Potentiation of antifungal activity of sesquiterpene dialdehydes against *Candida albicans* and two other fungi

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Abstract. The antifungal activity of two drimane sesquiterpene dialdehydes, polygodial (**1**) and warburganal (**2**), alone and in combination with several other substances, was examined against three fungi, *Candida albicans*, *Saccharomyces cerevisiae* and *Pityrosporum ovale* employing a broth dilution method. Anethole significantly synergized the activity of the two sesquiterpenoids against *C. albicans* and *S. cerevisiae*; however, it had only an additive effect against *P. ovale*. By contrast, two antioxidants, ascorbic acid (vitamin C) and BHA (butylated hydroxyanisole), noticeably enhanced the activity of the sesquiterpenoids against *P. ovale*, but had no effect against *C. albicans* and *S. cerevisiae*.
Key words. Polygodial; warburganal; antifungal activity; *Candida albicans*; *Saccharomyces cerevisiae*; *Pityrosporum ovale*; enhancing effect; antioxidants; vitamin C; BHA; anethole.

Systemic infections caused by filamentous microorganisms, especially in patients with impaired host defense mechanisms, have become increasingly serious all over the world. A drimane sesquiterpene dialdehyde, polygodial (**1**), isolated from various plants[1-3], was found to exhibit potent antifungal activity against *C. albicans*, one of the most important fungi responsible for human systemic infections[4]. The same sesquiterpene dialdehyde was also reported to enhance the antifungal activity of antibiotics such as actinomycin D and rifampicin against *S. cerevisiae* and *Candida utilis*, but not vice versa[5]. This combination effect seems to be caused by an increase in the permeability of the plasma membrane to the antibiotics brought about by polygodial[6].

A large number of phytochemicals have already been isolated as antifungal agents. However, their activity is usually not potent enough for them to be considered for practical application. Hence, studies of methods of enhancing their activity, for example, of their use in combination with other substances to enhance the total biological activity, are needed. However, a rational basis for choosing 'other substances' is still in a preliminary stage, although synergisms between antibiotics have been previously described[7]. In the experiments described here, polygodial was combined with selected substances in an attempt to enhance its antifungal activity against *C. albicans* and *S. cerevisiae*. These combinations were also tried against the dermatomycotic fungus *Pityrosporum ovale*.

The substances used were chosen from among those whose safety had been established, such as those isolated from food sources or compounds which have been used as food additives. For example, polygodial was chosen not only because of its potent antifungal activity, especially against *C. albicans* and *S. cerevisiae*[8], but also because it was first isolated from the sprouts of *Polygonum hydropiper* (Polygonaceae), which has long been consumed as a food spice[9]. In addition to polygodial, the closely related congener, warburganal (**2**) isolated from the barks of *Warburgia ugandensis* and *W. stuhlmannii* (Canellaceae), was also examined.

Materials and methods

Chemicals. Polygodial (**1**) and warburganal (**2**) were prepared as described in our previous work. Polygodial (**1**) was isolated from the sprouts and seeds of *P. hydropiper*[9], and warburganal (**2**) was isolated from the barks of *W. ugandensis* and *W. stuhlmannii*[10]. Anethole was isolated from the seeds of *Pimpinella anis* (Umbelliferae)[8].

Vitamins C and E, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). N,N-Dimethylformamide (DMF) was obtained from EM Science (Gibbstown, NJ, USA).

Antimicrobial Assay. All the microorganisms used for the assay (table 1) were purchased from the American Type Culture Collection (Rockville, MD, USA). The appropriate media and cultivation conditions have been previously described[11,12]. The highest concentration tested was 100 µg/ml, unless otherwise specified, because of limited availability and solubility in the water-based media of some of the substances used. The assay was performed by the broth dilution method as previously described[11,12]. The minimal inhibitory concentration (MIC) was measured by the twofold serial broth dilution method with incubations for 48 h, unless otherwise specified. The low-

Table 1. Antimicrobial activity of polygodial and warburganal

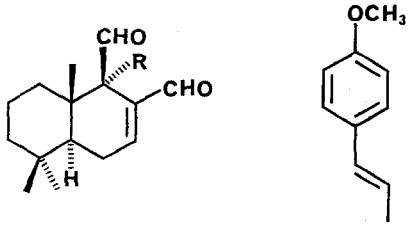
Microorganisms tested	MIC (µg/ml) Polygodial	Warburganal
Bacillus subtilis ATCC 9372	25	25
Brevibacterium ammoniagenes ATCC 6872	50	100
Staphylococcus aureus ATCC 12598	100	100
Streptococcus mutans ATCC 25175	>100	>100
Escherichia coli ATCC 9673	>100	>100
Pseudomonas aeruginosa ATCC 10145	>100	>100
Enterobacter aerogenes ATCC 13048	>100	>100
Saccharomyces cerevisiae ATCC 7754	3.13	6.25
Candida albicans ATCC 18804	3.13	6.25
C. utilis ATCC 9226	6.25	6.25
Pityrosporum ovale ATCC 14521	50	25
Penicillium chrysogenum ATCC 10106	3.13	6.25

est concentration of the test compounds in which no growth occurred was defined as the MIC. The combination data were obtained by the broth dilution checkerboard method[13]. The twofold dilutions of polygodial and warburganal were tested in combination with concentrations of twofold dilutions of the other. Each fungus was tested at least twice with the checkerboard method.

Results and discussion

The antimicrobial activity of two drimane sesquiterpene dialdehydes, polygodial (**1**) and warburganal (**2**) is listed in table 1. Both exhibited antimicrobial activity against all the fungi and some of the Gram-positive bacteria tested. In particular, they were highly active against *C. albicans* and *S. cerevisiae*. Thus, the potency of polygodial against these two fungi was comparable to that of amphotericin B, one of the most potent antibiotics against filamentous microorganisms[4].

In order to enhance the antifungal activity of these two sesquiterpene dialdehydes, their combination effects against three fungi, *C. albicans*, *S. cerevisiae* and *P. ovale*, with several selected substances, were examined. To start with, polygodial and warburganal were combined with several antioxidants, since both molecules possess two easily oxidizable aldehyde groups. One of the aldehydes is conjugated with a double bond, called an enal group, which is even more easily oxidized than an aldehyde group alone. The fact that oxidation is, in general, one of the most important detoxification (or metabolic) pathways in many living organisms also supports this approach. Thus, antioxidants presumably defend the sesquiterpene dialdehydes against oxidative detoxification, and this results in an extension of their period of activity.



1 R=H
2 R=OH

3

Four common food additive antioxidants[14], two natural ones (vitamins C and E) and two synthetic ones (BHA and BHT) were chosen to examine their combination effect with polygodial and warburganal. Vitamin E and BHT exhibited no antifungal activity up to 800 µg/ml against any of the three fungi tested. By contrast, BHA showed activity against all three, while vitamin C exhibited activity only against *P. ovale*, as listed in table 2.

Table 3 shows the MICs of polygodial (**1**) and warburganal (**2**) in combination with the antioxidants. Against *C. albicans* and *S. cerevisiae*, neither natural antioxidant exhibited any enhancing activity of either sesquiterpenoid. Their original antifungal activity was even antagonized by vitamin E. Thus, the antifungal activity of polygodial was decreased in proportion to the increasing amount of vitamin E. In the case of *P. ovale*, ascorbic acid (vitamin C) at a concentration of 200 µg/ml (equivalent to $^1/_2$MIC) increased the antifungal activity of polygodial 16-fold; the MIC was lowered from 50 to 3.13 µg/ml. However, this MIC was increased to 12.5 and 25 µg/ml when the cultivation was continued for 96 and 120 h, respectively. This indicated that vitamin C extended the period of activity of polygodial against *P. ovale*, presumably by retarding its oxidative destruction and, more importantly, that the combination was not fungicidal. As expected, ascorbic acid (vitamin C) also significantly synergized the activity of warburganal against *P. ovale* but not *C. albicans* and *S. cerevisiae*. Thus, by combining warburganal with 200 µg/ml of vitamin C, its MIC against *P. ovale* was reduced from 50 to 3.13 µg/ml. A similar result was obtained with the synthetic antioxidants. Thus, against *C. albicans* and *S. cerevisiae*, neither BHA nor BHT showed any enhancement of the activity of polygodial and warburganal. However, BHA synergized the activity of polygodial against *P. ovale* 32-fold when it was combined with 100 µg/ml of BHA (equivalent of $^1/_2$MIC for *P. ovale*), reducing the MIC from 50 to 1.56 µg/ml. Similarly, BHA also significantly enhanced the activity of warburganal against *P. ovale*. Its MIC was lowered from 25 to 1.56 µg/ml. Although this result was almost comparable to that for vitamin C, BHA prolonged the activity for a longer period. Interest-

Table 2. Antifungal activity of common food additive antioxidants

Fungi tested	MIC (µg/ml) Vitamin C	Vitamin E	BHA	BHT
C. albicans	>800	>800	200	>800
S. cerevisiae	>800	>800	200	>800
P. ovale	400	>800	200	>800

Table 3. Antifungal activity of polygodial in combination with common food additive antioxidants

Antioxidants combined	MIC (µg/ml) *C. albicans*	*S. cerevisiae*	*P. ovale*
None	3.13	3.13	50
Vitamin C	3.13	1.56	3.13[a]
Vitamin E	6.25	3.13	12.5
BHA	3.13[a]	0.78[a]	1.56[a]
BHT	6.25	6.25	25

The concentration of antioxidants was 800 µg/ml. [a] The concentration was $^1/_2$MIC.

Table 4. Antifungal activity of warburganal in combination with $^1/_2$MIC of anethole against *C. albicans*, *S. cerevisiae* and *P. ovale*

MIC (µg/ml) *C. albicans*	*S. cerevisiae*	*P. ovale*
6.25 → 0.024	6.25 → 0.20	25 → 3.13

ingly, despite its structural similarity to BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene) showed no enhancement of the activity of either polygodial or warburganal against any of the three fungi tested. In short, none of the antioxidants tested had enhancing activity for either of the sesquiterpene dialdehydes against *C. albicans* and *S. cerevisiae*. However, vitamin C and BHA exhibited significant enhancing activity against *P. ovale*.

Recently, we found that anethole (**3**) significantly increased the antifungal activity of polygodial against *C. albicans* and *S. cerevisiae*[8]. For example, by combining polygodial with 100 µg/ml of anethole ($^1/_2$MIC for *S. cerevisiae*) its antifungal activity against *S. cerevisiae* was increased 64-fold. Thus, its MIC was lowered from 3.13 to 0.049 µg/ml. Following this discovery, warburganal was also examined in combination with anethole to see if anethole had the same enhancing activity. As expected, anethole also significantly increased the activity of warburganal against both *C. albicans* and *S. cerevisiae*. In this combination, the activity against *C. albicans* and *S. cerevisiae* was enhanced 32- and 256-fold when warburganal was combined with 100 µg/ml of anethole (equivalent of $^1/_2$MIC for both *C. albicans* and *S. cerevisiae*). In other words, the MIC of warburganal against *C. albicans* was lowered from 6.25 to 0.20 µg/ml and that against *S. cerevisiae* from 6.25 to 0.024 µg/ml. Anethole also enhanced the antifungal activity of both polygodial and warburganal against *P. ovale*, but not as much as against *C. albicans* and *S. cerevisiae*. The MICs were reduced only from 50 to 6.25 µg/ml of polygodial, and from 25 to 3.13 µg/ml of warburganal.

In summary, anethole significantly synergized the antifungal activity of both polygodial and warburganal against *C. albicans* and *S. cerevisiae*, but not much against *P. ovale*. In contrast to anethole, both vitamin C and BHA had a noticeable enhancing effect on the activity of polygodial and warburganal against *P. ovale*, but not against *C. albicans* and *S. cerevisiae*. The combination efficacy of anethole and vitamin C and BHA varied with the species of fungal microorganism, despite structural similarities between anethole and BHA. Interestingly, these compounds enhanced the antifungal activity of the sesquiterpene dialdehydes, but not vice versa. The mechanisms for these combination effects are currently under investigation.

On the basis of observations described in the preceeding paragraph, we suggest that stabilization of sesquiterpene dialdehydes, resulting in extended periods of activity, and the ability to act synergistically with polygodial (**1**) and warburganal (**2**), represent two distinctly different antioxidant effects. Thus far, it would appear that the former is a rather general phenomenon, whereas the latter is more genus-specific and may depend on structural characteristics of particular antioxidants. In searching for other substances for an appropriate combination, the following considerations can be taken into account. First, knowledge of the mode of action of each compound is extremely helpful. Second, microorganisms often develop the ability to overcome this mode of action. Obviously, the knowledge of this interaction is also useful. With regard to a possible mechanism of synergy, a plausible explanation can be derived from several pieces of information. Polygodial and warburganal enhance the uptake of antioxidants by fungi. Once inside the cell, these compounds inhibit one or more physiological or biochemical processes. It is not illogical to suppose that these compounds can also interfere with microbial enzyme systems once the permeability barrier has been overcome. More work is needed to confirm these hypotheses. Needless to say, an additional benefit from the use of antioxidants in combination with antimicrobial compounds is their antioxidant properties.

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