Research Article

Fungistatic Property of *Eugenia caryophyllus* Bullock & Harrison and *Acorus calamus* Linn. Extracts Against *Candida albicans* and *Cryptococcus neoformans*

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Abstract

Objective: To investigated the anti-fungal activity of two medicinal plant extracts comparing with eugenol and amphotericin B (AmB) by using the National Committee for Clinical Laboratory Standards (NCCLs) M27-P broth microdilution method.

Materials and methods: The ethanol extracts of clove (*Eugenia caryophyllus* Bullock & Harrison) and sweet flag (*Acorus calamus* Linn.), eugenol and amphotericin B were determined for minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) against 28 clinical isolates of *Candida albicans* and 25 clinical isolates of *Cryptococcus neoformans.*

Results: 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 or antifungal drugs, which tested against *C. albicans* were also determined 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* were statistically significant ($p<0.01$) more susceptible to the extract of clove than sweet flag, whereas *C. neoformans* were not statistically significant 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* and *C. albicans* than eugenol.

Conclusion: These data indicated that the extracts of clove and sweet flag were potential fungistatic agents against yeasts whereas AmB and eugenol showed fungicidal effects. Bull Chiang Mai Assoc Sci 2001; 34: 89-97.

Key words: Clove, sweet flag, antifungal susceptibility, *Candida albicans*, *Cryptococcus neoformans*

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บทคัดย่อ : คุณสมบัติต้านเชื้อราของสารสกัดกานพลูและว่านน้ำต่อเชื้อ *Candida albicans* และ *Cryptococcus neoformans*

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วัตถุประสงค์ : เพื่อศึกษาคุณสมบัติต้านการเจริญเติบโตของเชื้อราของสารสกัดจากพืชสมุนไพรสองชนิดเปรียบเทียบกับยูจีนอลและยาแอมโฟเทอริซิน บี โดยใช้วิธี Broth dilution ของ National Committee for Clinical Laboratory Standards (NCCLs) M27-P

วัสดุและวิธีการ : ทำการสกัดพืชสมุนไพรสองชนิดได้แก่ กานพลูและว่านน้ำด้วยแอลกอฮอล์ แล้วนำสารสกัดทั้งสองชนิดมาทดสอบหาค่าความเข้มข้นต่ำสุดที่ยับยั้งการเจริญเติบโต (MIC) และทำลายเชื้อรา (MFC) เปรียบเทียบกับยูจีนอลและยาแอมโฟเทอริซินโดยศึกษาคุณสมบัติดังกล่าวกับเชื้อ *Candida albicans* และ *Cryptococcus neoformans* จำนวน 28 และ 25 สายพันธุ์ ตามลำดับ

ผลการทดลอง : พบว่าค่า MICs และ MFCs ของสารสกัดจากกานพลูและว่านน้ำด้วยแอลกอฮอล์ ยูจีนอลและแอมโฟเทอริซิน บี ต่อเชื้อ *C. albicans* ได้แก่ 17.41±8.64 mg/mL, 28.8±16.32 mg/mL, 12.16±4.53 mg/mL, 0.23±0.1 µg/mL และ 67.5±15.39 mg/mL, >75 mg/mL, 15.4±6.47 mg/mL, 0.47±0.21 µg/mL ตามลำดับ สำหรับค่า MICs และ MFCs ของสารสกัดจากกานพลูและว่านน้ำด้วยแอลกอฮอล์ ยูจีนอลและแอมโฟเทอริซิน บี ต่อเชื้อ *C. neoformans* มีค่าเป็น 2.43±0.95 mg/mL, 3.02±1.97 mg/mL, 6.28±3.4 mg/mL, 0.28±0.15 µg/mL และ 22.22±12.71 mg/mL, 30.82±27.11 mg/mL, 10.06±4.9 mg/mL, 0.51± 0.25 µg/mL ตามลำดับ

จากผลการทดลองดังกล่าวแสดงให้เห็นว่าสารสกัดจากกานพลูมีฤทธิ์ดีกว่าสารสกัดจากว่านน้ำในการยับยั้งการเจริญเติบโตอย่างมีนัยสำคัญทางสถิติต่อเชื้อ *C. albicans* ($p<0.01$) และไม่มีความแตกต่างกันอย่าง

มีนัยสำคัญทางสถิติสำหรับเชื้อ *C. neoformans* ($p>0.05$) นอกจากนั้นยังพบว่า สารสกัดจากกานพลูมีประสิทธิภาพในการยับยั้งการเจริญเติบโตของเชื้อ *C. albicans* และ *C. neoformans* ดีกว่ายูจีนอลอย่างมีนัยสำคัญทางสถิติ ($p<0.01$) อีกด้วย

สรุปผลการทดลอง : สารสกัดจากกานพลูและว่านน้ำด้วยแอลกอฮอล์มีคุณสมบัติในการยับยั้งการเจริญเติบโตของเชื้อราที่นำมาใช้ในการทดสอบ ขณะที่ยูจีนอลและยาแอมโฟเทอริซิน บีมีคุณสมบัติ ดีกว่าในการทำลายเชื้อราว วารสารเทคนิคการแพทย์เชียงใหม่ 2544; 34: 89-97.

คำหัส : กานพลู, ว่านน้ำ, การทดสอบความไวต่อสารต้านเชื้อรา, *Candida albicans, Cryptococcus neoformans*

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Introduction

Since the incidence of fungal infections has increased significantly in the last 20 years[1]. In immunocompromised patients, the emergence of candida infections with both primary drug resistance and the secondary development of azole-resistant Candida spp. isolates have been described[2,3,4]. Amphotericin B has been provided for the standard treatment of the most systemic fungal infections[5]. Unfortunately, treatment with amphotericin B, especially for long-term periods, can lead to its adu/rse effects to patients, or development of resistant organisms during the course of therapy[6]. 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 component. These medicinal plants have been used for the traditional medicine in Thailand and certain medical applications. Both plants have been reported to possess inhibitory properties to filamentous fungi *in vitro*[7,8].

Only limited knowledge is available regarding the antifungal activities of plant. Therefore, the aim of this study was to determine the antifungal activities of *Eugenia caryophyllus* Bullock & Harrison (Clove) and *Acorus calamus* Linn. (Sweet flag) extracts against clinical isolates of *C. neoformans* and *C. albicans* by broth microdilution method.

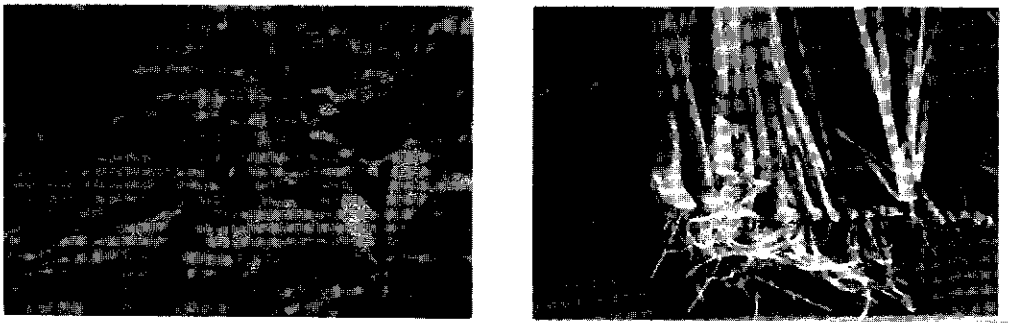
Materials and methods

Plant materials

Flowers of *Eugenia caryophyllus* Bullock

& Harrison (Clove) and rhizomes of *Acorus calamus* Linn. (Sweet flag) were selected to study (Fig.1).

Plant extracts were prepared as follows. Air dried plant materials (200 g) were finely ground. Then, the plant materials were infused in 95% ethanol and sonicated with 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 under rotary vacuum evaporator[8,9]. The concentrated plant material was then soaked in 10 ml of 95% ethanol. Finally, the ethanolic extracts were dried, weighted and kept at -20°C in steriled bottles.



A) B)

Fig. 1 The medicinal plants were used in this study.
A) Flowers of *Eugenia caryophyllus* Bullock & Harrison (Clove)
B) Rhizomes of *Acorus calamus* Linn. (Sweet flag)

Fungal isolation

Fifty-three clinical isolates (28 of *C.albicans* and 25 of *C. neoformans*) were isolated from oral, vaginal, urine and cerebro-spinal fluid of 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 standard procedure[10] 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[11,12]

RPMI 1640 powder (BIOCHOM KG) was prepared in distilled water and adjusted pH to 7.0 with 0.165 M morpholinopropane-sulfonic 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 Amphotericin B (AmB), eugenol and two medicinal plant extracts were prepared from stock solution and arranged in rows as well as a growth control well (Without drug) and a purity control well which contained yeast-free medium[11]. Stock solution of AmB deoxycholate (Fungizone, Squibb Industria Farmaceutica S.A.) and eugenol were prepared at 16,000 µg/mL and 500 mg/mL in dimethyl sulfoxide (Sigma), respectively. The final concentration ranges 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 medicinal plant extracts.

Inocula preparation

Yeast inocula were prepared as previously described[11,13,14]. Briefly, yeast was grown in SDA plate for 24 h *(C. albicans)* or 48 h *(C. neoformans)*. For each isolate, five colonies were grown until the diameter of colonies was at least 1 mm. Then, the colonies were picked 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 count was determined on SDA to verify the inoculum size. Testing of antifungal activity was performed in 96-well round-bottom microtitration plates (Nunc™). Microdilution wells were inoculated with 100 µL of yeast suspension in RPMI 1640 medium. The final inoculum concentration of approximately $5.0X10^2$ to $2.5X10^3$ blastoconidia/mL for *C. albicans* and 5.0X103 to $2.5X10^4$ blastoconidia/mL for *C. neoformans* after dilution with 100 µL of the drug solutions or extracts. The inoculated plates were incubated at 35°C, 24 h for *C. albicans* and 72 h for *C. neoformans*. Two replicated plates were used for each treatment.

Time of reading[11]

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[13] as, 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[15]. Briefly, 100 µL aliquots from tubes that showed their 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 compared using student's t-test analysis.

Results

In this report, the various concentrations of ethanol crude extracts of clove, sweet flag and commercial eugenol tested were comparable to the standard AmB. The results of the experiments were summarized in Table 1 and 2. Both medicinal plant extracts have shown antifungal activities against *C. albicans* and *C. neoformans*. In Table1, the average MICs (Mean±SD) 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 tested against *C. albicans* were also determined against *C. neoformans*. The average MICs (Mean±SD) 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 activity against *C. albicans*, whereas eugenol showed a broad range activity against *C. neoformans*. Generally, the average MICs of eugenol, clove and sweet flag showed less effective in inhibiting the growth of *C. albicans* than *C. neoformans* (Table 1).

Moreover, the average MFCs (Mean±SD) 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 (Mean±SD) 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).

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

Organisms	MICs (Mean ± SD)			
	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 MFCs of AmB, eugenol, clove and sweet flag extracts against *C. albicans* and *C. neoformans.*.

Organisms	MICs (Mean ± SD)			
	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

Discussion

Studies reported by Pabla *et al*[16] and Teissedre *et al*[17] were indicated that an essential oil was natural products extracted from plant materials, which can be used as antibacterial, antifungal, antioxidants, and anti-carcinogenic agents or to preserve and give specific flavors to foods. The susceptibility of yeast to ethanol crude extracts of clove, sweet flag was expected from eugenol, which is a mainly constituent component in both medicinal plants. The ethanol crude extracts of both plant were separated by thin layer chromatography (TLC) comparable to eugenol. The data showed that the plants and eugenol had the same Rf, 0.4. Therefore, this result indicated that eugenol was constituent in both plant. Eugenol is a derivative of phenol that effectively kills vegetative cells of bacteria and spores by causing membrane damage, leakage of cytoplasmic contents in the cells [18], inhibition of enzyme activities and denaturation of protein[19].

The results showed that *C. albicans* were statistically significant ($p<0.01$) more susceptible to the extract of clove than sweet flag, whereas *C. neoformans* were not statistically significant 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 standard antimycotic agent; amphotericin B to extracts showed that extracts from both medicinal plants had low antimycotic activity. However, it is important to point out that the extracts of clove and sweet flag were potential fungistatic agents against yeasts whereas AmB and eugenol showed fungicidal activities.

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