Antifungal Activity of Some Medicinal Plant Extracts Against *Candida albicans* and *Cryptococcus neoformans*

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Abstract

The ethanol extracts of clove (*Eugenia caryophyllus* Bullock & Harrison) and sweet flag (*Acorus calamus* Linn.) were investigated for their antifungal activity in comparison with eugenol and amphotericin B (AmB) by using the National Committee for Clinical Laboratory Standards (NCCLs) M27-P broth microdilution method. Two medicinal plant extracts, eugenol and amphotericin B were used to determine their minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) against 28 clinical isolates of *Candida albicans* and 25 clinical isolates of *Cryptococcus neoformans*. The MICs of clove, sweet flag, eugenol and AmB against *C. albicans* were 17.41±8.64 mg/ml, 28.8±16.32 mg/ml, 12.16±4.53 mg/ml and 0.23±0.1 μg/ml respectively. The MFCs were 67.5±15.39 mg/ml, >75 mg/ml, 15.4±6.47 mg/ml and 0.47±0.21 μg/ml respectively. The same extracts and antifungal drugs which were tested against *C. albicans* were also tested against *C. neoformans.* The MICs were 2.43±0.95 mg/ml, 3.02±1.97 mg/ml, 6.28±3.4 mg/ml and 0.28±0.15 μg/ml, respectively. The MFCs were 22.22±12.71 mg/ml, 30.82±27.11 mg/ml, 10.06±4.9 mg/ml and 0.51±0.25 μg/ml respectively. The results showed that *C. albicans* was significantly ($p<0.01$) more susceptible to the extract of clove than sweet flag, whereas *C. neoformans* was significantly susceptible to the clove extract ($p>0.05$). Moreover, the extract of clove showed significantly ($p<0.01$) more potent inhibitory activity against *C. neoformans* than eugenol, while it showed significantly ($p<0.01$) less inhibitory activity against *C. albicans* than eugenol. AmB, the drug of choice for invasive infection treatment, remains as one of the most effective antifungal drugs. These data indicate that the extracts of clove and sweet flag were potential fungistatic agents against yeasts, whereas AmB and eugenol showed fungicidal effects.

INTRODUCTION

The incidence of fungal infections has increased significantly in the last 20 years (Poeta et al.,1999). In immunocompromised patients, the emergence of candida infections with both primary drug and azole-resistance have been described (Willocks et al., 1991; Cameron et al., 1993; Pfaller et al., 1994). Amphotericin B has been provided for the standard treatment of the most systemic fungal infections (Medoff and Kobayashi, 1980). Unfortunately, treatment with amphotericin B, especially for long-term periods, can lead to adverse effects in patients, or to the development of resistant organisms during the course of therapy (Kovacicova et al., 2001). In the quest for new antifungal agents, low toxicity and broad spectrum fungicidal activities are needed for effective management of the infections.

Eugenia caryophyllus Bullock & Harrison (clove) and *Acorus calamus* Linn. (sweet flag) have eugenol as a major constituent. These medicinal plants have been used in traditional medicine in Thailand and certain medical applications. Both plants have been reported to possess inhibitory properties to filamentous fungi in vitro (Hitokoto et al., 1980; Tragoolpua, 1996).

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Only limited knowledge is available regarding the antifungal activities of the plant, which is also used for other technological purposes. Therefore, the aim of this study was to determine the antifungal activities of some medicinal plant extracts against clinical isolates of *C. neoformans* and *C. albicans* by using the broth microdilution method.

MATERIALS AND METHODS

Plant Material

Flowers of *Eugenia caryophyllus* Bullock & Harrison (clove) and rhizomes of *Acorus calamus* Linn. (sweet flag) were selected for study.

Plant extracts were prepared as follows. Air dried plant materials (200 g) were finely ground before being infused in 95% ethanol and sonicated in an ultrasonic bath (Bandelin Sonorex super RF 510H) for 30 min. The extracts were then filtered through Whatman filter paper No.1. The filtrate was evaporated and concentrated using a rotary vacuum evaporator (Fabry et al., 1996; Tragoolpua, 1996). The concentrated plant material was then soaked in 10 ml 95% ethanol. Finally, the ethanolic extracts were dried, weighed and kept at -20°C in sterile bottles.

Fungal Isolation

Fifty-three clinical isolates (28 of *C. albicans* and 25 of *C. neoformans*) were isolated from oral, vaginal, urine and cerebrospinal fluid of human immunodeficiency virus (HIV) – positive or – negative patients from Maharaj Nakorn Chiang Mai and Chiang Rai regional hospital, Thailand. The reference strain, *C. albicans* ATCC 90028, was included in all susceptibility tests as a control. The isolates were identified according to a standard procedure (Mahon and Manuselis, 1995) and cultured on Sabouraud dextrose agar (SDA) plates (BBL, Cockeysville, Md) at 35 °C for 24-48 h to ensure optimal growth before testing.

Assay Medium (Cormican and Pfaller, 1996; National Committee for Clinical Laboratory Standards,1997.)

RPMI 1640 powder (with L-glutamine, without bicarbonate; BIOCHOM KG, Leonorenstr.2-6. D-12247 Berlin) was prepared in distilled water and adjusted to pH 7.0 with 0.165 M morpholinopropanesulfonic acid (MOPS, Sigma). This assay medium was filter sterilized by using 0.2 μm millipore size filters (Acrodisc® 32, Gelman Sciences), aliquoted and stored at 4 °C until use.

Drug and Medicinal Plant Extracts Preparation

Ten serial twofold dilutions in RPMI 1640 of AmB, eugenol and two medicinal plant extracts were prepared from stock solutions and arranged in rows, as well as a growth control well (without drug) and a purity control well, which contained yeast-free medium (Cormican and Pfaller, 1996). Stock solutions of AmB deoxycholate (Fungizone, Squibb Industria Farmaceutica S.A., Esplugues-Barcelona, Spain) and eugenol were prepared at 16,000 μg/ml and 500 mg/ml in dimethyl sulfoxide (Sigma), respectively. The final concentration ranges used were 1.6 to 0.003 μg/ml for AmB, 50 to 0.98 mg/ml for eugenol and 75.0 to 0.15 mg/ml for both ethanol plant extracts.

Inocula Preparation

Yeast inocula were prepared as previously described (Archiesi et al., 1994; Anaissie et al., 1996; Cormican and Pfaller, 1996). Briefly, yeast was grown on Sabouraud dextrose agar plates for 24 h (*C. albicans*) or 48 h (*C. neoformans*). For each isolate, five colonies were grown until their diameters were at least 1 mm. Then, the colonies were picked off and suspended in 0.85% saline solution. The suspension was adjusted to the turbidity of a 0.5 McFarland standard at a wavelength of 530 nm. Quantitative colony plate counts were determined on SDA to verify the inoculum size. Testing of antifungal activity was performed in 96-well round-bottomed microtitration

plates (Nunc^{TM}, Denmark). Microdilution wells were inoculated with 100 µl of yeast suspension in RPMI 1640 medium. The final inoculum concentrations were approximately 5.0 X 10^2 to 2.5 X 10^3 blastoconidia/ml for *C. albicans* and 5.0 X 10^3 to 2.5 X 10^4 blastoconidia/ml for *C. neoformans* after dilution with 100 µl of either the drug solution or extract. The inoculated plates were incubated at 35°C for 24 h for *C. albicans* and 72 h for *C. neoformans*. Two replicate plates were used for each treatment.

Time of Reading (Cormican and Pfaller, 1996)

MICs were determined after incubation for 24 and 72 h at 35°C for *C. albicans* and *C. neoformans,* respectively

Endpoint Determination

Growth of yeasts in each well was estimated visually and then scored as previously described (Anaissie et al., 1996). Briefly, 0, optically clear; 1+, slightly hazy, i.e., turbidity of more than 0-25% compared to the drug-free growth control; 2+, turbidity of more than 25 to 50% of growth control; 3+, turbidity of more than 50 to 75% of growth control; and 4+, turbidity of more than 75 to 100% of growth control.

Minimum fungicidal concentration (MFC) experiments were adapted from the method of McGinnis (1980). Briefly, 100 µl aliquots from tubes that showed growth inhibition were plated on to SDA plates. The lowest drug concentration that yielded fewer yeast colonies was recorded as the MFC.

Statistical Analysis

Data were analyzed and treatments compared using student's t-test analysis.

RESULTS AND DISCUSSION

In this report, the various concentrations of crude ethanol extracts of clove and sweet flag, and eugenol tested were compared to the standard AmB. The results of the experiments are summarized in Tables 1 and 2. Both medicinal plant extracts showed antifungal activities against *C. albicans* and *C. neoformans.* In table 1, the average MICs of clove, sweet flag, eugenol and AmB against *C. albicans* were 17.41±8.64 mg/ml, 28.8±16.32 mg/ml, 12.16±4.53 mg/ml and 0.23±0.1 µg/ml, respectively. Likewise, the same extracts, eugenol and antifungal drugs which were tested against *C. albicans,* were also tested against *C. neoformans.* The average MICs were 2.43±0.95 mg/ml, 3.02±1.97 mg/ml, 6.28±3.4 mg/ml and 0.28±0.15 µg/ml, respectively. Sweet flag showed a broad range of activity against *C. albicans*, whereas eugenol showed a broad range of activity against *C. neoformans*. Generally, the average MICs of eugenol, clove and sweet flag showed that they were less effective in inhibiting the growth of *C. albicans* than *C. neoformans* (Table 1).

Moreover, the average MFCs of clove, sweet flag, eugenol and AmB against *C. albicans* were 67.5±15.39 mg/ml, >75 mg/ml, 15.4±6.47 mg/ml and 0.47±0.21 µg/ml respectively. Similarly, the MFCs against *C. neoformans* were 22.22±12.71 mg/ml, 30.82±27.11 mg/ml, 10.06±4.9 mg/ml and 0.51±0.25 µg/ml, respectively (Table 2).

Studies reported by Pabla *et al* (1997) and Teissedre *et al* (2000) indicated that essential oils were natural products extracted from plant materials, which can be used as antibacterial, antifungal, antioxidant, and anti-carcinogenic agents or to preserve and give specific flavors to foods. The susceptibility of yeast to ethanol crude extracts of clove and sweet flag was expected because of eugenol, which is the major constituent of both medicinal plants. The ethanol crude extracts of both plants were fractionated by thin layer chromatography (TLC) and compared to eugenol. The data showed that the plant extracts had a component, which, like eugenol, had an Rf value of 0.4. Therefore, this result indicated that eugenol is a constituent of both plants. Eugenol is a derivative of phenol that effectively kills vegetative cells of bacteria and spores by causing membrane damage and leakage of cytoplasmic contents in the cells (Lim, 1998), inhibition of enzyme activities and denaturation of protein (Sooksringam, 1985).

The results showed that *C. albicans* was significantly ($p<0.01$) more susceptible to the extract of clove than sweet flag, whereas *C. neoformans* was significantly susceptible to the clove extract ($p>0.05$). Moreover, the extract of clove showed significantly ($p<0.01$) more potent inhibitory activity against *C. neoformans* than eugenol, whilst it showed significantly ($p<0.01$) less inhibitory activity against *C. albicans* than eugenol.

In conclusion, comparison of the standard antimycotic agent, amphotericin B, to the extracts showed that the latter had low antimycotic activity. However, it is important to point out that the extracts of clove and sweet flag were potential agents against yeasts whereas AmB and eugenol showed fungicidal activities.

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Tables

Table 1. Average MIC of AmB, eugenol, clove and sweet flag extracts against *C. albicans* and *C. neoformans.*

Organisms	MIC			
	AmB (μg/ml)	**Eugenol (mg/ml)**	**Clove (mg/ml)**	**Sweet flag (mg/ml)**
C .albicans	0.23±0.10	12.16±4.53	17.41±8.64	28.80±16.30
C. neoformans	0.28±0.15	6.28±3.40	2.43±0.95	3.02±1.97

Table 2. Average MFC of AmB, eugenol, clove and sweet flag extracts against *C. albicans* and *C. neoformans.*

Organisms	MFC			
	AmB (μg/ml)	**Eugenol (mg/ml)**	**Clove (mg/ml)**	**Sweet flag (mg/ml)**
C. albicans	0.47±0.21	15.40±6.47	67.50±15.39	>75.0
C. neoformans	0.51±0.25	10.06±4.90	22.22±12.71	30.82±27.11