatest percentage of inhibition on mycelia growth of *C. eragrostidis* followed by *C. aeria*, *C. lunata* and least effective on *F. fujikuroi*. Mancozeb were able to completely inhibit growth of *C. aeria*, *C. lunata* and *C. eragrostidis* at lowest concentration of 50 ppm but only inhibited 59.4% growth of *F. fujikuroi*. This could imply *F. fujikuroi* is a more prevalent species compared to other three fungi strains as it showed lower inhibition after undergoing the same treatment. Overall results also show that fungicide thiram gave a lower inhibition on mycelia growth of all four strains when compared to fungicide mancozeb. Both water and methanol crude extracts at four tested concentration also showed a lower inhibition percentage compared to synthetic fungicides. This may be due to the limited amount of active compound which are antifungal specific in the crude extracts used as they may not contain the specific chemicals as synthetic fungicides. Further separation need to be done to isolate the specific acting compounds contributing to fungal growth inhibition as well as determining the effective dosage. Fungal inhibition percentage of seed extract are very much dependant on the prevalence of target fungi species, concentration of extract used along with the type of specific active compounds which may all differ depending of target fungi (Satish *et al.*, 2006).

Plant based fungicides could be a better alternative for their greener approach towards non-target organism and environmental health. Findings from current study shows promising results of the seed extract as antifungal agent which prepares an essential direction for further isolation and identification of antifungal constituents that need to be further investigated to protect rice grains along its production pathway. Results obtained prove *M. elengi* plant crude water and methanol extracts can be a great candidate against pathogenic fungi that causes diseases. However, before direct application of the crude extracts as fungicide, further investigation needed to be done to identify the responsible acting compounds and substantiating its effectiveness.

CONCLUSION

Mimusops elengi is an invaluable plant source which have been used traditionally for its drug effect. This study has shown both water and methanol crude extracts of the seeds contain promising antifungal properties on seed borne fungi which is as good as the synthetic fungicides compared in this study. The botanical extracts treatment did not cause any change in grains colour or odour and has no adverse effect on seed germination which could have aided in protecting grains value and seedling quality. A broad range of active phytochemical properties it possesses may be the contributing factor for the fungal growth inhibition. Further exploration into this plant extracts as synthetic pesticides alternatives through isolation and identification of active compounds which can serve not only as a more environmental friendly choice in

management of paddy diseases but also as useful drugs in a broad range of industrial applications.

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