Anethole, a Synergist of Polygodial against Filamentous Microorganisms

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Anethole, isolated as an antimicrobial principle from the seeds of *Pimpinella anisum*, was found to exhibit a significant synergistic effect on the antifungal activity of polygodial against *Candida albicans* and *Saccharomyces cerevisiae*.

INTRODUCTION

The seeds of *Pimpinella anisum* L. (Umbelliferae), known as aniseed, have long been used as a food spice worldwide. In addition, the aniseed has also widely been used as a folk medicine. For example, in South American countries, it has been used as expectorant, anodyne, anticonvulsion agent, etc. (Barriga, 1975; Balme, 1979; White, 1985). In Brazil, the aniseed is known as "erva dose". In our preliminary antimicrobial screening against four typical microorganisms (Taniguchi et al., 1978), the methanol extract of the erva dose exhibited rather broad antimicrobial activity. Although the antibacterial activity of the aniseed extract was previously reported (Morris et al., 1979), no specific active principle has yet been identified.

We can control many human and animal pathogens by currently available antibiotics. However, the need for new antibiotics still exists. For example, systemic infections caused by fungi, especially in patients with impaired host defense mechanisms, have become increasingly serious. Various antifungal agents have been explored, but the control of many of the fungal diseases has not yet been achieved.

Bioassay-guided fractionation led to the isolation of an active principle that was one of the most abundant compounds in the erva dose. This sweet-tasting substance was identified as anethole (1) by spectroscopic data. The results of a bioassay of purified anethole against 13 selected microorganisms (Himejima and Kubo, 1991) exhibited moderate but broad antimicrobial activity that included five fungi. In a previous paper (Capek, 1956), anethole was reported to exhibit antifungal activity against a food-borne fungus, *Aspergillus niger*, but activity against any other microorganism was not described.

Although antifungal activity of anethole was not potent enough to be considered of practical use, it is worthy of further investigation as a natural product isolated from a food spice (Boelens, 1991). An attempt to enhance its antifungal activity was made by combining it with one or more substances. This approach seems to be a promising strategy for efficient utilization of renewable natural resources.

We have recently described a sesquiterpene dialdehyde, polygodial (2), isolated from various plant sources (Barnes and Loder, 1962; Kubo et al., 1976; McCallion et al., 1982), that synergized the antifungal activity against *S. cerevisiae* and *Candida utilis* of several antibiotics such as actinomycin D and rifampicin (Kubo and Taniguchi, 1988). Therefore, anethole was initially combined with polygodial to test if polygodial had the same synergistic effect to anethole against one of the most important opportunistic yeast pathogens, *C. albicans*, as well as two additional yeasts, *S. cerevisiae* and *Pityrosporum ovale*. For comparison purposes, amphotericin B, one of the most potent antifungal agents against filamentous microorganisms, was also tested in combination with anethole to determine if it had the same enhancing activity.

MATERIALS AND METHODS

Chemicals. Polygodial was from our previous work (Kubo et al., 1976). An authentic anisaldehyde, anisketone, and amphotericin B used for the bioassay were purchased from Sigma Chemical Co. (St. Louis, MO). *N,N*-Dimethylformamide (DMF) was purchased from EM Science (Gibbstown, NJ).

Plant Material. Erva dose, the seeds of *P. anisum*, were purchased in Belem, Para, Brazil, and identified by Dr. M. E. van den Berg at Museu Paraense Emilio Goeldi, Belem, Para, Brazil.

Extraction and Identification. The seeds of *P. anisum* (188 g) were extracted with methanol at ambient temperature. The methanol was removed under reduced pressure to give a brown crude extract (15 g), which was suspended in water (100 mL). The suspension was successively partitioned between water and *n*-hexane, chloroform, ethyl acetate, and 1-butanol, yielding 7.00, 1.27, 0.45, and 0.79 g, respectively. The subsequent bioassay revealed the *n*-hexane fraction to be active. A portion of the bioactive *n*-hexane fraction (4.0 g) was steam-distilled to yield a distillate (3.1 g) and residue (0.4 g). The following bioassay indicated that the distillate maintained the original broad antimicrobial spectrum, while the residue exhibited almost no activity up to 800 μg/mL. According to the GC–MS analysis, the distillate consisted of 71% anethole. Besides anethole, other compounds identified in the distillate included three aromatic compounds, anisaldehyde (9.0%), anisketone (6.7%), and methyleugenol (0.23%); four monoterpenes, pulegone (1.4%), camphor (0.12%), cymene (0.05%), and β-terpinyl acetate (0.03%); and one fatty acid, stearic acid (trace). A combination of repeated crystallization and chromatography of the distillate (1.28 g) on normal-phase silica gel led to the isolation of anethole (0.65 g).

GC–MS Analysis. A Hewlett-Packard 5890 gas chromatograph equipped with direct coupling to a JEOL DX303 HF mass spectrometer was used with a DB-1 column (J&W Scientific, Folsom, CA). The conditions were as follows: temperature, isothermal from 50 to 230 °C at 2 °C/min; carrier gas flow rate, 2.5 mL/min of helium; temperature of ion source, 200 °C; electron energy, 70 eV.

Antimicrobial Assay. *Test Microorganisms.* Thirteen microorganisms, namely, *Bacillus subtilis* ATCC 9372, *Brevibacterium ammoniagenes* ATCC 6872, *Propionibacterium acnes* ATCC 11827, *Staphylococcus aureus* ATCC 12598, *Streptococcus mutans* ATCC 25175, *Escherichia coli* ATCC 9637, *Pseudomonas aeruginosa* ATCC 10145, *Enterobacter aerogenes* ATCC 13048, *Saccharomyces cerevisiae* ATCC 7754, *Candida albicans* ATCC 18804, *Trichophyton mentagrophytes* ATCC 18748, *Pityrosporum ovale* ATCC 14521, and *Penicillium chrysogenum* ATCC 10106, were utilized as test organisms. All of the microorganisms were purchased from American Type Culture Collection (Rockville, MD).

The freeze-dried microorganisms purchased from ATCC were reactivated as follows: *B. subtilis*, *S. cerevisiae*, *C. albicans*, *T.*

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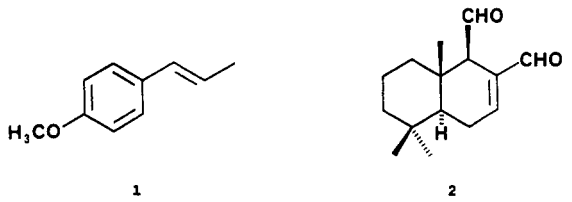

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Figure 1. Structures of anethole (1) and polygodial (2).

mentagrophytes, *P. ovale*, and *P. chrysogenum* were cultured with shaking at 30 °C; *B. ammoniagenes* and *E. aerogenes* were cultured stationarily at 30 °C; and all of the other microorganisms were cultured stationarily at 37 °C.

Media. The culture media for the bacteria consisted of 0.8% nutrient broth (BBL), 0.5% yeast extract (Difco), and 0.1% glucose, except for the case of *S. mutans*. For the culture of *S. mutans*, 3.7% brain heart infusion broth (Difco) was utilized. The culture media for the fungi consisted of 2.5% malt extract broth (BBL), except for the cases of *P. ovale* and *T. mentagrophytes*. For the culture of *P. ovale*, 1% bactopeptone (Difco), 0.5% yeast extract, 1% glucose, and 0.1% corn oil were used. For the culture of *T. mentagrophytes*, 1% peptone and 4% glucose were used.

Methods. The bioassays were performed according to a broth dilution method. Thus, the test compound was first dissolved in 3 mL of DMF. Then, 1% of the sample solution (30 μL) was added to 3 mL of the appropriate medium. Finally, 30 μL of 2-day-old culture of the test microorganism (5 days old of *T. mentagrophytes* and *P. chrysogenum*) was inoculated. The highest concentration used for the assay was 800 μg/mL, unless otherwise specified, because of solubility limitation in the water-based media. The concentration of DMF in the medium did not exceed 1%. The growth of any of the microorganisms tested was not affected by DMF up to 1%. For the antimicrobial assay, all mocroorganisms were cultured stationarily, except *P. chrysogenum* and *T. mentagrophytes*, which were cultured with shaking. After 2 days (5 days for *T. mentagrophytes* and *P. chrysogenum*), the growth of microorganisms was examined as turbidity (O.D. at 660 nm), except for *P. chrysogenum*, *T. mentagrophytes*, and *P. ovale*, which were observed with the naked eye. The minimal inhibitory concentration (MIC) was measured according to the twofold serial broth dilution method (Taniguchi and Satomura, 1972). The lowest concentration of the test compounds in which no growth occurred was defined as the MIC.

The combination data were obtained according to the checkerboard method (Norden et al., 1979). Thus, the twofold dilutions of the anethole were tested in combination with concentrations of twofold dilutions of the other. Each yeast was tested at least twice with the checkerboard method.

RESULTS AND DISCUSSION

In our preliminary routine antimicrobial screening against four typical microorganisms (Taniguchi et al., 1978), the methanol extract of the erva dose, the seeds of *P. anisum*, exhibited rather broad antimicrobial activity at 800 μg/mL. Thus, it inhibited the growth of *B. subtilis*, *E. coli*, and *S. cerevisiae* but not *P. chrysogenum*. To isolate the active principles, the methanol extract was sequentially partitioned between water and *n*-hexane, chloroform, ethyl acetate, and 1-butanol. The subsequent bioassay identified the *n*-hexane portion to be active. The bioactive *n*-hexane fraction was then steam-distilled to give a distillate and residue. The following bioassay demonstrated that the distillate maintained the original broad antimicrobial activity, while the residue exhibited no activity up to 800 μg/mL. According to the GC–MS analysis, the distillate contained 71% anethole (1). The content of anethole in the aniseed is usually more than 80% (Bauer et al., 1990), but repeated rotary evaporation under reduced pressure during the isolation procedure may have resulted in loss of some of volatile anethole. The distillate consisted of almost 90% aromatic compounds.

Table I. Antimicrobial Activity of Anethole

microorganism	MIC, μg/mL	microorganism	MIC, μg/mL
B. subtilis	400	*E. aerogenes*	>800
B. ammoniagenes	200	*S. cerevisiae*	200
P. acnes	100	*C. albicans*	200
S. aureus	>800	*T. mentagrophytes*	800
S. mutans	200	*P. ovale*	100
E. coli	200	*P. chrysogenum*	>800
P. aeruginosa	>800		

Besides anethole (1), other compounds identified in the distillate in order of decreasing concentrations were anisaldehyde, anisketone, pulegone, methyleugenol, camphor, cymene, β-terpinyl acetate, and stearic acid. A combination of crystallization and column chromatography on normal-phase silica gel of the *n*-hexane extract also led to the isolation of anethole in significant quantity. Nevertheless, the presence of a large amount of anethole in the *P. anisum* seeds is well documented (Bauer et al., 1990).

The antimicrobial activity of anethole against 13 selected microorganisms is listed in Table I. In addition to anethole, two major compounds, anisaldehyde and anisketone, were also bioassayed, but neither demonstrated any antimicrobial activity at 800 μg/mL. Anethole exhibited a moderate but broad antimicrobial spectrum which could explain the original antimicrobial activity of the methanol extract. The MICs against *C. albicans* and *S. cerevisiae*, 200 μg/mL for each, and *P. ovale*, 100 μg/mL, were not potent enough to warrant its practical application. However, it is still worthwhile to investigate the possibility of its practical use as an antifungal agent, since anethole was isolated from a food spice which has long been consumed by many people. Therefore, an attempt to enhance the antifungal activity of anethole by combining it with other substances was investigated. However, the rationale for the selection of "other substances" is still in an embryonic stage.

The initial selection of other substances was based largely on our previous study. We have recently reported that a sesquiterpene dialdehyde, polygodial (2) isolated from the sprouts of *Polygonum hydropiper* (Polygonaceae) (Barnes and Loder, 1962) and the barks of *Warburgia ugandensis* and *W. stuhlmanii* (Canellaceae) (Kubo et al., 1976; Nakanishi and Kubo, 1978), increased the antifungal activity of several antibiotics such as actinomycin D and rifampicin against *S. cerevisiae* and *C. utilis* (Kubo and Taniguchi, 1988; Taniguchi et al., 1988). The rationale for this combined effect appears to be based on an increase in the permeability of the antibiotics through the plasma membrane by polygodial, since its primary mode of action seems to be damage of the plasma membrane structure. Therefore, to enhance the antifungal activity of anethole, it was first combined with polygodial.

Polygodial, unexpectedly, did not enhance the antifungal activity of anethole. In contrast, a dramatic increase in the antifungal activity of polygodial occurred when it was combined with a sublethal amount of anethole, as shown in Table II. Thus, the activity of polygodial against *S. cerevisiae* and *C. albicans* was increased 64- and 32-fold, respectively. In other words, the MIC against *S. cerevisiae* was lowered from 1.56 to 0.024 μg/mL, and in the case of *C. albicans*, from 3.13 to 0.098 μg/mL, when polygodial was combined with 100 μg/mL anethole (half MIC for both *C. albicans* and *S. cerevisiae*). Anethole also enhanced the activity of polygodial against *P. ovale* but not as much as against *C. albicans* and *S. cerevisiae*.

Table II. Antifungal Activity of Polygodial in Combination with Anethole

antifungal agent	MIC against fungi tested, μg/mL		
	S. cerevisiae	*C. albicans*	*P. ovale*
polygodial	1.56	3.13	50
polygodial + anethole[a]	0.024	0.098	6.25

[a] The concentration of anethole was half MIC for each fungus.

Table III. Antifungal Activity of Amphotericin B in Combination with Anethole

concn of anethole, μg/mL	MIC against fungi tested, μg/mL		
	S. cerevisiae	*C. albicans*	*P. ovale*
0	0.78	0.78	3.13
6.25	1.56	1.56	3.13
12.5	3.13	3.13	0.78
25	6.25	12.5	0.78
50	12.5	6.25	0.39
100	3.13	0.20	

Thus, anethole increased the antifungal activity of polygodial 8-fold against *P. ovale*; the MIC was lowered only from 50 to 6.25 μg/mL when polygodial was combined with 50 μg/mL anethole (half MIC for *P. ovale*).

Although the rationale for this combination effect is still embryonic, anethole was also combined with other antifungal agents, such as amphotericin B, to see if it had the same enhancing activity. Amphotericin B, one of the most potent antibiotics known against filamentous microorganisms, is also known to damage the plasma membrane by interacting with sterols (Hamilton-Miller, 1974) in fungal cell membranes (Kinsky, 1970). However, anethole did not enhance the antifungal activity of amphotericin B against *C. albicans* and *S. cerevisiae*. In fact, the antifungal activity of amphotericin B was somewhat antagonized by anethole as shown in Table III. Increasing the amount of anethole increased its MICs. More specifically, the activity of amphotericin B against *C. albicans* and *S. cerevisiae* was decreased 16-fold when it was combined with 25 and 50 μg/mL of anethole, respectively. Thus, the MICs were increased against both fungi from 0.78 to 12.5 μg/mL. In contrast, anethole did increase the antifungal activity of amphotericin B against *P. ovale* 8-fold. Thus, the MIC was lowered from 3.13 to 0.39 μg/mL when amphotericin B was combined with 50 μg/mL anethole. These results do not seem to indicate that a chemical reaction between anethole and amphotericin B occurred prior to the assay.

In short, anethole remarkably enhanced the antifungal activity of polygodial against *C. albicans*, *S. cerevisiae*, and, to a lesser extent, amphotericin B. In the case of the latter, anethole somewhat antagonized its activity. The enhancing activity of anethole depends on the species of fungi and antifungal agents tested. The rationale for this combination effect is currently under investigation.

Lastly, it should be emphasized that both the seeds of *P. anisum* and the sprouts of *P. hydropiper* have long been used as food spices by many people. Therefore, their potential for human oral toxicity either is not serious or has been overlooked. Furthermore, a large amount of anethole has also been isolated from various plants, including two other spice plants, *Foeniculum vulgare* M. (Umbelliferae) and *Illicium verum* Hook (Magnoliaceae) (Bauer et al., 1985). In addition, polygodial has also been isolated from several other spice plants.

In conclusion, since the control of opportunistic yeast pathogens is becoming increasingly important, the current study to enhance the total biological activity by combining two or more substances may provide a new approach to solve this problem. In particular, two phytochemicals isolated from common food spices, anethole (**1**) and polygodial (**2**), may be considered for practical application.

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Registry No. 1, 104-46-1; 2, 6754-20-7; amphotericin B, 1397-89-3; anisaldehyde, 123-11-5; anisketone, 122-84-9.