行政院國家科學委員會補助專題研究計畫成果報告

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計畫類別：☒個別型計畫　　□整合型計畫
計畫編號：NSC89－2320－B－040－023　－
執行期間：88　年　8　月　1　日至　89　年 7　月 31　日

計畫主持人：殷梅津
共同主持人：曹世明

本成果報告包括以下應繳交之附件：
- □赴國外出差或研習心得報告一份
- □赴大陸地區出差或研習心得報告一份
- □出席國際學術會議心得報告及發表之論文各一份
- □國際合作研究計畫國外研究報告書一份

執行單位：中山醫學院

中　華　民　國　89　年　10　月　20　日

國科會八十九年度專題研究計畫成果報告

計畫名稱:食用植物抑制麴菌及念珠菌生長及臨床應用之研究
編號: NSC89-2320-B-040-023
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執行機關: 中山醫學院營養學系

一、中文摘要:

本實驗室旨在探討數種食用植物抑制院內黴菌感染的臨床效應。過去的研究發現許多蔥科植物、香辛料植物及酚類化合物都可以有效的抑制數種臨床常見的麴菌及念珠菌的生長。因而認為這些日常食用的蔥科植物、香辛植物及其成份應該有臨床應用的可能。而且這些食用植物在臨床應用上也有著高安全性、高接受性、低副作用、低成本等優點。

關鍵字: 食用植物、黴菌感染

Abstract

Candida species and Aspergillus species are two major fungi responsible for the nosocomial fungal infections occurred in many hospitals. The clinical antifungal agents, amphotericin B and azole compounds, are seldom effective because of severe adverse reactions such as renal toxicity. Therefore, our laboratoty are looking for the edible plant foods with antifungal activity. The dependence, required dose and side-effect of these drugs can be reduced if patients receive the antifungal components from foods.

Keywords: plant foods, fungal infection

二、緣由與目的:

由於抗生素的大量濫用使得院內黴菌感染(如念珠菌血症 Candidemia,麴菌瘤 Aspergilloma)的機會大增。引發院內黴菌感染較常見的菌種為念珠菌(*Candida*)及麴菌(*Aspergillus*)兩大類。其中又以 *Candida krusei*, *C. glabrata*, *Aspergillus flavus*, *A. fumigatus* 最為頑強。用來治療這些黴菌感染的藥物為 amphotericin B 及 azole 類的藥物,但是由於這些藥物因副作用(如腎毒性)較強而在使用時有許多限制。因此,本實驗室旨在探討數種食用植物及其成份抑制院內黴菌感染之念珠菌及麴菌的療效。

本研究的目的有二: 1) 找尋具有抑制黴菌生長的食用植物,確認其抑菌成份,並研討食物及其抑菌成份與藥物間的交互作用。2) 將

食用植物及其抑菌成份應用於臨床之感染患者以肯定其單獨療效或與藥物間的輔助療效。

三、結果與討論:

本研究的結果顯示許多食用植物的萃出液及其內的特定成份，如薑黃素(curcumin)不但可以殺死這些黴菌，還可以與 amphotericin B、fluconazole (臨床上用來治療黴菌感染的藥物)產生加成(additive)或加乘(synergistic)效果(附件一)。如此不但可以抑制黴菌生長，還可以減少藥物的使用劑量。

本研究中發現大蒜和韭菜的主要抑黴菌成份是出現在其萃出液的油層。於是，本實驗室以大蒜和韭菜製作的 steam-distilled oil 來作抑菌的實驗。結果發現，這些 steam-distilled oil 對這些黴菌都有很強的抑制效果。然後，再經由成份分析確認這些 steam-distilled oil 中含有大量的 sulfides 及 thiosulfinates，其中 diallyl sulfide, diallyl disulfide, diallyl trisulfide, diallyl tetrasulfide 等成份都被確認具有抑制黴菌生長的功效(附件二)。

上述研究成果的臨床應用(即研究目的的第二項)，目前尚進行當中。

四、計畫成果自評:

在預定的時間內達成了擬定的研究目的的第一項。並將具體成果撰寫為兩份 manuscripts，並投稿至 SCI journals，其中一份已經刊登，另一份也已經被 revised。因而自評：成果尚佳。

五、參考文獻

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Journal of Food and Drug Analysis, Vol. 8, No. 3, 2000, Pages 208-212 藥物食品分析 第八卷 第三期

Enhanced Inhibitory Effect from Interaction of Curcumin with Amphotericin B or Fluconazole against *Candida* Species

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(Received: March 16, 2000; Accepted: June 27, 2000)

ABSTRACT

The antifungal activity of curcumin against seven *Candida* species was studied by investigating the growth of 200 clinical isolates from patients with fungal infections. The MICs of curcumin against *Candida* species were in the range of 32 to 128 μg/mL. The interaction of curcumin with amphotericin B or fluconazole against these fungi was determined by FIC index and % reduction in turbidity. Synergistic effect was shown in all combinations of curcumin and amphotericin B; whereas both synergistic and additive effects were observed in the combinations of curcumin and fluconazole. This evidence suggests that when curcumin is combined with amphotericin B or fluconazole, it could provide greater fungicidal effects for the treatment of systemic fungal infections such as candidiasis and candidemia.

Key words: curcumin, amphotericin B, fluconazole, *Candida* species

INTRODUCTION

Candidiasis and candidemia are very common nosocomial fungal infections occurring in many hospitals. Immunocompromised patients such as those with organ transplants, cancer, human immunodeficiency virus (HIV) infection or prolonged antibiotic treatments are susceptible to fungal infections[1-3]. These fungal infections might be fatal if antifungal treatment is not prescribed. The common isolates of candidiasis or candidemia are *Candida albican, C. krusei, C. glabrata, C. tropicalis*, and *C. guilliermondii*, in which *C. albican* is the most common; however, *C. krusei* and *C. glabrata* have become increasingly important for hospitalized patients[2-4].

Amphotericin B belongs to the class of polyenes and is a clinically popular antifungal agent. However, the clinical use of amphotericin B is limited because of severe adverse reactions such as diarrhea, malnutrition and progressive renal toxicity[5-8]. Azole compounds such as fluconazole (FCZ), itraconazole (ICZ) are another class of antifungal agents used for systemic fungal infections. These azoles are less toxic than amphotericin B[7,8]; however, some side effects of azoles have been reported[9]. In order to cure fungal infections successfully and to lower the dose of amphotericin B or azoles, there is a need for the development of less toxic antifungal agent, or to find one that is able to work with amphotericin B or azoles additively or synergistically.

Curcumin, a yellow phenolic compound isolated from turmeric (*Curcuma longa*), is responsible for the yellow color of turmeric and curry. Based on its safe property, it has long been used as a spice, food preservative and food coloring agent in India and Southeast Asia[10,11]. The content of curcumin in turmeric is 1-5% (or 4-8% of dry weight); and 40% in turmeric oleoresin[11]. Many studies have proven that curcumin has several important pharmacological properties such as antioxidant, antimutagenic and antitumor activities[12-14]. Therefore, it is being evaluated as a chemopreventive agent by the National Cancer Institute. Li *et al.*[15] indicated that curcumin could block HIV-1 replication by inhibiting the activity of its long terminal repeat; moreover, curcumin could work with a reverse transcriptase inhibitor (e.g. dideoxyinosine) on HIV-1 synergistically. Although curcumin is a potent anti-viral agent[15,19], it remains unknown whether curcumin is an antifungal agent for *Candida* species.

This study was aimed to assay the *in vitro* inhibitory effect of curcumin against seven *Candida* species. The interactions of curcumin with amphotericin B or fluconazole against these fungi were also studied.

MATERIALS AND METHODS

I. *Fungi Strains and Medium*

Seven *Candida* species (*Candida albican, C. krusei, C. tropicalis, C. kefyr, C. guilliermondii, C. parapsilosis, C. glabrata*) were isolated from patients with fungal infections such as candidiasis or candidemia in the Chungshan Hospital (Taichung, Taiwan). A total of 200 isolates were tested in this study. All isolates were identified by conventional methods[16]. All cultures were routinely maintained on Sabouraud dextrose agar (Difco, Detroit, MI) at 25°C before use.

II. *Antifungal Agents*

Curcumin was purchased from Sigma Chem. Co. (St. Louis, MO). Amphotericin B (AMB) and fluconazole (FCZ)

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were prepared from pharmaceutical solutions in sterile water. All solutions were filtered through 0.22 μM filter for sterilization.

III. *Antimicrobial Assays*

All agents were further diluted with RPMI 1640 medium (1:5, v/v). The broth macrodilution method was performed as described in National Committee for Clinical Laboratory Standards (NCCLs) document M27-A[17]. The final inoculum was 2×10^3 CFU/mL and was confirmed by plating 10 and 100 μL from the agent-free control tube onto Sabouraud dextrose agar. The final volume was 1 mL. The agent concentrations ranged from 256 to 0.0625 μg/mL. Agent-free and fungi-free controls were included. The turbidity was measured at 530 nm by a spectrophotometer after 48 hr incubation at 35°C in RPMI 1640 medium containing 0.165 M morpholinepropanesulfonic acid (MOPS) (pH 7.0). The MIC was defined as the concentration which produced an 80% reduction in turbidity, compared with that of controls. According to the standard of NCCLs, the isolates were classified as susceptible if the MIC was ≦ 8 μg/mL; resistant if the MIC was ≧ 64 μg/mL; susceptible but dose dependent if the MIC was 8~64 μg/mL.

IV. *Interaction of Curcumin with AMB or FCZ*

The effects of combinations of amphotericin B or fluconazole with curcumin were evaluated by the checkerboard method recommended by the NCCLs. One hundred μL aliquots of each drug at 10X the targeted final concentration was used. Drug interaction was classified as synergistic, additive or less-than-additive based on the fractional inhibitory concentration (FIC) index, which is the sum of FICs for each drug. The FIC of each drug was calculated as the MIC of this drug in combined treatment divided by that of the drug used alone. Drug-drug interactions are considered synergistic if the FIC index was less than 1.0; additive if the FIC was equal to 1.0; less-than-additive if the FIC index was greater than 1.0. The interaction of curcumin with amphotericin B or fluconazole were examined by combining 0.25, 0.5, 0.75 MIC AMB (or FCZ) with curcumin at various MIC values. The total MIC values in each combination were ≦ 1. The final inoculum was 2×10^3 CFU/mL and the final volume was 1 mL. The turbidity of each combination was then measured at 530 nm by a spectrophotometer after 48 hr incubation at 35°C in RPMI 1640 medium containing 0.165 M (MOPS) (pH 7.0).

RESULTS AND DISCUSSION

The MICs of curcumin, amphotericin B and fluconazole against *Candida* species are presented in Table 1. The inhibitory effect of amphotericin B and fluconazole against these *Candida* species has been studied[9,18]. It was reported that the MICs of amphotericin B and fluconazole were in the range of 0.125-2 and 0.25-128 μg/mL, respectively. The observed MICs (Table 1) of amphotericin B and fluconazole in our present study were close to those of previous studies. It was reported that fluconazole is inactive to *C. krusei* and the MIC90 was 128 μg/mL[9]. In our present study, the MIC80 of fluconazole against *C. krusei* was 128 μg/mL. This result supported that *C. krusei* was resistant to fluconazole.

The MIC80 of curcumin against the tested *Candida* species were in the range of 32-128 μg/mL (Table 1). Curcumin was found to be weaker when compared with amphotericin B or fluconazole. Although curcumin is a food component and amounts of up to 100 mg/day have been taken by certain people for long time[11], it remains unknown whether curcumin could achieve the blood concentrations of 32-128 μg/mL via oral or i.v. administration. Moreover, further *in vivo* studies are needed to prove the safety of curcumin at these concentrations. The interaction of curcumin with amphotericin B or fluconazole, determined as FIC index, is presented in Table 2. All interactions of curcumin and amphotericin B were synergistic because the FIC indexes were less than 1. Several interactions of curcumin with fluconazole were additive because the FIC indexes were equal to 1. These observed synergistic effects showed that the interaction of curcumin with either amphotericin B or fluconazole exhibited greater effect against *Candida* species. Both synergistic and additive effects observed in these combinations also suggest that the dosage of amphotericin B or fluconazole could be decreased. The interactions of curcumin with amphotericin B or fluconazole, determined as % reduction in turbidity, are presented in Tables 3 and 4. Many combinations of amphotericin B (or fluconazole) plus curcumin demonstrated ≧ 80% reduction in turbidity. In this study, the MIC of each agent against each tested fungi was defined as 80% reduction in turbidity. Therefore, the greater turbidity reduction observed in these combinations suggests that these combinations exhibited greater anti-*Candidal* effects than each

Table 1. MIC (μg/mL) of curcumin, amphotericin B (AMB) and fluconazole (FCZ) against *Candida* species

Fungal species (number of isolates)	Curcumin	AMB	FCZ
C. albican (52)	32 ± 2	0.125 ± 0.06	0.5 ± 0.5
C. krusei (30)	128 ± 8	1.0 ± 0.5	128.0 ± 16.0
C. tropicalis (27)	48 ± 2	0.125 ± 0.06	1.0 ± 0.25
C. kefyr (25)	96 ± 4	0.25 ± 0.125	4.0 ± 0.5
C. guilliermondii (21)	108 ± 8	0.5 ± 0.25	32.0 ± 2.0
C. parapsilosis (20)	64 ± 4	0.25 ± 0.125	2.0 ± 0.5
C. glabrata (25)	80 ± 4	0.5 ± 0.25	16.0 ± 2.0

MIC was determined according to the macrodilution method recommended by NCCLs and was defined as 80% reduction in turbidity. The concentration is expressed as mean ± standard deviation (n=5).

Table 2. Interaction of curcumin with amphotericin B (AMB) or fluconazole (FCZ), determined as FIC index

Fungal species (number of isolates)	FIC			FIC		
	AMB	Curcumin	Index	FCZ	Curcumin	Index
C. albican (52)	0.5	0.25	0.75	0.25	0.5	0.75
C. krusei (30)	0.75	0.125	0.875	0.5	0.5	1
C. tropicalis (27)	0.5	0.25	0.75	0.25	0.5	0.75
C. kefyr (25)	0.25	0.5	0.75	0.75	0.25	1
C. guilliermondii (21)	0.25	0.5	0.75	0.5	0.5	1
C. parapsilosis (20)	0.5	0.25	0.75	0.25	0.5	0.75
C. glabrata (25)	0.5	0.25	0.75	0.25	0.625	0.875

The interaction of curcumin with AMB or FCZ was evaluated by the checkerboard method recommended by the NCCLs and expressed as the sum of fractional inhibitory concentration (FIC) index for each agent. The FIC of each agent is calculated as the MIC of this agent in combination divided by the MIC of this agent alone.

Table 3. Interaction of curcumin with amphotericin B (AMB), determined as % reduction in turbidity.

Fungal species (numberof isolates)	AMB 0.75 MIC			AMB 0.5 MIC			AMB 0.25 MIC		
	Curcumin			Curcumin			Curcumin		
	0.0625 (MIC)	0.125	0.25	0.125	0.25	0.5	0.25	0.5	0.75
C. albicans (52)	80 ± 3	90 ± 2	97 ± 3	71 ± 4	80 ± 3	96 ± 2	64 ± 4	85 ± 2	96 ± 2
C. krusei (30)	68 ± 4	79 ± 3	90 ± 2	62 ± 5	77 ± 2	88 ± 2	57 ± 5	75 ± 3	89 ± 3
C. tropicalis (27)	78 ± 2	87 ± 2	96 ± 2	68 ± 4	79 ± 3	94 ± 2	67 ± 3	84 ± 2	95 ± 2
C. kefyr (25)	75 ± 3	84 ± 2	93 ± 2	67 ± 3	76 ± 3	91 ± 3	57 ± 5	81 ± 3	93 ± 3
C. guilliermondii (21)	70 ± 3	82 ± 3	92 ± 3	63 ± 2	74 ± 2	93 ± 2	55 ± 4	80 ± 5	90 ± 1
C. parapsilosis (20)	78 ± 3	84 ± 2	95 ± 3	69 ± 4	80 ± 3	95 ± 2	62 ± 3	85 ± 3	94 ± 2
C. glabrata (25)	72 ± 4	82 ± 2	94 ± 2	66 ± 4	78 ± 3	92 ± 1	59 ± 4	83 ± 2	92 ± 3

AMB at 0.25, 0.5, 0.75 MIC was combined with curcumin at various MIC values. The total MIC values in each combination were ≦ 1. The turbidity of each combination was measured and expressed as mean ± standard deviation (n=5).

Table 4. Interaction of curcumin with fluconazole (FCZ), determined as % reduction in turbidity

Fungal species (number of isolates)	FCZ 0.75 MIC			FCZ 0.5 MIC			FCZ 0.25 MIC		
	Curcumin			Curcumin			Curcumin		
	0.0625 (MIC)	0.125	0.25	0.125	0.25	0.5	0.25	0.5	0.75
C. albicans (52)	77 ± 3	85 ± 2	90 ± 3	68 ± 4	76 ± 3	91 ± 2	61 ± 4	82 ± 3	91 ± 2
C. krusei (30)	57 ± 4	73 ± 2	82 ± 2	53 ± 4	68 ± 2	80 ± 2	50 ± 5	71 ± 2	82 ± 1
C. tropicalis (27)	76 ± 4	84 ± 3	91 ± 3	64 ± 3	72 ± 2	87 ± 3	59 ± 3	78 ± 3	90 ± 3
C. kefyr (25)	73 ± 2	80 ± 2	88 ± 3	61 ± 3	69 ± 2	86 ± 3	54 ± 4	75 ± 3	87 ± 3
C. guilliermondii (21)	68 ± 3	76 ± 4	85 ± 2	59 ± 4	88 ± 3	85 ± 2	51 ± 2	74 ± 2	86 ± 2
C. parapsilosis (20)	75 ± 3	80 ± 3	87 ± 3	67 ± 3	78 ± 2	88 ± 1	58 ± 2	83 ± 2	88 ± 3
C. glabrata (25)	70 ± 3	78 ± 2	86 ± 2	65 ± 4	75 ± 2	86 ± 2	53 ± 3	80 ± 3	87 ± 2

FCZ at 0.25, 0.5, 0.75 MIC was combined with curcumin at various MIC values. The total MIC values in each combination were ≦ 1. The turbidity of each combination was measured and expressed as mean ± standard deviation (n=5).

agent at 1 MIC. The various combinations included 0.75 MIC AMB (or FCZ) plus 0.25 MIC curcumin; 0.5 MIC AMB (or FCZ) plus 0.5 MIC curcumin; 0.25 MIC AMB (or FCZ) plus 0.75 MIC curucmin. It should be pointed out that the sum of MICs in the above combinations was ≦ 1. Since these combinations offered a similar or greater inhibitory effect than 1 MIC AMB or 1 MIC FCZ, the use of these combinations not only enhanced the overall fungicidal effect but also lowered the dosage of AMB or FCZ, which could reduce the risk of drug-induced cytotoxicity. These advantages should be beneficial in the treatment of candidiasis or candidemia.

An interesting finding is that 0.75 MIC AMB plus 0.25 MIC curcumin, 0.5 MIC AMB plus 0.5 MIC curcumin, and 0.25 MIC AMB plus 0.75 MIC curcumin resulted in similar fungicidal effects; indicating these combinations resulted in ≧ 85% reduction in turbidity for *C. krusei* and ≧ 90% reduction in turbidity for other tested *Candida* species (Table 3). As shown in Table 4, 0.25 MIC FCZ plus 0.75 MIC curcumin also offered similar inhibitory effect as 0.25 MIC AMB plus 0.75 MIC curcumin. Accordingly, in order to decrease the side effects of AMB (or FCZ) and to enhance the overall fungicidal effect against these *Candida* species, 0.25 MIC AMB (or FCZ) plus 0.75 MIC curcumin would be the best choice for clinical use, since the dosage of AMB (or FCZ) was very low.

The fungal cytotoxicity of amphotericin B is due to the interaction of this drug with fungal membrane ergosterol over the mammalian cell counterpart, cholesterol[6]. Like other azole compounds, the fungal cytotoxicity of fluconazole results from its binding to cytochrome p-450 molecules

involved in the synthesis of fungal ergosterol[20,21]. The failure of ergosterol synthesis then leads to the death of fungi. It has been reported that the anti-tumor effect of curcumin was due to the fact that this agent blocked arachidonic acid metabolism by inhibiting cycloxygenase and/or lipoxygenase activities[22,23]. The action mode of curcumin against fungi might be also due to its enzyme inhibitory effects, which is apparently different from that of amphotericin B. This different action mode of curcumin from amphotericin B could account in part for the enhanced inhibitory effect observed in these combinations. Nevertheless, it is not the only determinant because the effect of combined therapy was not simply additive. Further study is necessary to elucidate the fungicidal mechanism when these two agents cooperate.

Pharmacokinetic studies have indicated that following oral administration to rats and humans, curcumin was poorly absorbed and was transformed into metabolites during absorption through the intestine[24]. The major metabolites of curcumin in mice are curcumin glucuronide, dihydrocurcumin glucuronide, tetrahydrocurcumin[25]. It remains unknown whether these metabolites still possess antifungal activity like curcumin. However, the work of Shoba *et al.*[26] reported that piperine (20 mg), a major component of black pepper (*Piper nigrum* L.), remarkably enhanced the bioavailability of curcumin in humans with no adverse effects. Therefore, when curcumin is orally administrated as an antifungal agent, the concomitant use of piperine might be considered. Otherwise, i.v. administration of curcumin should be a better route for its efficacy because amphotericin B or fluconazole could be administrated via this method.

In conclusion, the combination of curcumin with amphotericin B or fluconazole exhibited a stronger fungicidal activity than monotherapy with curcumin, amphotericin B or fluconazole, respectively. The enhanced fungicidal effect observed in combined therapy suggests that the interactions between curcumin and these two agents were more than additive. These results suggest that the combined therapy of curcumin with one of these two agents may benefit the treatment of clinical fungal infections.

ACKNOWLEDGMENT

This research was supported by a grant from the National Science Council, ROC [NSC 89-2320-B-040-023].

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薑黃素與Amphotericin B或Fluconazole共同使用增強抑制念珠菌之功效

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摘 要

七種院內感染的念珠菌（共200隻取自臨床黴菌感染病患的菌株）被使用來探討薑黃素的單獨抑菌能力，及其與amphotericin B或fluconazole共同使用時的抑菌效果。此一共同使用時的抑菌效果以FIC index及turbidity降低的%來表示。結果發現，薑黃素對這七種念珠菌的最低抑制濃度為32-128 μg/mL。薑黃素與amphotericin B共同使用時則表現出加乘效果;而薑黃素與fluconazole共同使用時，對某些菌表現出加乘效果，但是對某些菌卻表現出加成效果。由於薑黃素與amphotericin B或fluconazole共同使用時可以因這些加乘或加成效果而減少amphotericin B或fluconazole的使用劑量，如此也可降低因這兩種藥物所誘發的副作用。本研究結果支持薑黃素與amphotericin B或fluconazole共同使用將有助於院內念珠菌感染的治療。

關鍵詞：薑黃素，amphotericin B，fluconazole，念珠菌

附件二

In Vitro Anti-MRSA and Antifungal Activities of Four Diallyl Sulfides Naturally Occurring in Garlic and Chinese Leek Oils

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running title: antimicrobial activity of four diallyl sulfides

Abstract

In vitro antimicrobial activities of garlic oil, Chinese leek oil and four diallyl sulfides naturally occurring in these oils against methicillin-resistant *Staphylococcus aureus* (MRSA), three *Candida* and three *Aspergillus* species (total 236 clinical isolates) were studied. Both anti-MRSA and antifungal activities of four diallyl sulfides followed the order diallyl tetrasulfide > diallyl trisulfide > diallyl disulfide > diallyl monosulfide ($p<0.05$). These results suggested that the involvement of disulfide bond is an important factor in determining the antimicrobial capabilities of these sulfides. The concentration of four diallyl sulfides in garlic and Chinese leek oils was in the range of 52.7~41.7% of total sulfides. Garlic oil with higher concentration of four diallyl sulfides showed greater antimicrobial activity than Chinese leek oil ($p<0.05$). These results provided laboratory evidence to support that diallyl disulfide, diallyl trisulfide, diallyl tetrasulfide and the oils rich in these sulfides may contribute to the prevention or treatment for nosocomial MRSA or fungal infections.

Keywords: MRSA, fungal infection, garlic oil, diallyl trisulfide, diallyl tetrasulfide

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA), *Candida* and *Aspergillus* species were the most commonly identified bacterial and fungal species responsible for the severe nosocomial infections occurred in Taiwan [1-3]. These infections not only require expensive antibiotic treatments but also increase the morbidity and mortality in hospitalized patients. In order to control these infections, there is a need for other agents with greater antimicrobial activity and less toxicity.

The antimicrobial activity and other medical benefits of garlic oil have been widely recognized [4-6]. These benefits were attributed to the presence of sulfides in garlic oil [5, 6]. Based on the advantages of easy to obtain or prepare as well as good stability, the medical properties of diallyl monosulfide and/or diallyl disulfide has been focused in many studies [7-10]. The chemical analysis of garlic oil showed that 54.5% of total sulfides were the sum of diallyl monosulfide, diallyl disulfide, diallyl trisulfide and diallyl tetrasulfide [11]. Although diallyl trisulfide and diallyl tetrasulfide were 26.6% of total sulfides in garlic oil, so far, little attention was paid to the medical benefits of these two agents.

The inhibitory effect of diallyl disulfide, not diallyl monosulfide, against *Candida albican* was observed in an *in vitro* study [12]. However, the information regarding the anti-*Aspergillus* and anti-MRSA activities of these two agents is limited. The inhibitory effect of garlic extract against wild type *Staphylococcus aureus* was observed [13]. However, it remains unknown whether garlic oil can inhibit MRSA and fungal pathogens. If so, which compounds in garlic oil responsible for this effect.

Like garlic, Chinese leek is a member of *Allium* family; and it is a vegetable commonly used in the oriental society. Besides garlic bulb and onion bulb, little attention was paid to the

medical contribution of other *Allium* plants. Recently, the antioxidant and antifungal activities of water extracts from Chinese leek and other *Allium* plants have been studied in our laboratory [14, 15]. Therefore, a continuous work regarding the antimicrobial activities of essential oils prepared from garlic and Chinese leek was processed.

This study was aimed to examine and compare the anti-MRSA and antifungal activities of four diallyl sulfides naturally occurring in garlic and Chinese leek oils. The results would be helpful for the development of new antibiotic agents or new functional foods.

Materials and methods.

Sample preparation

Garlic bulb (Allium sativum L.) and Chinese leek (Allium odorum L.) were directly purchased from farms. The method of Ravid and Putievsky [16] was used to prepare essential oil. Fresh plant materials were steam-distilled for 3 h in a 100 L direct steam pilot plant apparatus. The recovered oil (2.2~4.3 g oil / kg garlic bulb; 1.1~3.5 g oil / kg Chinese leek) was stored at -80℃ until used.

Standard preparation

Diallyl monosulfide (purity 97%) and crude diallyl disulfide (purity 80%) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Diallyl disulfide was further purified by fractional distillation and its final purity was > 98%, which was examined by HPLC. Diallyl trisulfide and diallyl tetrasulfide were obtained by fractional distillation from crude diallyl disulfide. The identification of diallyl trisulfide and diallyl tetrasulfide was confirmed by ^{1}H-NMR spectroscopy ($CDCl_3$, 300 MHZ) and it was coincide with the published data of Sparnins et al [17]. The prepared standards was stored at -80℃ until used.

Analysis of four diallyl sulfides in garlic and Chinese leek oil

One mg of essential oil prepared from each plant was redissolved in 10 ml of acetonitrile right before composition analysis. The method of Lawson et al. [11] was used to analyze the content of four diallyl sulfides in oils. Two μl sample was injected into C18-HPLC, which was conditioned as follow. Supelco LC-18, 250 mm x 4.6 mm x 5 μm column, acetonitrile/water/tetrahydrofuran (70/27/3), 1 ml/min, 240 nm. Prepared standards were used to identify and quantify these diallyl sulfides in oils prepared from garlic and Chinese leek.

Strains and medium

Methicillin-resistant *S. aureus*, three *Candida* species (*Candida albican*, *C. Krusei*, *C. glabrata*) and three *Aspergillus* species (*Aspergillus niger*, *A. flavus*, *A. fumigatus*) were isolated from patients with MRSA and/or fungal infections (candidiasis or aspergillosis) in Chungshan Hospital (Taichung, Taiwan). The total clinical isolates of MRSA and fungi were 60 and 176, respectively in this study. All isolates were identified by conventional methods [18]. All cultures were routinely maintained on nutrient agar or Sabouraud dextrose agar (Difco, Detroit, MI) at 25℃ until used.

Anti-MRSA tests

Prepared standards of diallyl monosulfide, diallyl disulfide, diallyl trisulfide, diallyl tetrasulfide and two essential oils were used for anti-MRSA test. Methicillin, penicillin, cefotaxime and tetracycline were purchased from Sigma Chem. Co. (St. Louis). Microdilution MIC was determined with strains grown in cation-adjust Mueller-Hinton broth according to National Committee for Clinical Laboratory Standards (NCCLs) guidelines [19]. The agent concentrations ranged from 128 to 0.125 μg/ml. MIC80, at

which 80% was inhibited, was determined. All incubations were at 35 ℃. Clavulanic acid at the concentration of 2 mg/L in the medium was used to verify the involvement of β-lactamase activity in these clinical isolates.

Antifungal tests

Prepared standards of diallyl monosulfide, diallyl disulfide, diallyl trisulfide, diallyl tetrasulfide and essential oils were also used for antifungal assay. Four diallyl sulfides and two essential oils were diluted 10-fold in polyethylene because of the poor solubility of these agents. All agents were further diluted 1:5 in RPMI 1640 medium. The broth macrodilution method was performed as described in NCCLs document M27-A [20]. The agent concentrations ranged from 128 to 0.125 μg/ml. Agent-free and fungi-free controls were included. The turbidity were measured by a spectrophotometer at 530 nm after 48 h incubation at 35 ℃ in RPMI 1640 medium containing 0.165 M morpholinepropanesulfonic acid (MOPS) (pH 7.0). The MIC was defined as the concentration which produced 80% reduction in turbidity, compared with that of agent-free controls after 48 h incubation in RPMI 1640 medium. Amphotericin B, purchased from Sigma Chem. Co. (St. Louis), was used for comparison in this antifungal test. Isolates were classified as susceptible if the MIC was $\leqq$ 8 μg/ml; resistant, if the MIC was > 64 μg/ml.

Statistical analysis

MIC was expressed as mean ± standard deviation of five experiments (n=5). Data were treated by analysis of variance (ANOVA) and computed using the SAS General Model procedure [21]. Difference among means was determined by the Least Significance Difference Test with significance defined at $p \leq 0.05$.

Results

The content of diallyl monosulfide, diallyl disulfide, diallyl trisulfide, diallyl tetrasulfide in garlic and Chinese leek oils is present in Table 1. The concentration of the four diallyl sulfides in garlic oil was higher than that in Chinese leek oil ($p<0.05$). The MIC values of four antibiotics, garlic oil, Chinese leek oil and four diallyl sulfides against 40 wild type *S. aureus* and 60 MRSA are present in Table 2. All test agents can inhibit the growth of wild type *S. aureus*, in which four antibiotics showed greater anti-*S. aureus* effects than two essential oils and four diallyl sulfides. However, the MIC values of four antibiotics (methicillin, penicillin, cefotaxime and tetracyline) against MRSA were > 64 μg/ml; and diallyl disulfide, diallyl trisulfide and diallyl tetrasulfide showed greater inhibitory effects than these antibiotics. The MIC values of two essential oils and four diallyl sulfides against six fungal pathogens are present in Table 3. The MIC values of amphotericin B against the 176 clinical fungal isolates were in the range of 0.25~4 (μg/ml) (data not shown). Therefore, the 176 fungal pathogens were not amphotericin B-resistant. Two essential oils, diallyl monosulfide and diallyl disulfide showed less antifungal activities than amphotericin B; however, diallyl trisulfide and diallyl tetrasulfide showed similar inhibitory effects as amphotericin B.

Discussion

It is reported that garlic possessed anti-*S. aureus* effect [13]. The results of the present study extended the antimicrobial activity of garlic oil to MRSA and six medically important fungi. Furthermore, the essential oil prepared from the other member in *Allium* family,

Chinese leek, also possessed similar antimicrobial capabilities. The MIC values of garlic and Chinese leek oils against MRSA and six fungal pathogens were < 64 μg/ml (Tables 2 and 3); therefore, these two oils could be considered as potent functional foods for clinical MRSA and fungal infection's prevention or therapy. Further study is necessary to examine the recover rate and concentration in circulation after these two agents are admitted orally.

Lawson et al. [11] reported that the sum of diallyl monosulfide, disulfide, trisulfide and tetrasulfide in garlic oil was 54.5% of total sulfides. In our present study, similar method was used to quantify the four diallyl sulfides and found that the sum of these sulfides in garlic oil was 52.7%, which was close to that of Lawson et al. [11]. Our present study also extended the four diallyl sulfides analysis to Chinese leek oil and conferred that these two oils contained high concentrations of these four diallyl sulfides. It should be pointed out that the concentration of diallyl monosulfide in these oils is very low (2.6~2.0 %) and the antimicrobial activity of this agent is not marked (Tables 2 and 3). Apparently, the contribution of this agent to the overall antimicrobial activities of these oils was mild. The concentration of other three diallyl sulfides (diallyl disulfide + diallyl trisulfide + diallyl tetrasulfide) in garlic oil was still significantly higher than Chinese leek oil. This may explain why garlic oil showed greater antimicrobial activities than Chinese leek oil in this study.

It is known that *S. aureus* can produce a serine protease called β-lactamase, and this enzyme can inactivate penicillin by hydrolyzing the β-lactam ring of this antibiotic [22, 23]. In our present study, β-lactamase was present in all clinical MRSA isolates. This may explain the resistance of these isolates to β-lactam agents such as penicillin, cefotaxime and tetracyclines. On the other hand, it is indicated that the resistance to methicillin confers resistance to all penicillinase-resistant penicillins and cephalosporins; and this resistance is due to the presence

of the *mec* gene that encodes penicillin-binding proteins (PBPs) [24, 25]. So far, altered forms of PBPs such as PBPs 1a, 2b have been implicated in the development of penicillin and cephalosporin resistance [24, 26]. In our present study, the six test agents showed weaker inhibitory effects against 40 wild type *S. aureus* than four antibiotics; however, showed greater inhibitory effects against MRSA than these antibiotics. Although it remains uncertain that the penicillin-binding proteins of these MRSA were denatured or destroyed by these six agents, it is sure that these six agents are not the substrate of β-lactamase. The MIC values of these agents against MRSA were significantly higher than against wild type *S. aureus*. Apparently, the presence of β-lactamase and/or penicillin-binding proteins in these MRSA also increased the bactericidal difficulty for these agents.

The inhibitory effect of diallyl monosulfide and diallyl disulfide against *Klebsiella pneumoniae*, an opportunist pathogen for nosocomial infection, has been observed [27]. These authors indicated that this inhibitory effect was due to the fact that these agents inhibit arylamine N-acetyltransferase activity of this organism, in which diallyl disulfide showed greater inhibitory effect than diallyl monosulfide. In the study of Naganawa et al. [12], the anti-*C. albican* activity of diallyl diulfide was significantly greater than diallyl monosulfide, too. These authors indicated that the disulfide bond of diallyl disulfide was important for its antifungal activity. In the present study, both diallyl monosulfide and diallyl disulfide showed anti-MRSA and antifungal effects, in which the antimicrobial activity of diallyl disulfide was also greater than diallyl monosulfide. Our results agreed those previous studies and supported that daillyl disulfide possesses stronger antimicrobial activity than diallyl monosulfide. The antifungal effect of diallyl trisulfide against *Cryptococcus neoformans* has been observed [28]. Our present study extended the antimicrobial activity of this agent to MRSA, *Candida* and

Aspergillus species (Tables 2 and 3) and observed that the antimicrobial activity of diallyl trisulfide was greater than diallyl disulfide. Furthermore, diallyl tetrasulfide showed the greatest antimicrobial activity in our present study. The number of disulfide bond in the four diallyl sulfides is 0, 1, 2 and 3, respectively. It is likely that the anti-MRSA or antifungal activity is correlated with this disulfide bond; thus, more disulfide bonds, greater antimicrobial activities. These results supported that the disulfide bond in these sulfides was an important factor in determining the antimicrobial activities.

The MICs of diallyl disulfide, diallyl trisulfide and diallyl tetrasulfide against MRSA, *Candida* and *Aspergillus* spp. were ≤ 12 μg/ml. Although these concentrations are higher than 8 μg/ml, a breakpoint for susceptible, these agents are the components naturally occur in certain foods such as garlic and Chinese leek. Therefore, these agents at these concentrations may be still in the safe range. Further *in vivo* study is necessary to evaluate the clinical application of these three agents for controlling MRSA or fungal infections.

In conclusion, diallyl disulfide, diallyl trisulfide, diallyl tetrasulfide and essential oils rich in these three sulfides possessed strong antimicrobial activities. These diallyl sulfides or oils were potent agents to prevent or treat MRSA or fungal infections.

Acknowledgement

This research was supported by a grant from the National Science Council, ROC [NSC 89-2320-B-040-023].

1 Table 1. Content[a] (μg/g) of four diallyl sulfides in garlic oils (GO) and Chinese leek oils
2 (CLO). Data were expressed as mean ± standard deviation (n=5).
3

	GO	CLO
DAS[b]	112 ± 7	104 ± 11
DADS	1183 ± 42	943 ± 45
DAT	751 ± 24	494 ± 37
DATS	368 ± 19	341 ± 21
Sum[c]	2414 ± 227	1882 ± 187
Total sulfides	4581 ± 383	4508 ± 432
%[d]	52.7	41.7

4 [a]Limit of detection is 5 μg/g.
5 [b]DAS=diallyl monosulfide; DADS=diallyl disulfide; DAT=diallyl trisulfide; DATS=diallyl
6 tetrasulfide.
7 [c]Sum = DAS + DADS + DAT + DATS.
8 [d]% = sum of four diallyl sulfides x 100 / total sulfides.

Table 2. Minimum inhibitory concentration (μg/ml) of four antibiotics, garlic oil, Chinese leek oil and four diallyl sulfides against 40 wild type *S. aureus* and 60 MRSA. Data were expressed as mean ± standard deviation (n=5).

Agents	*S. aureus*	MRSA
Methicillin	0.5 ± 0.25	> 64.0
Penicillin	0.5 ± 0.125	> 64.0
Cefotaxime	1.0 ± 0.25	> 64.0
Tetracycline	1.0 ± 0.25	> 64.0
Garlic oil	24.0 ± 4.0	32.0 ± 8.0
Chinese leek oil	36.0 ± 6.0	48.0 ± 8.0
Diallyl monosulfide	20.0 ± 4.0	32.0 ± 8.0
Diallyl disulfide	4.0 ± 1.0	12.0 ± 2.0
Diallyl trisulfide	2.0 ± 1.0	8.0 ± 2.0
Diallyl tetrasulfide	0.5 ± 0.125	2.0 ± 0.5

Table 3. Minimum inhibitory concentration (μg/ml) of garlic oil (GO), Chinese leek oil (CLO) and four diallyl sulfides[a] against three *Candida* and three *Aspergillus* species. Data were expressed as mean ± standard deviation (n=5).

Specie (# of isolate)	GO	CLO	DAS	DADS	DAT	DATS
C. albican (39)	16.0 ± 2.0	24.0 ± 2.0	32.0 ± 4.0	4.0 ± 1.0	1.0 ± 0.25	0.5 ± 0.125
C. Krusei (27)	24.0 ± 4.0	48.0 ± 8.0	72.0 ± 8.0	12.0 ± 2.0	8.0 ± 1.0	4.0 ± 0.5
C. glabrata (25)	32.0 ± 4.0	40.0 ± 8.0	54.0 ± 8.0	8.0 ± 2.0	4.0 ± 1.0	2.0 ± 0.5
A. niger (31)	20.0 ± 2.0	32.0 ± 4.0	40.0 ± 4.0	8.0 ± 2.0	2.0 ± 0.5	1.0 ± 0.25
A. flavus (26)	40.0 ± 8.0	64.0 ± 8.0	64.0 ± 8.0	12.0 ± 4.0	4.0 ± 2.0	2.0 ± 1.0
A. fumigatus (28)	32.0 ± 4.0	56.0 ± 8.0	54.0 ± 4.0	12.0 ± 2.0	8.0 ± 2.0	4.0 ± 2.0

[a]DAS=diallyl monosulfide; DADS=diallyl disulfide; DAT=diallyl trisulfide; DATS=diallyl tetrasulfide.

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