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Antimicrobial activity of endophytic fungi isolated from different plant parts of *Curcuma mangga* Valeton and Zijp

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Abstract

The endophytic fungi isolated from different plant parts including leaf, stem and rhizome of *Curcuma mangga* were screened for antimicrobial activity by employing agar plug diffusion assay and disc diffusion assay for primary screening and secondary screening, respectively. A total of 127 endophytic fungi that were successfully isolated from various plant parts were cultured to examine their antimicrobial activities. Qualitative screening using agar plug diffusion assay revealed that 118 isolates (92.9%) showed antimicrobial activity against at least on one test microorganisms and suggested that the rhizome part exhibited the highest percentage of antiyeast (58.3%) and antifungal (91.7%) activities compared to leaf and stem parts. Quantitative screening using disc diffusion assay indicated that ethyl acetate extract from fermentative broth (extracellular compound) demonstrated better antimicrobial activity compared to methanol extract derived from fungal biomass (intracellular compound) against all the four classes of pathogenic microorganisms tested (Gram-positive bacteria, Gram-negative bacteria, yeast and fungi). The future of endophytic fungus study is very promising as it possesses hidden potential to be developed as natural antimicrobial agent.

1. INTRODUCTION

Endophytic fungi are considering ubiquitous organisms that are colonizing intracellularly or intercellularly of healthy plant tissues without causing any adverse effects to the host plant (Petrini, 1991). The existence of fungal endophytes in the plant contributes to the symbiotic relationship between fungi and host plant in which endophytic fungi play a major role in promoting fitness and resistance of the plant towards herbivore, insects, pathogen and abiotic stress tolerance (Gimenez *et al*., 2007).

Traditionally, endophytic fungi are regarded as an essential component of plant defence system against pathogenic organisms due to its ability to produce antibacterial, antiviral, antifungal, antioxidant, anticancer agents and immunosuppressive substances. Their role is resembled as chemical synthesizer in the plant since they are responsible in controlling the selection system for microbes to secrete bioactive compounds that possess a lower toxicity towards plants and other higher organisms (Strobel, 2003). Undoubtedly, many researches have been directed their investigations to the endophytic fungi studies due to their promising future in producing bioactive compounds that are pharmaceutically important

Previous report reveals that about 25% of thenewly introduced drugs to the market are plant derivatives. Hence, medicinal plant that has been exploited by human for centuries in traditional medicine is an outstanding source for the endophytic fungi isolation due to its potential in therapeutic values. *Curcuma mangga*, one of the local medicinal plant is widely used by the communities to treat various ailments including backache, asthma, wounds, sprains, fever, bronchitis, gastric ulcer and womb healing (Muchtaromah *et al.* 2017). Pharmacological studies of *C. mangga* also proved that the bioactive compounds found in this plant possess antitumour, antifungal, antiviral, insecticidal, antioxidant and anti-inflammatory properties (Liu and Nair, 2011; Tewtrakul and Subhadhirasakul, 2007). Considering all the potential and medicinal values of this plant, it can be concluded that this medicinal plant harbours plethora of endophytes with ability to produce valuable bioactive substances. Hence, the objectives of this study were to isolate the endophytic fungi from various plant parts of *C. mangga* and screening its antimicrobial activity.

2. MATERIALS AND METHODS

2.1. Sample collection

Healthy and fresh plant parts including leaf (young, mature, old and senescent), stem, and rhizome with no visible symptom of disease were selected in this study. The plant samples were collected from Kampung Bukit Lakota, Jeli, Kelantan, were kept in zip lock bags and transported to the laboratory for further processes. The determination of leaf age stages was done based on the colour observation and relative chlorophyll content as shown in Table 1.

Table 1: The different age stages of *C. mangga* leaves

Leaf age stages	Young	Mature	Old	Senescent
Colour	Light green	Dark green	Dark green with yellowish at the backside	Yellow
Chlorophyll content (SPAD unit)	20	40	30	10

The Soil Plant Analysis Development, SPAD 502 meter (Konica Minolta, Japan) was used to measure the relative chlorophyll content of the leaves and the contents were recorded in SPAD unit in triplicate readings.

2.2. Isolation of endophytic fungi

The collected samples were processed within 4 hours of collection. Firstly, all the plant parts were washed under running tap water to remove dust and debris. For the sterilization purpose of plant samples, all the samples were immersed in 1% sodium hypochlorite (1 min for young leaf; 3 min for mature leaf; 9 min for old leaf; 12 min for senescent leaf; 2 min for stem and 4 min for rhizome) followed by rinsing 3 times with sterile distilled water. These samples were then aseptically cut into 5 x 5 mm^2 pieces and placed onto agar plate containing 200 mg/L chloramphenicol to suppress the bacterial growth. The treated samples were imprinted on the agar plate to check the effectiveness of surface sterilization. Sterilization is regarded as successful if no fungal colony grew from the imprinted agar plate (Schulz *et al*., 1998) Media used for isolation purpose was Potato Dextrose Agar (PDA) supplemented with host plant powder (5 g/L). The host plant powder was prepared by grinding the sample of dried leaf into a powder form. The inoculated plates were then incubated at 30 ^oC up to 3 weeks and observed daily to check the exuding of fungal hyphal tips from the plant samples. Small cuts of these growth were then transferred onto the fresh PDA media and repetitive re-plating of this fungal isolate was continued until a pure culture was successfully obtained. Different fungal isolates were identified based on its morphologies, shapes and colours. Pure isolates of endophytic fungi were kept in glycerol stock containing 5 g/L leaf powder at -20 ^oC. The cultures were routinely subcultured on fresh PDA supplemented with host plant powder every 6 months to ensure its viability.

2.3. Preparation of test microorganisms

The test microorganisms used in this study were 4 Gram-positive bacteria (*Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 33591, *Pseudomonas aeruginosa, Bacillus cereus* ATCC 10876 and *Bacillus subtilis* IBRL A3), 6 Gram-negative bacteria (*Proteus mirabilis, Yersenia enterocolitica, Escherichia coli* IBRL0157, *Klebsiella pneumoniae* ATCC13883, *Salmonella typhimurium, Acinetobacter anitratus* and *Pseudomonas aeruginosa* ATCC 27853), 3 yeasts (Candida albicans IBRL, *Candida utilis* IBRL and *Cryptococcus* sp.) and 4 fungi (*Trichophyton rubrum* IBRL SD3, *Aspergillus niger* IBRL S1, Microsporum fulvum IBRL SD3 and *Microsporum gypseum*). The cultures of the test microorganisms were provided by Industrial Biotechnology Research Laboratory, Universiti Sains Malaysia, Penang, Malaysia. The inoculums were prepared by transferring single colonies or spores into 5 mL of sterile physiological saline and mixed well to get cell or spore suspensions. The bacterial inoculums (approximately 1 x 10^8 CFU/mL) and yeasts (approximately 1 x 10^6CFu/mL) were prepared by comparing its turbidity with 0.5 McFarland standards, whereas fungal inoculums were prepared as 1 x 10^6 spores/mL by using a hemocytometer slide (Neubauer, Germany). The test bacterial and yeast inoculums were then seeded on Mueller Hinton Agar (MHA) (Merck, Germany) and fungal inoculums were seeded on Sabouraud Dextrose Agar (Merck, Germany) by adopting the swab method.

2.4. Agar plug diffusion assay (Primary screening)

The antimicrobial activity of fungal endophyte isolates was investigated by employing the modified agar plug method (Radakrishnan *et al.,* 2011). Agar plug was prepared by inoculating fungal cultures onto PDA plates supplemented with host plant powder. After 20 days of incubation at 30 ^oC, the cultures were cut into plugs 10 mm in diameter and 4 mm in thickness using a cork borer. These agar plugs were placed properly on agar medium seeded with test microorganisms. The plates were initially kept at 4 ^oC for 7 days for the bioactive compounds diffusion purpose prior to incubated at either 37 ^oC (bacteria and yeast) or 30 ^oC (fungi). Ketoconazole served as a positive control for yeast and fungi whereas chloramphenicol is used as a positive control for bacteria. After incubation for 16-18 hours (bacteria), 24-48 hours (yeast) or 72-96 hours (fungi), the diameter of inhibition zone surrounded the fungal agar plugs were measured. Fungal isolates that demonstrate significant activities were then chosen for secondary screening.

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2.5 Culture medium

Yeast extract sucrose broth (YES) (Merck, Germany) was used to cultivate the fungal endophytes isolates containing (g/L): yeast extract, 20; sucrose, 40; magnesium sulphate, 0.5 with the addition of 1000 mL of plant water extract of *C. mangga* (pH 5.8). The preparation of plant water extract was done by boiling 2.5 g of the dried plant materials powder in 500 mL distilled water for 30 minutes. The extract was then filtered and mixed with freshly prepared culture media and autoclaved at 121 °C for 15 minutes.

2.6 Cultivation and extraction

The preparation of inoculum was done by introducing 2 mycelial agar plugs (1.0 cm in diameter and 4.0 mm thickness) that were excised from the periphery of 7 days old fungal culture into 250 mL Erlenmeyer flasks containing 100 mL of YES broth with addition of plant water extract as a culture medium. The cultures were cultivated at 30 °C with rotational speed of 120 rpm for 20 days. The fermented broth and fungal biomass were separated by filtration using filter paper (Whatman No. 1, England). The fungal biomass was freeze-dried and macerated in methanol (1:50, w/v) overnight. The extracts were then concentrated to dryness using rotary evaporator to obtain a crude extract paste. Meanwhile, the filtered broth was extracted thrice with equal volume of ethyl acetate (1:1, v/v). The upper organic phase was concentrated to dryness under reduced pressure in a rotary evaporator to obtain a crude paste. Since *C. mangga* also exhibited significant antimicrobial activities, a set of control was prepared by extracting the sterile medium following exactly the same procedure as that for fungal endophyte cultures.

2.7 Disc diffusion assay (Secondary screening)

The disc diffusion assay was conducted according to the Clinical and Laboratory Standards Institute (CLSI) standard M2-A9 (Clinical and Laboratory Standard Institute, 2006) and M44-A (Clinical and Laboratory Standard Institute, 2004). The fungal extracts were initially dissolved in 1% DMSO. 20 µL of each extract with a concentration of 20 mg/mL were impregnated onto sterile Whatman antibiotic discs and placed on the surface of inoculated medium. One percent DMSO served as a negative solvent control, 30 µg chloramphenicol as a positive control for bacteria and 30 µg ketoconazole for yeast and fungi. The discs were then placed on agar plates seeded with test bacteria, yeast and fungi. The plates were incubated at 37 °C for 16 to 18 hours for bacteria, 24 to 48 hours for yeasts and 30 °C for 48 to 96 hours for fungi. The diameter of the inhibition zones formed around the discs was measured. All the experiments were done in triplicate.

2.8 Statistical analysis

The experiments were conducted in three replicate (n=3) and the test data were expressed as mean ± standard deviation (SD). The data was analyzed by means of the ONE-WAY ANOVA using SPSS 15.0. Duncan test was employed to access the difference between means. The results were considered statistically significant if $p<0.05$.

3. RESULT AND DISCUSSION

A total of 127 endophytic fungal isolates were successfully isolated from different leaves age stages (young, mature, old and senescent), stems and rhizomes (Table 2). Senescent leaves were the most densely colonized (47 isolates) by endophytic fungal isolates compared to the other plant parts. The number of fungal isolates recovered also increased with leaf age stages. The endophytic fungi can be isolated from various plant parts such as leaf, flower, seed, stem and rhizome with leaf abundantly harbor endophytes (Uzma *et al*., 2016) compared to stem, rhizome and root (Sun *et al*., 2013; Suphaphon *et al*., 2013). The diversity of endophytic fungi colonizing in the terrestrial plants is depending on the age of host plant and type of host plant tissue (Xing *et al*. 2010). Interestingly, the number of endophytic fungi that can be recovered from the leaves part increase with the leaves age as shown in a few plant hosts studied such as *Theobroma cacao* (Arnold and Herre, 2003), *Bauhinia brevipes* (Hilarino *et al.,* 2011), *Camellia japonica* (Osono, 2008) and *Rhizophora apiculata* (Kumaresan and Suryanarayanan, 2000). The higher colonization density in older leaves is due to the changes in leaves structure over time and also chemical properties changes during the leaf cycle (Arnold, 2005; Malinowski and Belesky, 2000).

Table 2: Endophytic fungal isolates isolated from various plant parts of *C. mangga*

Plant part	Young leaf	Mature leaf	Old leaf	Senescent leaf	Stem	Rhizome
Number of isolates	3	5	35	47	23	12
Total	125					

The density of colonized endophytes that possess antimicrobial activity in the leaves is influenced by leaves maturity as the constant changes occur in intrinsic environment of the leaves during the maturity causing the leaves to support different communities of endophytes (Toofanee and Dulymamode, 2002). Generally, younger leaves possess higher concentration of antifungal and anti-herbivore compounds compared to mature leaves (Coley, 1988; Coley and Barone, 1996) where this plant part is highly chemically defended for the colonisation purpose of endophytes. This could be the reason only a small number of endophytes that can be recovered from the young leaves of *C. mangga*. The density of fungal-leaf endophytes

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colonization can be affected by other factors including time of exposure, contact surface size and also natural opening numbers in the plant tissues (Nascimento *et al*., 2015). The longer the plant tissues being exposed to the environment, the more numbers of fungal endophytes can be recovered from those tissues (Arnold and Herre, 2003). Thus, a wax layer on the surface of young leaves of *C. mangga* can serve as a physical barrier to fungal entry (Lorenzi and Matos, 2002).

The antimicrobial activities result from the primary screening showed that 94.4% of 127 endophytic fungal isolates demonstrated significant inhibitory activity against at least on one test microorganisms. The percentage of isolates that exhibiting antimicrobial activities of leaf, stem and rhizome is shown in Figure 1. Stem and leaf are the best parts of *C. mangga* herb to isolate endophytic fungi with antibacterial activities. On the other hand, rhizome showed remarkably higher antiyeast and antifungal activities compared to the leaf and stem parts.

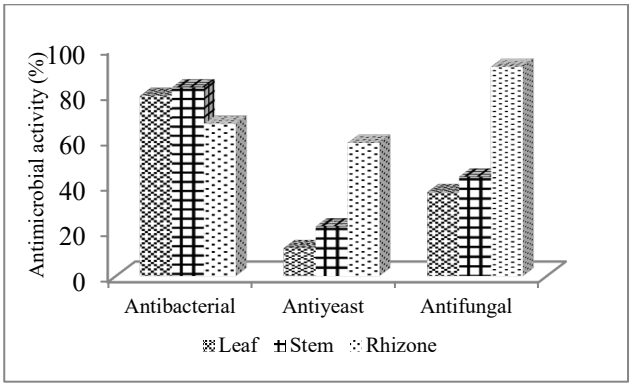

Figure 1: Antimicrobial activities of endophytic fungal isolates isolated from various plant parts of *C. mangga*

Fifteen isolates with prominent antimicrobial activities (produced diameter inhibition zones of 9 mm to 25 mm) in the agar plug diffusion assay were selected for secondary screening. Figure 2 shows the antimicrobial activities of ethyl acetate and methanol extracts of fungal isolates against pathogenic bacteria, yeasts and fungi. The ethyl acetate extracts obtained from fermentative broth exhibited greater inhibitory activity against all the four classes of test microbes (Gram-positive bacteria, Gram-negative bacteria, yeast and fungi) compared to methanol extracts derived from fungal biomass. The results indicated that the antimicrobial compounds of the fungal isolates were mostly associated with fermentative broth, of which extracellularly released by the fungal isolate into the broth. On the other hand, there were very little of antimicrobial compound synthesized intracellularly by the fungal biomass. The advantage of extracellular compound is the ability to easily obtain high extraction yield of antimicrobial compound compared to intracellular compound which requires proper destruction of fungal mycelia to gain high yield of antimicrobial compound (Taufiq and Darah, 2019).

Meanwhile, the antiyeast and antifungal activities as shown in Figure 2 were poorly observed for both ethyl acetate and methanol extracts for all the endophytic fungal isolates under investigation. The pattern of microorganism susceptibility towards the fungal extracts shows that the endophytic fungal isolates tend to inhibit gram positive bacteria rather than gram negative bacteria, yeast and fungi in which depicted that varying responses of pathogenic microorganisms towards antimicrobial agents. Gram positive bacteria are more susceptible to the extract because their cell membranes consist of greater volume of peptidoglycan but no lipopolysaccharides outer membrane. This thick peptidoglycan is capable to absorb a lot of foreign substances including antibiotics that can facilitate the access of antibiotics to their site of action. Ironically, the presence of thinner LPS outer membrane in Gram negative bacteria that almost high degree of infiltration rate serves as a barrier to the antibiotics to penetrate to their site of action (Silhavy *et al*., 2010), thus making Gram-negative bacteria are more resistant to the crude extract. The resistance of pathogenic yeast and fungi to the crude extract probably due to their thick cell with high percentage of chitin. Recent genomics and proteomics studies supported the evidence that yeast and fungal cell walls share a common origin. The chitin in yeast and fungal cell wall is laid down in microfibrillar bundles that form a thick and though cell wall structure that serve as an efficient permeability barrier (Michael and Martinko, 2006).

4. CONCLUSION

Traditional medicinal herb, *C. mangga* is the potential source to isolate endophytic fungi that exhibit a wide range of antimicrobial activity with the leaves part is the most colonizing part of these fungal endophytes. The bioactive compounds in the crude extracts of prominent fungal endophytes are secreted extracellularly. However, further studies should be carried out to identify the bioactive compounds present in the crude extract.

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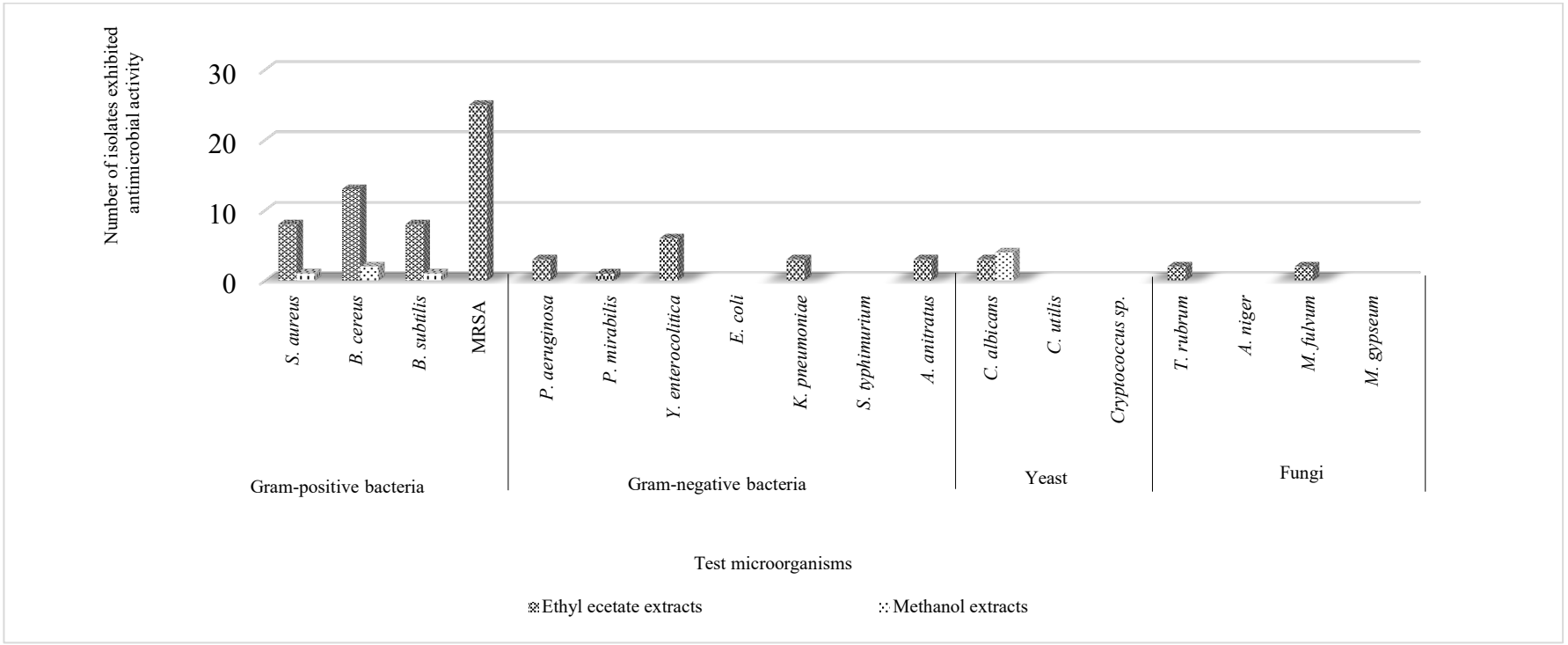

Figure 2: Antimicrobial activities of ethyl acetate and methanol extracts from 15 selected endophytic fungal isolates

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