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Ceratobasidium ramicola IBRLCM127, an endophytic fungus isolated from *Curcuma mangga* Valeton & Zijp with strong anti-candidal activity

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ABSTRACT

Ceratobasidium ramicola IBRLCM127, an endophytic fungus isolated from the rhizome of the local medicinal plant *Curcuma mangga* Valeton & Zijp, was found to possess significant anti-candidal activity. This fungal endophyte was cultivated in submerged fermentation system using yeast sucrose medium supplemented with host plant water extract and cultivated at 25°C, agitated at 120 rpm for 12 days. The ethyl acetate was used as a solvent to extract compounds in the fermentative broth. The fungal ethyl acetate extract demonstrated significant inhibitory zones toward cells of *Candida albicans* with minimum inhibitory concentration (MIC) value of 2.5 mg/ml, of which exerting yeastocidal effect. The time–kill study conducted at three distinct ethyl acetate concentrations (half MIC, MIC, and 2 MIC values) revealed that the growth of *C. albicans* cells was concentration-dependent. Yeastostatic activity was shown at lower concentration and yeastocidal activity was shown at higher concentration. The structural degeneration of the *C. albicans* cells after treated with ethyl acetate extract was observed under the scanning and transmission electron microscopes and the results exhibited various cell deformities including severe damage of the cell extracellularly and intracellularly which led to cell death beyond repair, thus suggesting that the extract could be a potential antifungal agent.

INTRODUCTION

The incidence of invasive and opportunistic fungal infections for the past few decades has shown a dramatic increase resulting in the higher rate of morbidity and mortality (Miceli *et al*., 2011). Approximately, 95% of fungal infections in human are caused by *Candida* species, particularly *Candida albicans* that can cause the life-threatening disease called candidiasis if it reaches human bloodstream (Kaufman, 2012).

Candida albicans is a polymorphic fungus with the ability to alternately switch in between a round and long straggly structure that enable the cell to be successfully adapting to various kinds of environmental conditions and making it extraordinarily harmful. Candidal infections or candidiasis is one of the antecedents in all major fungal infections of which these infections have been identified as the leading cause of death among immunocompromised patients and also including those that have been treated with broad-spectrum antibiotics, immunosuppressive agents, undergone organ transplantation, intensive care of low birth weight infants, and HIV-infected (Chapman *et al*., 2017). Some strains of *Candida* sp., particularly *C. albicans* have developed resistance towards conventional antifungal treatment. For example, in 2013, fluconazole-resistant *C. albicans* was reported to be the main threat of fungal infections with approximately 3,400 candidal infection cases annually (Centers for Disease Control and Prevention, 2013). Therefore, drug-resistant yeast and fungi could pose a serious threat to mankind which demands immediate alternative strategies to search for novel antibiotics to tame this problem. In this case, endophytes are depicted as an outstanding

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source of medicinally important compounds that can offer a new horizon of drug-resistance dilemma (Levy, 2005).

Endophytes are microorganisms (bacteria, yeast, and fungi) that live inside the healthy host plant tissues without posing any apparent symptoms or diseases (Yu *et al*., 2010). Due to the long co-evolution of endophytic microorganisms with its plant host, they possess the ability in synthesizing bioactive metabolites originally associated with their host plants and it has been acknowledged as an important source of novel drug discovery (Lam, 2007). The pharmacologically bioactive compounds produced by endophytes show a high level of structural diversity and have been proven to exhibit antimicrobial, antitumor, antioxidant, antiviral, and antifungal properties that offer a tempting source of drugs with less adverse effect to human (Alvin *et al*., 2014; Berger, 2009).

Based on the aforementioned facts above, the attempt to discover a novel anti-candidal agent from natural source is proceeded in this study by investigating the potential of secondary metabolites of *Ceratobasidium ramicola* IBRLCM127, a fungal endophyte previously recovered from the rhizome of local medicinally important plant *Curcuma mangga* Valeton & Zijp against *C. albicans*.

MATERIALS AND METHODS

Culture and maintenance of the endophytic fungus

The endophytic fungus *C. ramicola* IBRLCM127 which was previously isolated from the rhizome of *C. mangga* Val & Zijp was supplemented by the division of culture collection of Industrial Biotechnology Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. This isolate was cultured on Potato Dextrose Agar supplemented with host plant powder (5 gl^{-1}) and incubated at 30°C for 6 days. The isolates were then stored at 4°C prior to the usage. This isolate was cultured routinely on a fresh agar media once a month to ensure the cell is viable and pure.

Culture medium

Yeast extract sucrose broth was prepared containing (gl^{-1}): yeast extract, 20; sucrose, 40; and magnesium sulfate, 0.5 mixed uniformly with 1,000 ml distilled water supplemented with *C. mangga* extract. The preparation of extract was done via 30 minutes of boiling of 10 g of the plant powder in 500 ml distilled water, followed by filtration of the mixture using Whatman No. 1 filter paper and mixing the filtrate with fresh culture medium. The pH mixture was adjusted to 5.8 and autoclaved for 15 minutes at 121°C prior to the usage in cultivating fungal endophyte isolates using the shake-flask system.

Cultivation and extraction

Two mycelial agar plugs (diameter 1.0 cm and thickness 4.0 mm) from 6-day old culture of the fungal isolate were introduced into 250 ml Erlenmeyer flasks consisting of YES broth (100 ml). This culture was incubated at 30°C, 120 rpm for 20 days under the dark condition. Next, Whatman no 1 filter paper was used to separate out the fermentative broth and fungal biomass. The fermentative broth was subjected to the extraction process using the same volume of ethyl acetate (1:1; v/v) thrice. The organic phase (upper part) was collected and concentrated to dryness using rotary evaporator under the reduced pressure to get the paste form of ethyl acetate extract. A sterile medium was prepared as a control using a similar process as in endophytes culture preparation due to the biological activity possessed by *C. mangga*. The fungal biomass, on the other hand, was freeze-dried and macerated overnight in methanol (1:50; w/v). The mixture was then filtered using Whatman no 1 filter paper followed by concentrating using rotary evaporator to get crude methanolic extract in a paste form.

Test microorganisms and culture maintenance

The stock culture of *C. albicans* was obtained from the culture collection of IBRL, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. This cultural stock was stored as 20% glycerol stock in −20°C freezer and maintained routinely by sub-culturing once a month on sabouraud Dextrose Agar (SDA) followed by incubating process under designated conditions (37°C, 24 to 48 hours).

The preparation of *C. albicans* inoculum was done by picking up 3 to 5 pure colonies of 24- to 48-hours-old culture and transferred into universal bottle consisting of 10 ml of sterile physiological saline (0.85% NaCl, w/v), followed by vortex of a mixture for uniform mixing and adjusted the turbidity by using McFarland standard.

Disk diffusion assay

The assay method was conducted as proposed by Espinol-Ingroff (2007). A 100 µl of suspension containing 1×10^6 CFU/ml of *C. albicans* cells was spread onto SDA using a sterile cotton swab. Sterile antibiotic disk (6.0 mm diameter) impregnated with 20 µl of crude extract of both ethyl acetate and methanol at the concentration of 50 $mgml^{-1}$ (dissolving in 5% dimethyl sulfoxide) were placed onto the seeded agar surface. Negative control of 5% dimethyl sulfoxide used to detect the solvent effects, while positive control consists of 30 μgml^{-1} ketoconazole. These plates were incubated (37°C, 24 hours) and the diameter of clear zone around the disk was measured. This experiment was carried out in three replicates on separate occasions.

MIC and MYC determination

Broth microdilution technique was performed in sterile 96-well microtiter plate to determine the minimum inhibitory concentration (MIC) (Nor Afifah *et al*., 2010). The prepared inoculum was diluted using sterile physiological saline (0.85% NaCl. w/v) and adjusted to the inoculum size of 5×10^4 CFU/ml. The fungal extract was subjected to two-fold serial dilution using sterile Sabouraud dextrose broth (SDB) medium as a diluent to yield the final extract concentrations ranging from 0.0098 to 5.0000 $mgml^{-1}$. The control of an experiment consisting of the mixture of sterile SDB medium and inoculum. The plates were incubated for 24 hours at a temperature of 37°C. Next, 40 µl of 0.2 $mgml^{-1}$ p-iodonitrotetrazolium violet salt (INT) was added into each well as a growth indicator which is shown by the purple color of INT. The MIC value was determined as the lowest concentration of extract that inhibited the growth of yeast cell, while the determination of minimum yeastocidal concentration (MYC) performed by taking out a sample volume of 100 µL from each treatment well and diluted accordingly prior to streaking on SDA plate to determine the viability of the cells (Tong *et al*., 2012).

The MYC was defined as the lowest concentration of extract that was able to kill 99.9% of yeast cells as compared to control.

Time–kill curve study

The experiment was conducted following the protocol proposed by CLSI (2006) with some modifications. The *C. albicans* suspension was prepared by picking up 3–5 colonies from 16 to 24 hours old *C. albicans* culture and aseptically transferred them into the 20.0 ml SDB followed by incubation process under designated condition (37°C, 150 rpm, and 16 to 18 hours). The cells' turbidity during the log phase was measured using spectrophotometer (625 nm wavelength). The extract was mix with 25 ml of SDB in 50 ml Erlenmeyer flask to obtain the desired concentration of extract, viz., 0 (control), 1.25 (half MIC), 2.50 (MIC), and 5.00 (2 MIC) $mgml^{-1}$ after the addition of the yeast inoculum (Yogalatha *et al*. 2010). The inoculated flasks were incubated in an orbital shaker at 37°C with agitation speed of 150 rpm. At predetermined time intervals, a 100 µl aliquot from each treatment flask was pipetting out for viable cell count at every 4 hourly during the time intervals of 0–48 hours. The samples were diluted and spread onto fresh SDA plates prior to incubation process (37°C, 24 hours) to determine colony-forming unit per milliliter ($CFUml^{-1}$), only the plates with the number of colonies ranging from 30 to 300 were counted. A curve for time–kill study (log $CFUml^{-1}$ *vs*. time) was plotted for each concentration of extract including control. The experiment was carried out in triplicates on separate occasions.

Structural degeneration of the C. albicans cells treated with ethyl acetate extract

The preparation of *C. albicans* suspension was mentioned previously. The mixing of suspension with extract solution was performed to yield the final concentration of 2 MIC (5.00 $mgml^{-1}$) in the flask and these flasks were then incubated accordingly (37°C, 150 rpm). The harvesting process of cultures was carried out at 0 and 36 hours of incubation time prior to subjected to scanning electron microscope (SEM) (Leica Cambridge, S-360, UK) and transmission electron microscope (TEM) (LIBRA@120 EFTEM, Germany) observations as mentioned by Borges *et al*. (1989) and Mares (1989).

Statistical analysis

The data were analyzed by Student *t*-test to compare the effect of ethyl acetate extract on pathogen using SPSS Version 12.0. The tests were performed independently in three replicates. The results in the current study were analyzed using a one-way analysis of variance and presented as the mean ± SD.

RESULTS

The disk diffusion susceptibility assay was employed to investigate the anti-candidal activity of fungal endophyte *C. ramicola* IBRLCM127 recovered from the rhizome of *C. mangga* and the result was shown in Table 1. The result revealed that only ethyl acetate extract exhibited anti-candidal activity with 15.3 ± 0.6 mm diameter of inhibition zones while no activity was detected for methanolic extract, suggesting that the bioactive compound(s) with anti-candidal activity was secreted extracellularly into the fermentative medium.

The MIC and MYC values of ethyl acetate extract towards *C albicans* were recorded in Table 2. In most cases involving studies of microorganisms, the MYC values are always equal or higher than MIC values, meaning that the concentration of extract must be increased significantly to successfully kill the pathogen cells. In this case, the extract was exerted a bactericidal effect as the ratios of MYC/MIC were ≤ 4 (Table 2). The result from the broth microdilution technique demonstrated that the technique employed was more sensitive and very useful to evaluate the extract susceptibility against the tested microorganism.

The time–kill curve of the ethyl acetate extract from *C. ramicola* IBRLCM127 towards *C. albicans* was depicted in Figure 1, in which the growth curve for control shows four different growth phases consisting of lag, exponential, stationary, and death phases. However, this growth curve changed significantly with the addition of the fungal extract. As the concentration of extract increased, the growth of candida in term of colony-forming units was reduced. No post-antibiotic effect was observed for all the concentration tested in this study. At half MIC value (1.25 mg/ml) and MIC value (2.5 mg/ml), the effects were yeastostatic, whereas at the 2 MIC value (5.0 mg/ml), the effect observed was yeastocidal. At 2 MIC value, the extract was able to successfully reduce the cell number from the initial inoculum proving that the potency of the extract as anticandidal agent against *C. albicans*.

The detail observations of morphological changes of *C. albicans* cells caused by extract were done via SEM and TEM. Figure 2 depicts the photomicrographs from SEM observation representing the effect of ethyl acetate extract derived from fungal

Table 1. Anti-candidal activity of the crude ethyl acetate and methanolic extracts of *C. ramicola* IBRLCM127 against *C. albicans.*

Tested yeast	Diameter of inhibition zone (mm)			
	Ethyl acetate extract	Methanolic extract	Positive control	Negative control
C. albicans	15.3 ± 0.6	-	20.3 ± 0.6	-

Table 2. MIC and MYC values (mg/ml) of ethyl acetate extract from *C. ramicola* IBRLCM127.

Test microorganism	MIC (mg/ml)	MYC (mg/ml)	Ratio MYC:MIC
C. albicans	2.5	2.5	1 (Bactericidal)

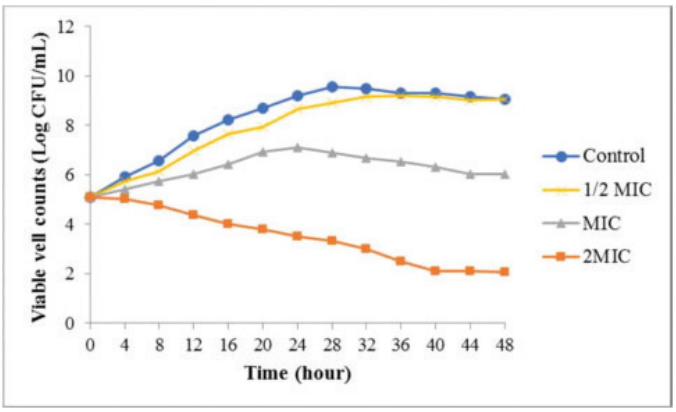

Figure 1. Time-kill curve study on *Candida albicans* cells by the ethyl acetate extract of *C. ramicola* IBRLCM127.

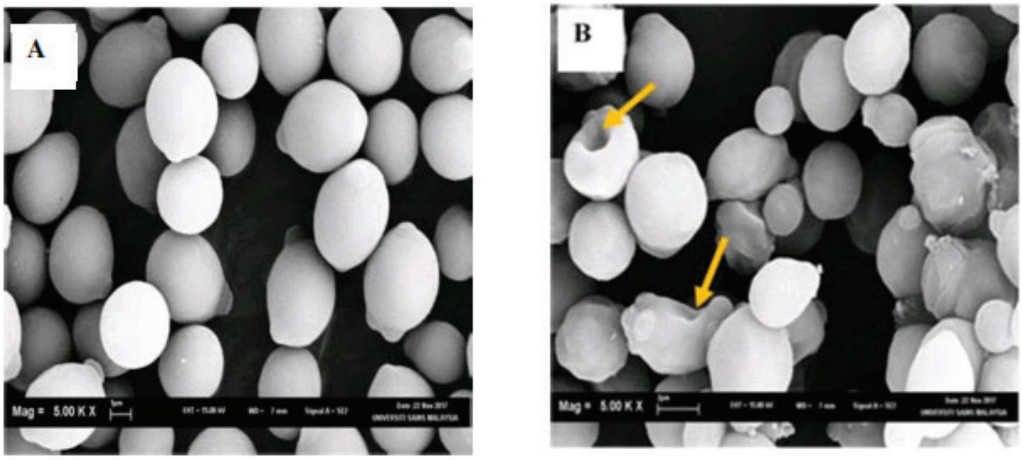

Figure 2. SEM photomicrographs of *C. albicans* cells. (A) untreated cell (control) and (B) treated cell with ethyl acetate extract of *C. ramilcola* IBRLCM127 at 2MIC value (5.0 mg/ml) and 36 hours of exposure time. Magnifications 5,000×. Arrows indicated the morphological and distinct feature changes occurred to the cells.

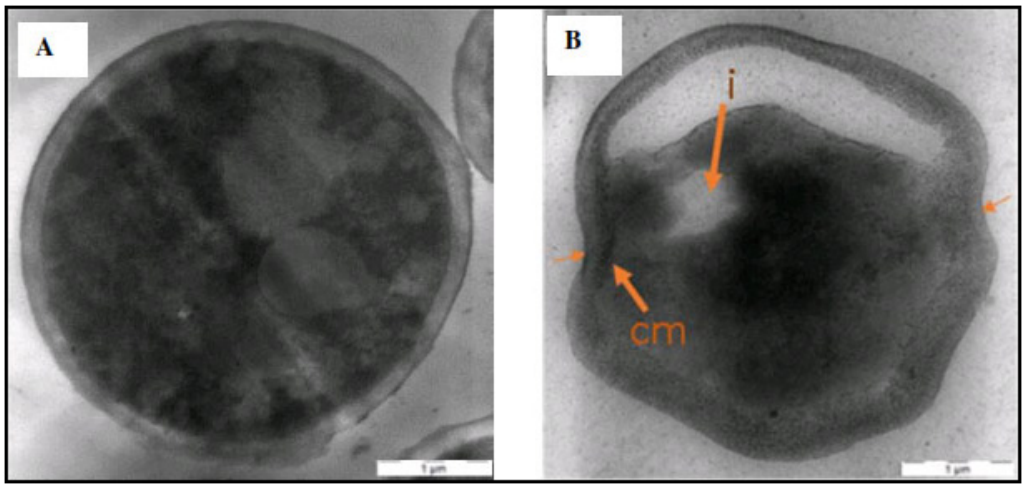

Figure 3. TEM photomicrographs of *C. albicans* cells. (A) untreated cell (control) and (B) treated cell with ethyl acetate extract of *C. ramicola* IBRLCM127 at 2MIC value (5.0 mg/ml) and 36 hours of exposure time. Damage at cell wall is indicated with small arrows in (B).

endophyte *C. ramicola* IBRLCM127 toward *C. albicans* cells. Figure 2A indicates most of the *C. albicans* cells were appeared to be in oval and smooth state with some at a budding stage (control or untreated cells). On the contrary, most of the treated cells with 36 hours of exposure time to the extract (Fig. 2B) were shrunken and starting to collapse, meaning that most of the cells lost their intact oval structures. At this stage, the cell damage was beyond repair and the cells completely lost their metabolic function.

For a closer view, a TEM study was performed (Fig. 3). Figure 3A shows a typical structure of a control or untreated *C. albicans* cells which possessed cytoplasm with cells organelles suspending within it, and surrounded by the cell membrane and cell wall. On the other hand, there was a deformation and stunted budding formation for the 36 hours extract treated cell (Fig. 3B). The cytoplasm had migrated to the periphery of the cell and leaked out probably due to the imbalance of osmotic pressure caused by the cell membrane permeability alteration. At this point, the cell membrane was partially disintegrated especially in the area close to the budding site. Inclusions also can be observed at a budding site of a damaged cell which then led to cell lysis.

DISCUSSION

There has been an increasing number of yeasts that are developing resistance towards antifungal drugs available in the market nowadays, particularly *C. albicans* that have been reported to develop resistance towards amphotericin B and fluconazole (Odds *et al*., 2003). *Candida albicans* serve as an important opportunistic pathogen and exist as commensal in healthy human but not in immunocompromised patients as they possess a potential systemic infection due to their remarkable adaptation in the distinct host (Rosenbach *et al*., 2010). *Candida albicans* often recalcitrant to most of the therapy due to their ability to morphologically switch from yeast to pseudohyphae and also mixed infections with bacteria. However, their ability to form drug-resistant biofilm is the main successful factor in becoming the main etiologic agent of candidiasis (Ramage and Lopez-Ribot,

2005). Further studies revealed that candida biofilms developed drug resistance towards the most commonly used antifungal agents, fluconazole and amphotericin B through the increasing of the maturation process and the presence of minimal matrix (Sardi *et al*., 2011). The prevalence of anti-candidal drug resistance demands an urgent search for new anti-candidal agents to tackle this bothersome situation. Despite the availability of numerous anti-candidal agents clinically in treating superficial and systemic candidiasis, there are very few classes that have been used widely including polyenes, flucytosine, and azoles due to their high toxicity (Kathiravan *et al*., 2012). Hence, endophytes from medicinal plants are depicted as an outstanding and safer source for antifungal drugs development as they might provide diverse and enormous phytochemical compounds that offer a great scope of novel antifungal lead (Turner *et al*., 2006), besides their lower toxicity as their compounds do not kill the eukaryotic host plant system (Alvin *et al*., 2014).

The intriguing finding from the current study is the fermentation broth of endophytic fungal isolate *C. ramicola* IBRLCM127 that was isolated from the rhizome of *C. mangga* exerted significant anti-candidal activity. The MIC and MYC values for *C. albicans* were found to be the same, viz., 2.5 mg/ml, thus giving the ratios of 1 for MYC/MIC. According to Levison and Levison (2009), bactericidal drugs are those with minimal lethality concentration value same or not more than fourfold greater than their MIC values. Therefore, the activity of ethyl acetate extract from the current study can be regarded as fungicidal due to the fact that the ratio of MYC/MIC was ≤4 (Table 2). Clinically, there has been an understandable concept that bactericidal drugs are more powerful antibiotic agents compared to bacteriostatic drugs as these classes of drugs can kill bacteria and rather effective in severely ill and immunocompromised patients (Fauci *et al*., 2008).

Candida albicans is one of the frequently isolated pathogenic fungi in human, especially those with immunodeficiency (Mohan, 2017). *Candida albicans* can be regarded as a polymorphic fungus as it able to grow in few distinct forms likes ovoid budding cell, pseudohyphae with ellipsoidal cells restricted at the septa, and true hyphae that possesses parallel walls (Berman and Sudbery, 2002). The pathogenicity and virulence of *C. albicans* are much related to the ability of cells to morph alternately between filamentous form and yeast. Both forms are found to be pathogenic, of which the filamentous form of hyphal cells plays a crucial role in infection process as it is more invasive than the yeast, while the yeast form plays key roles in dissemination (Dalle *et al*., 2010; Saville *et al*., 2003).

The complexity of lifestyle and virulence strategy in *C. albicans* has triggered profound research among the researchers to explore natural sources for the production of novel bioactive compounds with anti-candidal activity, especially the untapped endophytic fungi. Fungal endophytes have been acknowledged as an arsenal of novel biologically active substances for drug discovery (Okoye *et al*., 2015) of which only a handful from a million of fungal endophytes species have been explored so far. Therefore, it is believed that the further discovery of this untapped source might increase the possibility to discover novel bioactive natural products (Yu *et al*., 2010). The result from our study also indicated a significant anti-candidal activity exerted by ethyl acetate extract of *C. ramicola* IBRLCM127. There are also a few reports concerning this matter including the study conducted by Mittal *et al*. (2016) that was successfully isolated a novel compound of N- [(2S,3aR, 6S,7aS)-6- acetamido-octahydro-1,3-benzothiazol-2-yl]2-(adamantan-1-yl) which derived from endophytic fungal *Emericella* sp. recovered from *Azadirachta indica* A. Juss (Neem) tree that showed a great potential in inhibiting the growth of *C. albicans*.

The photomicrographs from SEM and TEM confirm the most noteworthy morphological changes in some organelles and structures of *C. albicans* cells are caused by the ethyl acetate extract of *C. ramicola* IBRLCM127 that possesses anti-candidal activity. The activity of the extract suggests a potential mechanism to alter the components of the cell wall and cell membrane, inhibit DNA and protein synthesis, as well as enhance membrane permeability and cytoplasmic content leakage (Torey *et al*., 2016). A crude extract of *C. ramicola* IBRLCM127 contains several distinctive active compounds in which each of the compounds possesses different mechanisms of actions towards *C. albicans* cells. Therefore, the rigid cell walls of *C. albicans* which are made up mainly from chitins and glucans (Chaffin, 2008) offer a notion that some crude extract compounds are responsible to penetrate the cell wall while the other compounds are accountable in accessing further into the cell in order to cause severe damage.

The genomics and proteomics advancement gives solid evidence that yeast and fungi are sharing a common origin of their cell walls. Generally, yeast and fungal cell walls like other eukaryotic cell walls are made up of fibrous components such as protein and polysaccharide as well as a cross-linked amorphous matrix, i.e., glycoprotein, of which these chemical components are highly diverse in plant and fungal cell walls (Gonzalez *et al*., 2009). Fungal cell walls mainly composed of mannoproteins, chitins, and glucans with different proportions according to the species with β-1,3-glucan serves as a main cell wall component (Brown & Gordon, 2003). However, there is slightly difference in the chitin concentration of fungi and yeast of which the chitin concentration in fungi is higher than in the yeast (Bernard and Latge, 2001). Both fungal and yeast cell walls are dynamic structure that gives protection to the cells for their viability during osmotic pressure and other environmental stresses, while at the same time permitting the cells interaction with their surrounding (Bowman and Free, 2006).

Up to now, many studies have been conducted to investigate the action mode of the endophytic fungal extract on the yeast and fungal cells with the emphasizing to the deformation or destruction of the cell wall and cell membrane (Kumari *et al*., 2018). The current results from showed that ethyl acetate extract of endophytic fungal *C. ramicola* IBRLCM127 impacted the cell wall synthesis and cell membrane permeability, congruent with Nath and Joshi (2017) that reported the presence of various cell deformities like wrinkled and disintegrated cell structures and disruption of cell wall after the exposure to the endophytic fungal extracts. Fungal cells are typically enclosed in a cell wall that serves as an essential structural support for cell protection. The fungal cell wall components of β-glucan and chitin contribute to the strengthening the cell wall shape of *C. albicans*, whereas mannoproteins responsible for permeability, antigenicity, and adherence (Cabib *et al*., 1988). Any disruption to the synthesis of these components can lead to the failure of the cell wall to

protect the cell (Cabib *et al*., 1982). Meanwhile, the distortion of the fungal cell membrane by the extract could possibly due to the disruption of the ergosterol synthesis as cell membrane of the fungi is made up of unique ergosterol that assists in maintaining the structure and function of the plasma membrane. Interestingly, the absence of some yeast and fungal cell wall and cell membrane components in human such as β-glucan, chitin, and mannoproteins of the cell wall, as well as ergosterol of the cell membrane making them a vulnerable target that provide some selective and toxicity advantages over the modes of action of antifungal agents (Akins, 2005; Tada *et al*., 2013).

CONCLUSION

The ethyl acetate extract of endophytic fungal *C. ramicola* IBRLCM127 isolated from the rhizome of *C. mangga* exhibited significant anti-candidal activity. The extract was proven to exhibit fungicidal effect on the *C. albicans* cells and the structures of the cells were starting to collapse after 36 hours of exposure to the fungal extract. Further investigation in regard to the isolation and identification of the biologically active compounds inside the extract is necessary considering the fact that the discovery and development of a new antifungal drug are at a very slow pace despite the technological advancement.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest relevant to the contents of this article.

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