Fungicidal Activity of Polygodial in Combination with Anethole and Indole against *Candida albicans*

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In our continuing search for antimicrobial agents from edible and medicinal plants, beverages, and food spices, we have reported the limited antimicrobial activity of various compounds. Combining two or more of these chemicals to increase the activity spectrum seems to be a promising approach to practical use, although a rational basis for this is still in an early stage. The combination of polygodial (1) and anethole (2) exhibited fungicidal activity against *Candida albicans* and *Saccharomyces cerevisiae*. In contrast, the combination of polygodial and indole (3) had fungistatic activity against *C. albicans*.

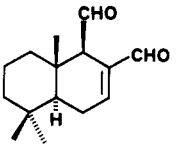
INTRODUCTION

Many infectious diseases caused by pathogenic microorganisms can be cured by antibiotics. However, since many uncontrolled pathogens still exist, new antimicrobial agents that either kill or inhibit the growth of microorganisms are needed. For example, fungal infections have become increasingly serious, especially when host defense mechanisms are impaired. Although various antifungal agents that either kill or inhibit the growth of fungi have been explored, many fungal diseases remain to be controlled.

In our continuing search for antimicrobial agents from edible and medicinal plants, beverages, and food spices, we have reported the antimicrobial activity of various compounds (Himejima and Kubo, 1991; Kubo et al., 1991a,b, 1992). Only limited antifungal activity has been found. Also, large numbers of phytochemicals were previously isolated and shown to have antifungal properties (Mitscher et al., 1972, 1987). Their antimicrobial activities are usually insufficiently potent for practical use. Combining two or more of these chemicals to enhance their activity seems to be a promising approach for practical use. In addition, antifungal natural products identified from edible and medicinal plants, beverages, and food spices are superior for this approach, because these compounds are generally recognized as safe due to their extensive use as food sources. However, a rational basis for this approach is still in an early stage. Therefore, we have tested a combination of two kinds of chemicals, identified in a food spice and a medicinal plant, to enhance their antifungal activity. We previously reported activities sufficiently potent to be considered for practical use (Kubo and Himejima, 1991; Himejima and Kubo, 1992).

We recently reported that anethole (2), isolated from the seeds of *Pimpinella anisum* L. (Umbelliferae), synergized the antifungal activity of polygodial (1), isolated from various plant sources (Barnes and Loder, 1962; Kubo et al., 1976; McCallion et al., 1982), against *Candida albicans* and *Saccharomyces cerevisiae*. Thus, the antifungal activity of polygodial against *C. albicans* and *S. cerevisiae* was increased 32- and 64-fold, respectively, by anethole (Kubo and Himejima, 1991).

In our antimicrobial assays, when the growth of any microorganism tested was not visible, it was not possible to discern if the microorganisms were viable. That is, it was unclear if activity was fungistatic, that is, able to inhibit the growth of fungi, or fungicidal, that is, able to kill fungi.



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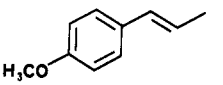

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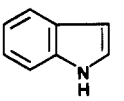

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It is well-known that *C. albicans*, one of most important opportunistic human pathogens, can cause candidiasis when the host microflora is suppressed by antibiotic therapy or defense mechanisms are impaired. In such a case, antimicrobial agents with fungistatic activity may be useful for controlling *C. albicans* in a preventive usage. In contrast, antimicrobial agents exhibiting fungicidal activity may be superior for controlling fungi after infection. Thus, it is particularly important to distinguish between agents that are fungistatic or fungicidal.

This paper describes the effect of combining anethole and polygodial on the viability of *C. albicans* and *S. cerevisiae*. The effects of combining indole and polygodial against *C. albicans* are described.

MATERIALS AND METHODS

Microorganisms and Media. Two fungi, *C. albicans* ATCC 18804 and *S. cerevisiae* ATCC 7754, were purchased from the American Type Culture Collection (Rockville, MD). For culturing these microorganisms, MEB [2.5% malt extract broth (BBL)] was used. For viable cell count experiments, MEA (2.5% malt extract broth and 2% agar) was used. The freeze-dried *C. albicans* and *S. cerevisiae* were reactivated with shaking culture at 30 °C.

Chemicals. Polygodial (1) was obtained previously (Kubo et al., 1976). Anethole (2) was isolated from the seeds of *P. anisum* as previously described (Kubo and Himejima, 1991). Authentic indole (3) was purchased from Sigma Chemical Co. (St. Louis, MO). *N,N*-Dimethylformamide (DMF) was purchased from EM Science (Gibbstown, NJ).

Antimicrobial Assay. The antimicrobial assays were performed by a 2-fold serial broth dilution method (Kubo and Himejima, 1991). For the antimicrobial assay, *C. albicans* and *S. cerevisiae* were cultured without shaking. After 2 days, the growth of these fungi was examined as turbidity (OD at 660 nm). The lowest concentration of the test compounds at which no growth occurred was defined as the minimum inhibitory concentration (MIC).

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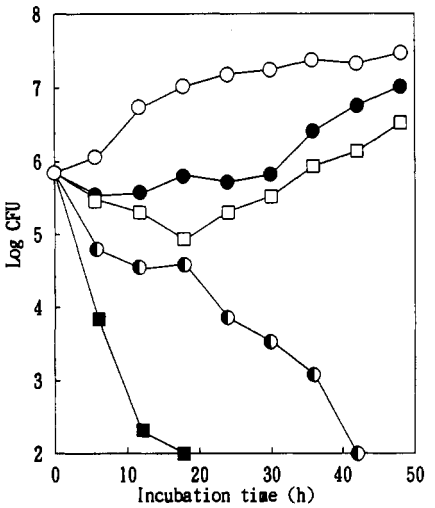

Figure 1. Fungicidal activity of anethole and polygodial against *C. albicans*: ○, control; ●, anethole (100 μg/mL); ◐, anethole (200 μg/mL); □, polygodial (1.56 μg/mL); ■, polygodial (3.13 μg/mL).

Viable Cell Count. The test compound was first dissolved in DMF, and then the sample solution was serially diluted 2-fold with DMF. Each sample dilution was added to MEB. MEB containing 1% DMF was used as the control. The concentration of DMF in MEB never exceeded 1%, and the growth of any microorganism tested was unaffected by 1% DMF. Subsequently, the microorganisms to be tested were inoculated into MEB and incubated with the test compounds at 30 °C without shaking. A portion of the broth was serially diluted 10-fold with sterilized saline solution every 6 h. Fifty microliters of diluted solution was then inoculated onto MEA and incubated at 30 °C. Inoculation of diluted solution onto MEA was duplicated. After 2 days, colonies on MEA plats were counted and the number of viable cells per 1 mL of the broth was defined as a colony forming unit (CFU) (Taniguchi et al., 1988).

RESULTS AND DISCUSSION

In our previous paper, anethole (2) synergized antifungal activity of polygodial (1) against *C. albicans* and *S. cerevisiae*. The antifungal activity of polygodial against *C. albicans* and *S. cerevisiae* was increased 32- and 64-fold, respectively by anethole. Thus, the MIC of polygodial against *C. albicans* was lowered from 3.13 to 0.098 μg/mL and in the case against *S. cerevisiae*, from 1.56 to 0.024 μg/mL, when polygodial was combined with 100 μg/mL (half-MIC for both *C. albicans* and *S. cerevisiae*) of anethole. Since this antimicrobial assay was performed by the 2-fold serial broth dilution method and the growth of microorganisms tested was examined as turbidity, it was not clear if the combination of anethole and polygodial had fungicidal or fungistatic activity (Kubo and Himejima, 1991). Here, we used the viable count method to analyze the growth curve of *C. albicans* and *S. cerevisiae* with a combination of anethole and polygodial. The growth curves of *C. albicans* and *S. cerevisiae* in the presence of anethole and polygodial are shown in Figures 1 and 2, respectively.

Both anethole and polygodial exhibited fungicidal activity against *C. albicans* at concentrations of 200 and 3.13 μg/mL, respectively, but did not show fungicidal activity at concentrations of 100 and 1.56 μg/mL, respectively (Figure 1). Thus, *C. albicans* was not viable within 42 and 18 h at 200 μg/mL of anethole and 3.13 μg/mL of polygodial, respectively. The lethal concentrations of both anethole and polygodial against *C. albicans* was the same as their MIC values (Table I).

In the case of *S. cerevisiae*, anethole and polygodial exhibited fungicidal activity at concentrations of 200 and 3.13 μg/mL, respectively (Figure 2). The lethal concentration of anethole was the same as its MIC, but that of polygodial was different. *S. cerevisiae* was viable at the MIC of polygodial (1.56 μg/mL) at which no growth occurred, as measured by turbidity. Thus, *S. cerevisiae* was not viable within 36 and 6 h at the concentrations of 200 μg/mL of anethole and 3.13 μg/mL of polygodial, respectively. Both anethole and polygodial had the same lethal concentration against *C. albicans* and *S. cerevisiae*, although these compounds exhibited fungicidal activity more drastically against *S. cerevisiae* than against *C. albicans*.

Fungicidal activity against *C. albicans* and *S. cerevisiae* with a combination of anethole and polygodial is shown

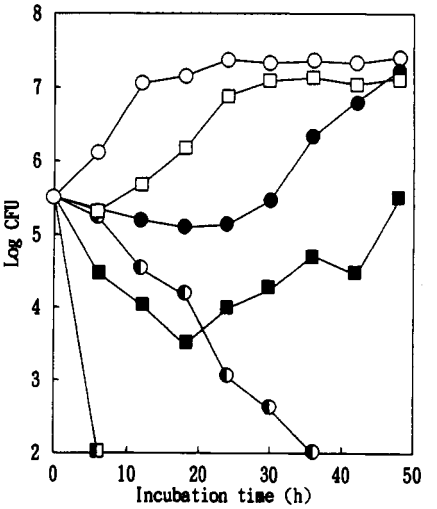

Figure 2. Fungicidal activity of anethole and polygodial against *S. cerevisiae*: ○, control; ●, anethole (100 μg/mL); ◐, anethole (200 μg/mL); □, polygodial (0.78 μg/mL); ■, polygodial (1.56 μg/mL); ◧, polygodial (3.13 μg/mL).

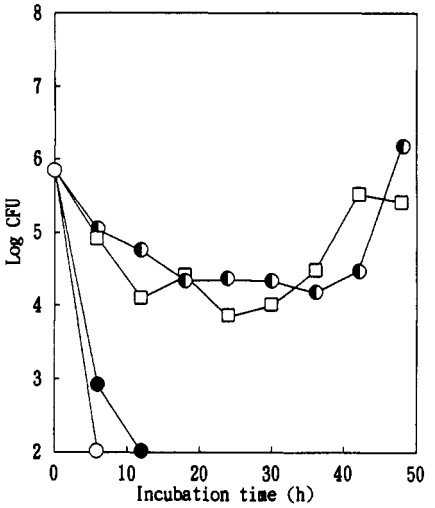

Figure 3. Fungicidal activity in combination of anethole and polygodial against *C. albicans*: ○, anethole (100 μg/mL) and polygodial (0.20 μg/mL), or anethole (50 μg/mL) and polygodial (1.56 μg/mL); ●, anethole (100 μg/mL) and polygodial (0.098 μg/mL); ◐, anethole (100 μg/mL) and polygodial (0.049 μg/mL); □, anethole (25 μg/mL) and polygodial (1.56 μg/mL).

Table I. Antifungal Activity of Polygodial, Anethole, and Indole against *C. albicans* and *S. cerevisiae*

compound	MIC against fungi tested, μg/mL	
	C. albicans	*S. cerevisiae*
polygodial	3.13	1.56
anethole	200	200
indole	800	>800

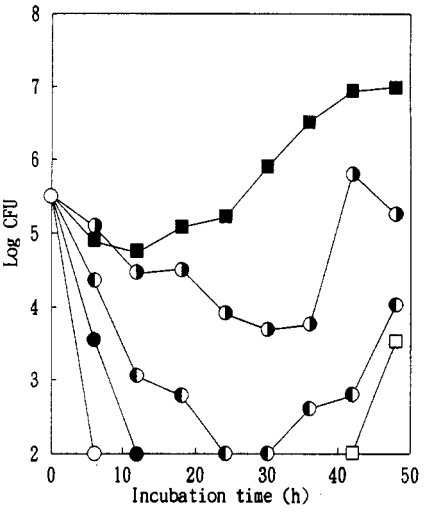

Figure 4. Fungicidal activity in combination of anethole and polygodial against *S. cerevisiae*: ○, anethole (100 μg/mL) and polygodial (0.098 μg/mL); ●, anethole (100 μg/mL) and polygodial (0.049 μg/mL); ◐, anethole (100 μg/mL) and polygodial (0.024 μg/mL); ◑, anethole (100 μg/mL) and polygodial (0.012 μg/mL); □, anethole (50 μg/mL) and polygodial (0.78 μg/mL); ■, anethole (25 μg/mL) and polygodial (0.78 μg/mL).

Table II. Fungicidal Activity of Polygodial in Combination with Half-MIC of Anethole

	fungi tested	
polygodial, μg/mL	*C. albicans*	*S. cerevisiae*
0.20	+[a]	+
0.098	+	+
0.049	–[b]	+
0.024	–	–

[a] Fungicidal activity. [b] No fungicidal activity.

Table III. Fungicidal Activity of Anethole in Combination with Half-MIC of Polygodial

	fungi tested	
anethole, μg/mL	*C. albicans*	*S. cerevisiae*
100	+[a]	+
50	+	–
25	–[b]	–

[a] Fungicidal activity. [b] No fungicidal activity.

in Figures 3 and 4, respectively. When anethole at a concentration of 100 μg/mL (half-MIC) was combined with 0.20 or 0.098 μg/mL of polygodial, *C. albicans* was not viable within 6 or 12 h, respectively. However, the combination of 100 μg/mL of anethole and 0.049 μg/mL of polygodial did not show fungicidal activity against *C. albicans* (Table II). On the other hand, 50 μg/mL of anethole had fungicidal activity against *C. albicans* in combination with 1.56 μg/mL of polygodial (half-MIC), but 25 μg/mL of anethole did not (Table III). Thus, fungicidal activity of polygodial against *C. albicans* was increased 32-fold by anethole, but that of anethole was increased only 4-fold by polygodial. These results indicate that the combination of anethole and polygodial had fungicidal rather than fungistatic activity against *C. albicans*.

In the case of *S. cerevisiae*, when anethole at the concentration of 100 μg/mL (half -MIC) was combined with 0.098 or 0.049 μg/mL of polygodial, polygodial exhibited fungicidal activity within 6 or 12 h, respectively. However, 0.024 μg/mL of polygodial no longer showed fungicidal activity in combination with 100 μg/mL of anethole (Table II). On the other hand, when 0.78 μg/mL of polygodial (half-MIC) was combined with 100 μg/mL

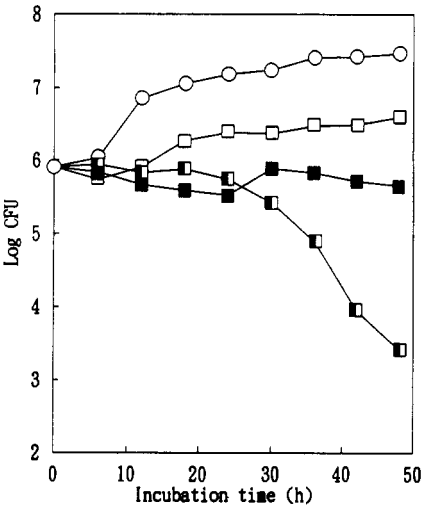

Figure 5. Fungicidal activity of indole against *C. albicans*: ○, control; □, indole (200 μg/mL); ■, indole (400 μg/mL); ⧈, indole (800 μg/mL).

of anethole, anethole showed fungicidal activity (Table III). In the case of the combination of 0.78 μg/mL of polygodial and 50 μg/mL of anethole, *S. cerevisiae* started growing after 42 h. That is, this combination showed fungicidal activity incompletely (Figure 4). Thus, fungicidal activity of polygodial against *S. cerevisiae* was increased 32-fold by anethole and that of anethole was increased only 2-fold by polygodial. The combination of anethole and polygodial also had fungicidal activity against *S. cerevisiae*.

We have reported that the MIC of polygodial against *C. albicans* was lowered from 3.13 to 0.098 μg/mL and in the case of *S. cerevisiae*, from 1.56 to 0.024 μg/mL, when polygodial was combined with 100 μg/mL of anethole (Kubo and Himejima, 1991). The lethal concentration of polygodial against *C. albicans* was lowered from 3.13 to 0.098 μg/mL and in the case of *S. cerevisiae*, from 1.56 to 0.049 μg/mL, when polygodial was combined with 100 μg/mL of anethole. The combined effect of anethole and polygodial against *C. albicans* with respect to fungicidal activity was the same as for antifungal activity, but with *S. cerevisiae* the fungicidal activity was weaker than the antifungal activity.

Thus, the combination of anethole and polygodial showed fungicidal rather than fungistatic activity. Since anethole significantly synergized the fungicidal activity of polygodial, it may be safer to use for controlling infectious fungi. Also, this combination may be useful for controlling *C. albicans* cases of candidiasis.

We reported that indole (**3**) synergized the bactericidal activity of linalool against *Streptococcus mutans*. The combination of 800 μg/mL of linalool and 400 μg/mL of indole (half-MIC against *S. mutans*) showed bactericidal activity, although indole itself did not have bactericidal activity against *S. mutans* (Kubo et al., 1993). Therefore, it was considered whether indole showed the same synergistic effect in combination with polygodial against *C. albicans* as in combination with linalool against *S. mutans*.

In our preliminary antimicrobial assay, the MIC of indole against *C. albicans* was 800 μg/mL (Table I). When the broth in which growth occurred was reinoculated into fresh medium, growth of *C. albicans* was normal. This result indicated that indole had fungistatic, rather than fungicidal, activity against *C. albicans*. Therefore, a growth curve of *C. albicans* in the presence of indole was generated to confirm fungistatic activity of indole (Figure 5). As

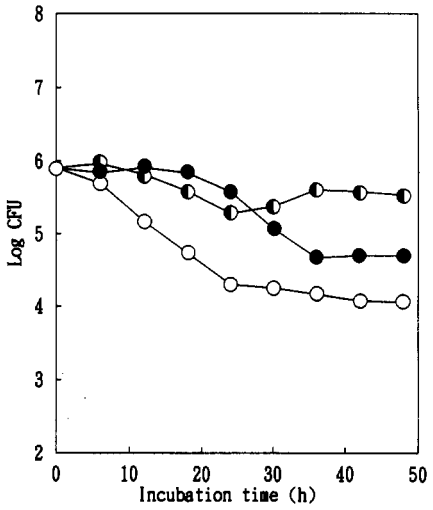

Figure 6. Fungicidal activity in combination of indole and polygodial against *C. albicans*: ○, indole (400 μg/mL) and polygodial (1.56 μg/mL); ●, indole (400 μg/mL) and polygodial (0.39 μg/mL); ◐, indole (400 μg/mL) and polygodial (0.098 μg/mL).

expected, the number of *C. albicans* viable cells decreased at a concentration of 800 μg/mL of indole, however, *C. albicans* was not killed by indole after 48 h of incubation. The number of viable cells of *C. albicans* did not change at 400 μg/mL. Interestingly, in the antimicrobial assay, turbidity increased at 400 μg/mL for 48 h of incubation (relative OD was 22% compared with the control). *C. albicans* may grow and die at a concentration of 400 μg/mL of indole, and dead cells may affect turbidity measurements.

The combination of anethole and polygodial, both of which had fungicidal activity, also exhibited fungicidal activity. We studied the combined effect of polygodial and indole, the latter of which had fungistatic activity (Figure 6). When 400 μg/mL of indole was combined with 1.56 or 0.39 μg/mL of polygodial, the number of viable cells of *C. albicans* decreased but remained static, and *C. albicans* was still viable after 48 h of incubation. The number of viable cells did not change when 400 μg/mL of indole was combined with 0.098 μg/mL of polygodial. Although 400 μg/mL of indole enhanced the antifungal activity of polygodial against *C. albicans*, its activity was fungistatic. In other words, *C. albicans* was controlled by 0.39 μg/mL of polygodial in combination with 400 μg/mL of indole; however, *C. albicans* was still viable at this concentration. This result indicated that the combination of indole and polygodial had fungistatic rather than fungicidal activity. Hence, combinations of indole and polygodial may be useful for controlling the growth of troublesome persistent fungi such as *C. albicans* when the constant body microflora is out of balance in antibiotic therapy. Thus, the synergistic effect of indole to polygodial was different from that of anethole.

Although polygodial itself is one of the most potent antifungal compounds, it becomes much more potent when combined with anethole. Thus, polygodial can be used at a much lower concentration and is safer with anethole to control infectious fungi and also *C. albicans* cases of candidiasis, since the combination of anethole and polygodial had fungicidal activity. In contrast, since the combination of indole and polygodial exhibited fungistatic activity, this combination may be useful for controlling troublesome persistent fungi such as *C. albicans* in a preventive usage. Thus, polygodial may be useful for controlling both troublesome infectious and persistent fungi by combination with other substances such as anethole and indole. The combination of polygodial with other substances may be a very effective way for controlling troublesome fungi such as *C. albicans*.

ACKNOWLEDGMENT

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