SCREENING OF ANTIBACTERIAL ACTIVITY OF ENDOPHYTIC FUNGI ISOLATED FROM DIFFERENT LEAF AGES OF CURCUMA MANGGA USING DIFFERENT GROWTH MEDIA

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ABSTRACT

Endophytic fungi are microorganisms that resided symbiosis in whole or a part of its life-cycle in the living plant tissue without causing any disease symptoms to their host plant. Nowadays, the use of endophytes for antimicrobial activity is emphasized due to their ability to produce variety of novel secondary metabolites which are almost same to their host plant. The isolation of endophytic fungi from medicinal plants was believed to have significant use in pharmacology field since their growth can be manipulated and have potential to substitute the role of their host plant as a source of antibiotic compounds. Hence, in the present study, the endophytic fungi were isolated from different leave stages of medicinal herbs, Curcuma mangga using different growth media. The agar plug diffusion assay was implemented as primary screening for antimicrobial activity and followed by disc diffusion assay. A number of 142 endophytic fungi were isolated and the most of them succeed be isolated from senescent leaves using potato dextrose agar supplemented with host plant. In the primary screening, 66 (46.48%) isolates exhibited antimicrobial activity against test pathogenic microorganism and the percentage of isolates exhibiting antimicrobial activity increased due to increment of leaf age. The result suggested PDA agar medium with supplement of host plant powder and the healthy leaves at older growth stage can be potential source to isolate endophytic fungi from C. mangga leaves for their antimicrobial compounds.

Keywords: Endophytic fungi, C. mangga, antimicrobial activity, host plant extract, host plant powder, malt extract agar, potato dextrose agar

1. INTRODUCTION

Curcuma mangga is one of medicinal herb that has been used in malay culture as traditional medicine. Most of them basically used the rhizome of the C. mangga to gain benefit. The plant is believed to have medicinal value such as antioxidant, antimicrobial, anticancer and so on. Endophytic fungi that has been isolated from C. mangga as their host plant was used in this study to isolate their antibacterial activity. Endophyte is defined as all microorganisms residing in their host plant tissue either rhizome, stems, leaves and flowers without causing any disease to their host. Endophytic fungi isolated from C. mangga leaves were used in this study and the antibacterial activity was tested on 8 different bacteria. Different media were used in the study in order to isolate endophytic fungi since effect of various media may affect the growth of endophyte. Basically, the study was initiated to investigate the effect of various growth media on number of endophytic fungal isolates. Besides, it also aimed to screen the effect of bioactive compound produced by endophytic fungal isolate on tested bacteria.

Medicinal plants play a crucial role, not only as a source of traditional medicines used in many cultures, but also as trade commodities which meet the demand of often distant markets [31]. Medicinal
plants now are being seen as a new alternative based on natural products for drug discovery \[18\]. They have become an interesting reservoir of medicines and chemicals for the researchers as 25% of the drugs used in modern medicines are from medicinal plants \[5\].

Plants and algae have been reported as hosts of endophytic fungi \[11,34,43\]. Endophytic fungi refers to a situation where one organism lives in host plant, reside in the tissues without causing any symptoms. In fact this endophytes have formed mutualistic symbiosis with host plant to bordering pathogens. Normally there could be more than one type of endophytes found within one host plant \[25\]. Among the host plants, the medicinal herbs are one of the important groups of hosts for endophytic fungi \[13,16,17,38,40\]. There are a lot of studies proved that endophytic fungi produce secondary metabolites such as enzyme and growth hormone that can be used to treat various diseases. These metabolites have anti-microbial activities that can be used to search for new antimicrobial agents as a solution to the global problem due to the development and spread of drug-resistant pathogens \[6,26\]. In Malaysia itself, many researches have been conducted to extract various part of local herbs for the sake of disease treatment \[21,22\].

_Curcuma mangga_ will be used as a sample study. The selection of this plant is based on its advantages to the human health and the potential of this herbs to the industry itself. Commonly, this herb is widely used in food preparation, supplements and traditional medicine \[15\]. Besides that, there are many studies suggested the useful pharmacological properties of this herb such as anti-inflammatory \[1\], anti-tumor \[24\] and immunological effects \[7\]. In our country, turmeric leaves is one of the ingredients added to various dishes for flavor and it is believed to be beneficial for general health. The aromatic leaves of _C. mangga_ also used to enhance the flavouring steamed and baked fish. The leaves contained labda-8 \[28\], 12-diene-15,16 dial with antifungal and mosquitocidal activity \[28\].

Other beneficial part of _C. mangga_ is rhizomes. The rhizomes that tastes a little spicy but delicious also suitable for use as a side dish. Even according to traditional healers, when you practice it as a side dish on the menu everyday, it is good to get rid of wind from the body. It rhizomes can be made juices to drink on a daily basis and also works to cure fever and reduce body heat. It leaves also useful to prevent the acceleration of oxidation process to the prepared food \[19\].

2. MATERIALS AND METHODS

2.1 Selection and preparation of plant materials

Fresh and healthy leaves with no visible of disease symptom at different age stages (young, matured, old and senescent) were chosen in this study. Plant materials were collected at Kg. BendangPauh, Kelantan (5°50’12.6”N 102°22’54.6”E) by hand picking method. Leaf samples were then stored in separate ziplock plastic bag and carefully transported to the Universiti Malaysia Kelantan. All leaf samples were processed within 4 hours after collection and kept at 10°C (polyester box containing ice) during transportation. For host plant powder preparation, leaf samples were washed carefully under tap water to remove any contaminants and air-dried until constant weight achieved. The dried plant samples were then ground using grinder into powder form. The host plant powder was stored in desicator to control humidity and kept for further experiment.

2.2 Isolation of endophytic fungi

The isolation of endophytic fungi was performed by adapting Okuda method with some modifications \[20\]. The leaf samples were cleaned thoroughly under running tap water to remove dust and then air-dried. To differentiate leaf age stages, the leaf colour was observed and the relative chlorophyll content was measured using a chlorophyll meter and determined in SPAD (Soil Plant Analysis Development) units. All age stages of leaves were measured in six replicates. Due to leaf surface sterilization, the leaves were washed with 10% ethanol for one minute followed by distilled water. Then, the samples were soaked in 1% sodium hypochlorite solution (2.5 minutes for young leaves, 5 minutes for matured, old, and senescent leaves) followed by rinsing three times with sterile distilled water. The effectiveness of leaf surface sterilization was examined by imprinting the treated leaf samples on the agar plate. The sterilization method was assumed as effective if there are no fungal colonies developed on imprinted agar plate.

After leaf surface sterilization process, the sterile leaves were cut aseptically into 2 x 2 mm² pieces and placed onto various agar plate containing 0.2 g/L chloramphenicol to inhibit bacterial growth. Six types of agar media were used for the isolation: Potato Dextrose Agar (PDA), PDA with
supplement of host plant extract, PDA supplemented by host plant powder, Malt extract agar (MEA), MEA with supplement of host plant extract and MEA supplemented by host plant powder (5.0 g/L). Host plant extract was prepared by soaking and sonicating 2.5 g of plant powder in 500 ml distilled water for 30 minutes. The inoculated plates were incubated in dark place at room temperature (25°C) for more or less 3 weeks to allow the growth of endophytes. The plates were observed daily until the fungal hyphal tips were seen exuding from the leaf samples and the contaminated plates were discarded. Small cuts of fungal hyphal fragments were then transferred onto new fresh MEA and PDA agar respectively. Repetitive re-plating of fungal isolates were conducted until the pure culture was obtained. Pure culture isolates were kept in glycerol stock containing 5.0 g/L host plant powder at -20°C prior to further experiment. The fungal pure culture was sub-cultured every 6 months on new fresh MEA and PDA respectively to ensure their viability.

2.3 Statistical analysis

Endophytic fungal assemblages of different leaf stages as well as growth media agar were compared and analysed using non parametric Kruskal-Wallis H test.

2.4 Culture media

Yeast extract sucrose broth was used to cultivate the endophytic fungal isolates which contained (g/L): Yeast extract, 20; sucrose, 40; magnesium sulphate, 0.5; with supplemented of Ocimumbasilicum extract (pH 5.8). The host plant extract was prepared by soaking and sonicating 2.5 g of dried host plant powder in 500 ml distilled water for 30 minutes. The extract was then filtered and mixed with freshly prepared culture media and autoclaved for 15 minutes at 121°C.

2.5 Cultivation and extraction

The inoculum was prepared by excising mycelial agar plug with 1 cm in diameter from the periphery of 7-days-old fungal pure culture. Two mycelial agar plugs were then introduced into 250 ml Erlenmeyer flasks containing 100 ml of yeast extract broth medium (supplemented with host plant extract) as mention above. The culture was incubated at 30°C with rotational speed of 120 rpm in a rotary shaker under dark condition for 20 days. The fungal culture was harvested by separating fungal biomass and fermented broth using filter paper (Whatman No. 1, England). The fungal biomass was then freeze-dried and macerated overnight in methanol (1:50, v/v). To obtain crude extract paste, the extracts were concentrated to dryness under reduced pressure in a rotary evaporator. The filtered fungal broth was extracted trice with equal volume of ethyl acetate (1:1; v/v). The extracts were then concentrated by using rotary evaporator in order to obtain a crude extract paste. A control was included by extracting sterile medium exactly the same step as for the endophytic fungal due to the antimicrobial activities of the host plant.

2.6 Evaluation of antimicrobial activity (Primary screening)

Primary screening of antimicrobial activity of endophytic fungal isolates were conducted according to agar plug method. The endophyte fungal culture were inoculated onto MEA and PDA plates, respectively containing host plant extract for agar plug preparation. The cultures were cut into plugs in diameter of 10 mm and 4 mm in thickness using a sterile cock borer after 20 days of incubation at 25°C. The agar plugs were then placed on the agar medium seeded with test pathogenic microorganisms. To allow diffusion of fungal bioactive compounds, the plates were kept and fumigated for 7 days at 4°C and subsequently incubated at 37°C overnight. Chloramphenicol (30μg) was used as positive control. The inhibition zones formed around the fungal agar plugs were measured after 16-18 hours of incubation period. Fungal isolates exhibiting significant antimicrobial activities against pathogenic microorganisms were subjected to secondary screening.

3. RESULTS AND DISCUSSION

3.1 Plant materials and isolation of endophytic fungi

Plant materials selection, collection procedure, sampling area are crucial factor determining the successful isolation of enormous endophytic fungal and medicinal herbs are believed to have numerous of endophytic fungi that live symbiotically in their host plant. The selection of leaves from C. mangga plant was prior to its extensive use in traditional medicine. The plants selection and sampling area were crucial factor determining the successful isolation of endophytes with pharmaceutical potentials. In the present study, fresh and healthy leaves were collected from 2 or more years of age at cultivated areas free of fungicides. Matured host plants and healthy leaves were
selected since the maturity of the host plants may affect the diversity of the isolates \[3\] and the chosen of healthy plant without disease symptoms was taken into consideration as well \[42\].

Many researchers intent to use the entire plant parts such as leaves, flowers, stems and roots due to their use in traditional medicine \[36\] as different part of the plant has variation bioactive compounds \[27\]. However, in this work C. mangga leaves were selected to be source of endophytic fungal isolates. Four different age stages of C. mangga leaves were used that is young, mature, old and senescent. The diversity of endophytic fungi at older stage of leaves was abundant with high potential source of antimicrobial properties \[14\]. Leaf colour was observed and relative chlorophyll content was determined to differentiate leaf age stages. Estimation of chlorophyll contents and assessment of the photosynthetic pigments in every leaf with different stages of maturity is very important indicator to measure the leaf senescence since the chlorophyll will loss due to the environmental stress \[39\]. The result was shown in Table 1. Based on the results, the leaf colour was vary from young to senescent and relative chlorophyll content was increased from young to mature but decrease from old to senescent leaves.

The surface sterilization of leaf samples can be performed by using sodium hypochlorite, ethanol and distilled water. The use of sodium hypochlorite for surface sterilization is enough to remove the epiphyte fungi, dirt or contaminants on leaf samples. These technique is the most frequently choice for surface sterilization in most laboratories \[23\]. The leave samples were soaked in ethanol for one minute since exceed soaking times (in ethanol) might kill the endophytic fungi. This is because ethanol is a sterilizing agent that extremely phytotoxic. Previous study stated that treatment of the plant samples with ethanol before sodium hypochlorite was more effective for reducing the surface contaminants compared with treated it first with detergents \[30\].

Table 1: Colour and relative chlorophyll content of Curcuma mangga leaves at different age stages.

<table>
<thead>
<tr>
<th>Leaf age stages</th>
<th>Young</th>
<th>Mature</th>
<th>Old</th>
<th>Senescent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour</td>
<td>Light green</td>
<td>Dark green</td>
<td>Dark green + yellowish</td>
<td>Yellowish</td>
</tr>
<tr>
<td>Relative chlorophyll content (SPAD unit)</td>
<td>25</td>
<td>40</td>
<td>35</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2: Immersion time of leaf at different age stages (Surface sterilization)

<table>
<thead>
<tr>
<th>Leaf age stages</th>
<th>Immersion time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>1</td>
</tr>
<tr>
<td>Mature</td>
<td>3</td>
</tr>
<tr>
<td>Old</td>
<td>9</td>
</tr>
<tr>
<td>Senescent</td>
<td>12</td>
</tr>
</tbody>
</table>

The use of sodium hypochlorite and ethanol are not accessible to the propagation of the microbial endophytes because those sterilants can cause less robust samples \[3\]. However, there is no other suitable method for surface sterilization of plant samples due to the isolation of endophytic fungus is a method dependent process in which this method used severely influence the fungal endophytes isolated \[9\]. Table 1 shows the preliminary study of optimization of immersion time in 1% sodium hypochlorite. Based on the result, the young leaves showed less growth of epiphytes since it need less immersion time to eliminate epiphytic fungi while senescent leaves showed high growth of epiphytes than the others leaf stages. This is because the senescent leaves have a high population density of epiphytes microorganisms rather than young leaves. The result was in agreement with the previous study which revealed that the diversity of epiphytes on young and old Cocconeis sp. of seagrass leaves is different and old leaves tend to be colonized by more epiphytes \[2\].

A total of 142 endophytic fungi were successfully isolated from young, mature, old and senescent leaves of C. mangga using six different agar medium viz; PDA, PDA supplemented with host plant powder, MEA, MEA supplemented with host plant powder and
MEA with host plant extract. The number of endophytic fungal isolates increased with the increment of leaf age stages (Table 2). The most densely endophytes colonized (59 isolates) were senescent leaves as compared to other leaf age stages. Contradictory, the young leaves were the least colonized by endophytes (18 isolates). The finding was in agreement with the previous study that stated the endophytic fungus mostly colonized in more ages leaves [12]. Various previous studies showed that endophytic fungi relatively tend to colonize old leaves rather than a young leaf [35,37]. Surface of old leaves are large rather than young, mature and senescent, tend to give the large surface area for endophytic fungal penetrated and colonized. The previous study stated that the old leaf has large diversity of endophytic fungi rather than mature and young. Result also revealed that the least number of endophytic fungi observed in young age stage of the leaf samples. This is due to high level of flavonoids that acts as an antifungal defences and at the age’s level, the endophyte started to commence [29].

Figure 1 shows the endophytic fungal isolated from Curcuma mangga leaves using PDA and MEA media. The result showed that endophytic fungi from C. mangga leaves are more tend to grow on Potato dextrose agar (PDA) media than Malt extract agar (MEA) media. PDA was best agar media for fungal isolation since potato starch, potato infusion and dextrose can support luxuriant growth of fungi[4]. In addition, the use of PDA is believed to promote the sporulation of fungi. The use of Potato dextrose agar in endophytic fungi isolation were diversified by using the mix of Potato Dextrose agar with host plant powder and host plant extract. The aims of different culture media used is to observe whether endophytic fungi are more tend to grow on the single media or with supplement of the host plant. The previous study stated that the media for endophytic fungal isolation should be supplemented with their host plant due to endophytic fungal nature and their symbiotically live with host plant which can promote and enhance their growth [41].

Result also revealed that endophytes from C. mangga leaves are more tend to grow on Potato dextrose agar supplemented with their host plant powder (PHP) rather than grow on PDA and PPE. Potato dextrose agar are more likely the most media to isolate and cultivate almost all genera of fungi where this media is the most used media for fungi isolation in the laboratories work. PDA contains 1.5% agar, 2% glucose, with nitrogen, phosphorus, vitamins and micronutrients being derived from a crudely filtered extract of macerated potatoes. The high carbon and nutrient ratio of Potato dextrose agar allows decent growth and more vital for sporulation and pigmentation for a broad range of fungal taxa [8].

The finding revealed that most of endophytes are like to grow on PDA media since PDA can support the growth of almost all endophytic fungi from wide range of fungal taxa whereas Malt Extract agar, the composition of this media might be generally restricted certain endophytic fungi to grow. Besides, Malt extract agar (MEA) also known as one of the semi synthetic media since it contains both natural and chemical substances. MEA usually used to isolate and cultivate fungi from soil and wood such as Ascomycota and Basidiomycota. The media supplemented with host plant are more tend to grow endophytic fungi since various numbers of fungi unable to grow on the artificial media where the fungi are too choosy and hardly to grow on. The finding revealed that endophytic fungi isolated from C. mangga need their host plant with to grow well. Therefore, powdered plant material which is C. manggae leaf powder were added to the isolation media to increase the growth of the endophytic fungi due to mutualistic interaction of endophytes and their host in nature [41].
Table 3: Endophytic fungal isolated from different leaf age stages of *C. mangga* using different culture media.

<table>
<thead>
<tr>
<th>Leaf age stages</th>
<th>Number of isolates</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PDA(^1)</td>
<td>PDA + HPP(^2)</td>
</tr>
<tr>
<td>Young</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Mature</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Old</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Senescent</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>20</td>
<td>42</td>
</tr>
</tbody>
</table>

\(^1\) Potato dextrose agar, \(^2\) Host plant powder, \(^3\) Host plant extract, \(^4\) Malt extract agar

Figure 1: Number of endophytic fungal isolated from *Curcuma mangga* leaves on different culture media

3.2 Primary screening (agar plug diffusion assay)

For primary screening, agar plug diffusion method was performed. The method was implemented to determine the presence of antimicrobial activity of the endophytic fungal isolates. The principle of this method is the bioactive compounds secreted by fungal isolates will be diffused into fresh agar medium seeded with test microorganisms and inhibition zone formed due to antimicrobial properties secreted. The bacteria used for primary screening are *Staphylococcus aureus*, *Entrococcusfaecalis*, *Bacillus subtilus*, Methicillin-resistant *S.aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiellapneumoniae* and *Shigellaboydii*.

Incubation time for fumigation was one week. A number of 142 isolates were proceeded in primary screening and 66 (46.48%) isolates exhibited antimicrobial activity against at least one test pathogenic microorganisms. Table 3 shows ten selected endophytic fungi isolates that exhibited antimicrobial activity against at least two of test pathogenic microorganism. Most endophytic fungi from the total of them endophytes gave a high positive result on gram-negative bacteria if compared with gram-positive bacteria. Bacteria cell wall composed of a substance called peptidoglycan and the layer of peptidoglycan of gram negative bacteria are much thinner than peptidoglycan layer of gram positive bacteria. Therefore, the bioactive compounds secreted by endophytic fungi easily to destroy the synthesis of cross linked peptidoglycan\(^{10,32}\). Most endophytic fungi from the total of ten endophytes gave high positive result on *E. coli* and
S. boydii. However, none of isolate able to inhibit E. faecalis and this may due to low concentration of extract. CMYL-PHP2 showed high inhibitory activity toward test bacteria since it inhibits seven out of eight pathogenic bacteria. Result also revealed that Methicillin resistant Staphylococcus aureus was susceptible to CMYL-PHP2, CMSL-PHP8, CMYL-PHP and CMSL-PHP10 isolates. These isolates are able to inhibit most resistant bacteria strain against all beta-lactam antibiotics that cause high fatality every year due to the infection caused by MRSA (Rory Clements, 2014). Plate 1 shows the inhibition zone of selected endophytic isolates.

Table 3: The present of antibacterial agent of endophytic isolates against selected microorganisms.

<table>
<thead>
<tr>
<th>No</th>
<th>Endophytic fungi</th>
<th>²S. a</th>
<th>²E. f</th>
<th>²B. s</th>
<th>⁴MRSA</th>
<th>³E. c</th>
<th>³P. a</th>
<th>⁷K. p</th>
<th>⁸S. b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CMYL-PHP1</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>CMSL-PHP3</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>CMML-PHP6</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>CMSL-PHP5</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>5</td>
<td>CMML-PHP8</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>CMSL-PHP1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>CMYL-PHP2</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>CMSL-PHP8</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>CMYL-PHP</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>CMSL-PHP10</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

(Keys: strong ++++, moderate ++, low +++)

4.0 CONCLUSION

The current study revealed that potato dextrose agar supplemented with host plant was the best medium for the isolation of endophytic fungal from Curcuma mangga leaves since most of the isolates grow well in the medium (142 isolates). Besides, the healthy leaf at older growth stage can be potential to isolate endophytic fungi from C. mangga leaves for their antimicrobial compounds. Primary screening shows that 10 selected endophytic fungal isolates have potential as antibacterial since all of them able to inhibit at least two of test bacteria.
5.0 REFERENCES


