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Antifungal Activity of *Rhizome coptidis* and *Alpinia galangal* against *Candida* species

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Candidiasis is a fungal infectious disease, caused by the opportunistic pathogens, *Candida* species. Incidences of candidiasis have become more common, as a result of prolonged antibiotic therapy and increased number of immunocompromised patients. This study was conducted to investigate the antifungal properties of *Rhizome coptidis,* Radix *Sophorae flavescentis,* Radix *Stemonae, Etlingera elatoir, Alpinia galangal* and *Cymbopogon citratus* extracts in terms of inhibition zone and minimum inhibitory concentration (MIC) of plant extracts against some of clinically prevalent species of *Candida* outlined by CLSI susceptibility testing guidelines for yeast cells. Our favourable results demonstrated that *R. coptidis* has potential to show the strong antifungal activity against all *Candida* species tested as ranged from 64 to >1024 µg/ml. *A. galangal* was also able to inhibit the growth of *Candida tropicalis* and *Candida glabrata*, although to a lower extent. Moreover, the MIC value of *A. galangal* was 64 µg/ml for both *Candida* tested. Meanwhile, the majority of plant extracts tested did not show significant antifungal activity. Nonetheless, *In vivo* testing needs to be performed to support these findings.

Key words: *Candida* species, *Rhizome coptidis, Alpinia galangal,* anti-*Candida* activity.

Candidiasis is a fungal infection which is caused by *Candida* species. Candidiasis thereby encompasses infections that range from superficial to systemic and potentially life-threatening diseases. *Candida* yeasts are usually present in most people, but uncontrolled multiplication may result in disease symptoms, especially in immunocompromised patients. However, due to wide usage of azole drugs and prolonged antifungal therapy, the number of azole-resistant isolates has increased in many institutions during the past decade. There are five most frequently isolated species of *Candida*, namely *C. albicans, C. tropicalis, C. krusei, C. parasilopsis* and *C. glabrata.* Among these, *C. albicans* is the most significant species causing numerous infections in human, especially in immunocompromised patients[1].

Clinically, candidiasis is normally treated with antimycotics, which are the antifungal drugs commonly used in hospitals. For non-severe clinical condition, the antifungal drugs used include topical clotrimazole, topical nystatin, fluconazole and topical ketonazole, whereas for severe infection, amphotericin B, caspofungin or variconazole may be used. Although different kinds of antifungals have been developed, resistant strains still exist, especially *C. glabrata. C. glabrata* has emerged as the second most common

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cause of blood stream and mucosal infection in many countries and exhibits intrinsically low susceptibility to fluconazole. This species is naturally about 8 fold more resistant to fluconazole than *C. albicans* and easily develops further fluconazole resistance following prolonged therapy of patients with fluconazole[2-5].

Rhizoma coptidis (called Huang Lian in Chinese) has been used in traditional Chinese medicine for clearing away heat and depriving dampness for treatment of diarrhoea, dysentery and jaundice, and clearing away toxic material for the cases of seasonal febrile diseases, carbuncle, and sore throat. The herbal medicine possesses broad-spectrum antibacterial and anti-protozoal effects[6]. The herb Radix *Sophorae flavescentis* (known as Ku Shen in Chinese), a famous traditional Chinese medicine herb, is cold by nature with bitter taste, entering the three channels of heart, spleen and kidney. It has been used as a diuretic and for the treatment of acute dysenteric gastrointestinal hemorrhage and eczema[7]. The pharmacological tests revealed that *Sophora flavescens* has strong antimicrobial and anticancer properties. The herb *Radix stemonae* (called Bai Bu in Chinese) has been used as an antitussive and antihelmintic in traditional Chinese medicine for some 2000 years and as an insecticide in the Orient[8]. The tuberous roots are well known for their antibacterial, antiparasitic and expectorant properties. They are prescribed in the therapy of cough, ascariasis and oxyuriasis [World Health Organization (WHO) Regional Publications]. The torch ginger or wax flower [*Etlingera elatior* (Jack) R.M. Smith] is believed to be native to Sulawesi (Celebes) and Jawa, Indonesia (Java). In Malaysia, it is called bungakantan. Plants of *Etlingera* have various traditional and commercial uses. In Sabah, Malaysia, the hearts of young shoots, flower buds, and fruits of *E. elatior, E. rubrolutea, and E. littoralis* are consumed by indigenous communities as condiment, eaten raw or cooked[9]. Leaves of *Etlingera* species exhibited antibacterial activity against Gram-positive but not Gram-negative bacteria[10].

This study was conducted to investigate the antifungal properties of *Etlingera elatoir*, *Rhizoma coptidis, Radix stemonae,* and *Radix sophorae flavescentis* extracts. The disc diffusion method was used whereby the length of inhibition zone was measured for each *Candida* species treated with different concentrations of plant extracts tested. Eventually, The MIC of significant extracts against *Candida* species was also determined using modified broth dilution method.

MATERIALS AND METHODS

Plant/Herbs extraction

The flower buds of *E. elatoir* were prepared from a local market, while the Chinese herbs Radix *Stemonae, Rhizoma coptidis,* Radix *S. flavescentis, Cymbopogon citrates* and *Alpinia galanga* were sourced from a Chinese traditional medicine shop. The specimens were identified by Institute of Tropical Agriculture of Universiti Putra Malaysia and confirmed. The flower buds and herbs were cleaned prior to usage, subsequently cut into small pieces and dried in the oven at 45°C for 1 to 3 days. The dried pieces were ground into powder form using grinder. *E. elatoir,* Radix *Stemonae*, lemongrass and Radix *S. flavescentis* were subjected to ethanolic extraction while *Rhizoma coptidis* was subjected to methanolic extraction. One hundred g of the powder was soaked in 200 ml of solvents in the conical flask and was left overnight. The mixture was filtered on the next day and the filtrate was subjected to evaporate the alcoholic solvent at 40°C at 140 to 160 rpm, and at 15 to 20 lbs of pressure using rotary evaporator. Eventually, the concentrated extracts were dried in air to remove residual water contents. The extracts were stored in -20°C prior to usage.

Determination of antimicrobial activity

According to the Clinical and Laboratory Standards Institute[11] for yeast cell with some slight modification, paper disks of 6 mm in diameter were prepared using Whatman filter paper. The paper disks were autoclaved at 121°C for 20 min before use. The stock solutions of 100 mg/ml of extracts were prepared by dissolving 100 mg of extract into 1 ml solvent. Serial ten-fold dilution was carried out to make different concentrations of plant extracts. Amphotericin B (1 mg/ml) was also used as a standard control antifungal drug. Eventually, 5µl of each concentration of plant extracts were impregnated on paper disks separately. The disks were left at room temperature for 30 min to dry any residue solvent which may have extra antifungal effect on *Candida*.

Four different *Candida* species including *C. albicans* ATCC 14053, *C. glabrata* ATCC 2001, *C. krusei* ATCC 6258 and *C. tropicalis* ATCC 750 were grown on separate Sabouraud's dextrose agar (SDA) (Difco Laboratories, Detroit, Michigan) and then passaged three times to ensure its viability and activity. Inocula were prepared by picking five colonies of ≥1 mm in diameter from 24 h culture of *Candida* species using 5 ml of PBS buffer. Following, the inocula were centrifuged and the supernatant was removed and the cell pellet was washed with PBS buffer and then centrifuged again for 5 min. These steps were repeated at least three times. The cell density was adjusted from 1×10^6 to 5×10^6 cells/ml using spectrophotometric method at 530 nm wavelength to achieve the turbidity equivalent to 0.5 McFarland standards. Working suspensions were prepared by the stock solution with ratio 1:100 with PBS followed by 1:20 dilution with same solution to produce $5 \times 10^2 – 2.5 \times 10^3$ yeast cells/ml. The inocula were spread on SDA using a sterile cotton swab. The culture plates were kept at room temperature for 15 min to dry. Subsequently, the plant extract disks were applied on the agar and kept again at room temperature for 15 min and then incubated at 37°C for 24 h. Eventually, the diameter of zone of inhibition was measured.

The plant extracts were examined in terms of antifungal activities through the determination of MIC, according to CLSI documents with slight modifications. In summary, 100 µl of the two fold dilution of the antifungal agents dissolved in Sabouraud's dextrose broth (SDB) (Fluka, Germany) were inoculated with 100 µl of inoculum containing between 5×10^2 to 2.5×10^3 yeast cells/ml using U-bottom 96-well microplates (Brand 781660, Wertheim, Germany)[12]. The microplates, including plant extracts and cells, were incubated at 35°C and MICs were measured at 530 nm using an EMaxs micro-plate reader after 24 h.

RESULTS AND DISCUSSION

Disk diffusion assay is a simple and reliable preliminary screening test to investigate the antifungal activity of extracts. The plants investigated in this study have been used in traditional medicine for their antimicrobial and detoxification properties. However the result of preliminary test using the disk diffusion method demonstrated that only *Rhizoma coptidis* could able to show the strong antifungal properties against all *Candida* species tested comparable to antifungal activity of amphotericin B. On the other hand, *Alpinia galanga* has slight anti-*Candida* activity against *C. tropicalis* and *C. glabrata* (Table 1 & 2). The other plant extracts have no significant potential in terms of anti-*Candida* activity. Moreover, *R.coptidis* was found to be more effective against *C. glabrata* ATCC 2001 and *C. tropicalis* ATCC 750 in terms of diameter of the zones of inhibition (Table 2).

Although *C. albicans* is the main cause of infection in clinical cases, *C. glabrata* has emerged as a common cause of bloodstream and mucosal infections in many countries, and it exhibits intrinsically low susceptibility to fluconazole[3,13]. This species is naturally about eight-fold more resistant to fluconazole than *C. albicans* and easily develops further fluconazole resistance following prolonged therapy of patients with fluconazole[4,5].

Table 1. Preliminary susceptibility testing of plant extracts on four *Candida* species

Extracts (at 100 mg/ml)	*Candida* species			
	C. albicans ATCC 14053	*C. krusei* ATCC 6258	*C. tropicalis* ATCC 750	*C. glabrata* ATCC 2001
Rhizoma coptidis	Susceptible	Susceptible	Susceptible	Susceptible
Radix *Stemonae*	Resistant	Resistant	Resistant	Resistant
Radix *S. flavescentis*	Resistant	Resistant	Resistant	Resistant
Etlingera elatoir	Resistant	Resistant	Resistant	Resistant
Cymbopogon citrates	Resistant	Resistant	Resistant	Resistant
Alpinia galanga	Resistant	Resistant	Susceptible	Susceptible

All *Candida* species were susceptible to *Rhizoma coptidis* extract at the concentration of 100 mg/ml (w/v)

Therefore, the discovery of antifungal properties of *R. coptidis* and *A.galanga* in this present study indicate a potential drug candidate for alternative treatment of azole-resistant candidiasis in which the causal strain is naturally resistant to the azoles.

Importantly, *R. coptidis* is a traditional Chinese medicine which is proven to possess broad-spectrum antibacterial and antiprotozoal effects[6]. Although the main mechanisms of antifungal activity of *R. coptidis* is not clear but may be due to the main compound of this plant which is named berberine. Berberine, the major active compound in *R. coptidis,* is an isoquinoline derivative alkaloid and has many pharmacological

Table 2. Antimicrobial activity of *Rhizoma coptidis* and *Alpinia galangal* against *Candida* species

Candida species	Inhibition zone (mm) of antifungals tested ± SD*			Concentration of
	Rhizoma coptidis	*Alpinia galanga*	Amphotericin B	antifungals (mg/ml)
C. albicans	12 ±0.2	-	16 ±0.6	100
ATCC 14053	11 ±0.6	-	15 ±1.1	90
	10 ±0.3	-	15 ±0.8	80
	9 ±0.7	-	15 ±0.3	70
	7 ±0.6	-	14 ±0.6	60
	7 ±0.5	-	13 ±1.2	50
	-	-	13 ±0.9	40
	-	-	13 ±0.2	30
	-	-	12 ±0.5	20
	-	-	12 ±0.4	10
C. tropicalis	25 ±0.6	8 ±0.2	16 ±0.1	100
ATCC 750	23 ±0.7	8 ±0.0	15 ±0.9	90
	22 ±0.6	7 ±0.8	15 ±0.8	80
	22 ±0.6	7 ±0.7	15 ±0.2	70
	22 ±0.5	7 ±0.2	15 ±0.1	60
	20 ±0.6	7 ±0.1	14 ±0.2	50
	20 ±0.2	6 ±0.9	13 ±0.8	40
	18 ±0.8	6 ±0.5	13 ±0.3	30
	18 ±0.5	6 ±0.4	12 ±0.9	20
	13 ±0.5	6 ±0.1	12 ±0.8	10
C. glabrata	23 ±0.9	8 ±0.5	7 ±0.0	100
ATCC 2001	23 ±0.1	8 ±0.4	6 ±0.3	90
	20 ±0.6	8 ±0.1	5 ±0.5	80
	19 ±0.1	7 ±0.6	2 ±0.9	70
	17 ±0.6	7 ±0.5	1 ±0.3	60
	19 ±0.1	6 ±0.3	-	50
	17 ±0.9	5 ±0.7	-	40
	16 ±0.9	5 ±0.5	-	30
	14 ±0.9	5 ±0.1	-	20
	11 ±0.6	4 ±0.8	-	10
C. krusei	9 ±0.9	-	15 ±0.5	100
ATCC 6258	9 ±0.5	-	15 ±0.1	90
	8 ±0.6	-	15 ±0.0	80
	8 ±0.2	-	14 ±0.3	70
	7 ±0.6	-	13 ±0.8	60
	7 ±0.3	-	13 ±0.7	50
	-	-	13 ±0.0	40
	-	-	12 ±0.9	30
	-	-	12 ±0.5	20
	-	-	12 ±0.2	10

*Values are mean ± SD of two independent experiments; SD: standard deviation.

effects including activation of the aryl hydrocarbon receptor[14] and inhibition of arylamine *N*-acetyltransferase activity[15]. It is active by inhibiting the basal and 12-otetradecanoylphorbol-13-acetate mediated prostaglandin E2 level and cyclooxygenase-2 expression[16]. Interestingly, *A. galanga*, a *Zingiberaceae* (Ginger family) tree, which also known as Galangal, has been used in South East Asia for many years in the treatment of several diseases in medicine and also in traditional cooking.Galanga contains a greenish-yellow volatile oil comprising cineol, eugenol, sesquiterpenes, isomers of cadinene, and a resin containing galangol, kaempferide, galangin, as well as starch and other constituents[17]. Galangal shows antitumor activity in mice[18] and has been found to be moderately effective as an antihelmintic against the human *Ascaris lumbricoides*[19]. It is demonstrated that *A. galanga* could enhance the antifungal activity of quercetin and chalcone against *C. albicans*[20] due to derived active compound of this plant, but our results indicated that *A. galangal* alone is not able to show antifungal activity against *C. albicans* and *C. krusei*. On the other hand, our findings show the slight anti-*Candida* activity of *A. galanga* against *C. glabrata* and *C. tropicalis*. Therefore, it is suggested to use this plant in combination with the other antifungals to enhance the potential of anti-*Candida* activity. More recently, it has also been demonstrated that the combination of galangal with rosemary or lemon could be able to show the

Table 3. Minimal inhibitory concentration of alcoholic extracts of *Rhizoma coptidis* and *Alpinia galangal* against *Candida* species

Candida species	MIC (µg/ml)	
	Rhizoma coptidis	*Alpinia galanga*
C. albicans ATCC 14053	>1024	-
C. tropicalis ATCC 750	128	64
C. glabrata ATCC 2001	64	64
C. krusei ATCC 6258	>1024	-

significant synergistic activity against some of bacteria such as *Escherichia coli* and *Staphylococcus aureus*[21].

Table 3 shows MIC value of *R. coptidis* and *A. galanga* against *Candida* species tested. Interestingly, the amount of absolute MIC was low against *C. glabrata* (64 µg/ml) for both significant plant extracts. These results are compatible with the susceptibility testing via disk diffusion method whereby *C. glabrata* had zones of inhibition at the lowest concentration of plant extracts tested. This may be due to high sensitivity of *C. glabrata* to both antifungals tested.

The MIC value of 64 µg/ml *R. coptidis* and *A. galanga* towards *C. glabrata* might not be considered as an effective antifungal drug compared to the MIC_{50} of fluconazole at 8 mg/ml that inhibited 50% of *C. glabrata* clinical isolates[2]. However, one should consider that the plant extracts used in this study is not a pure compound, but just a crude extract where the active compound (s) that possesses the antifungal property might be in lower concentration.

In future studies, investigation should be carried out to elucidate the active compound of *R. coptidis* and *A. galanga* that is responsible for antifungal activity against *Candida* species. This may lead to novel antifungal drug discovery, hence providing a solution to the limited choice of drugs available currently to combat the azole-resistant *Candida* species, especially *Candida glabrata.* Moreover, gene expression study on common drug target sites, such as *ERG11* and *CDR1,* should be explored to determine the mode of antifungal mechanism of *R. coptidis* and *A. galanga* at the molecular level.

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