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Naturally Occurring Antifungal Agents against *Zygosaccharomyces bailii* and Their Synergism

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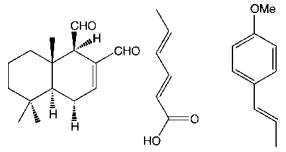
Polygodial was found to exhibit a fungicidal activity against a food spoilage yeast, *Zygosaccharomyces bailii*, with the minimum fungicidal concentration (MFC) of 50 μg/mL (0.17 mM). The time−kill curve study showed that polygodial was fungicidal at any growth stage. The primary action of polygodial comes from its ability to disrupt the native membrane-associated function of integral proteins as nonionic surface active agents (surfactants) followed by a decrease in plasma membrane fluidity. The fungicidal activity of polygodial was increased 128-fold in combination with a sublethal amount (equivalent of 1/2 MFC) of anethole and vice versa relative to the fungicidal activity of anethole. The fungicidal activity of sorbic acid was enhanced 512-fold in combination with 1/2 MFC of polygodial. Conversely, the fungicidal activity of polygodial was enhanced 128-fold in combination with 1/2 MFC of sorbic acid.

KEYWORDS: ***Zygosaccharomyces bailii*; antifungal activity; polygodial; anethole; sorbic acid; synergism; plasma membrane fluidity**

INTRODUCTION

The species of the yeast genus *Zygosaccharomyces* are notable for their ability to grow in a high osmoticum of sugar and/or salts and are involved in the spoilage of honey, syrups, and molasses and in the fermentation of soy sauce and some wines. Specifically, *Zygosaccharomyces bailii*, an osmophilic food spoilage yeast (*1*), is known for its capacity to survive in stressful environments and, in particular, in an acid media with ethanol, as in wine (*2*). In addition, spoilage of mayonnaise and salad dressing by *Z. bailii* is well-described (*3*, *4*). Despite the role of some *Zygosaccharomyces* species as agents causing spoilage of food and beverages, little is known of their genetics or of adaptations that allow them to grow in hyperosomotic environments. Hence, safe and effective fungicides are needed to safely control this organism.

In our continuing search for antifungal agents, polygodial (**1**; see **Figure 1** for its structure), a bicyclic sesquiterpene dialdehyde isolated as the principal pungent agent from the sprouts of *Polygonum hydropiper* (Polygonaceae) (*5*), known as "tade" and used as a spice in Japan, was previously described to exhibit potent fungicidal activities against yeasts such as *Saccharomyces cerevisiae* and *Candida albicans* (*6*−*8*). Here, we describe the antifungal activity of polygodial against *Z. bailii* followed by membrane damage such as the decrease in plasma membrane fluidity. In addition, the fungicidal action of polygodial against *S. cerevisiae* and *C. albicans* was dramatically enhanced through combination with phenylpropanoids, such as anethole and methyleugenol, which are mainly extracted from herb and spice oils of aniseed (*8*). For example, the minimum inhibitory concentration (MIC) of polygodial against *S. cerevisiae* was reduced from 3.13 to 0.049 μg/mL when combined with 1/2 MIC of anethole (*8*, *9*). Combining two or more phytochemicals in order to enhance total biological activity seems to be a most promising strategy. Thus, combining more than two compounds might, in general, be superior to the use of a single antimicrobial compound in order to make more unlikely the development of resistance mechanisms in microorganisms, in addition to enhancing and/or broadening the biological activity. However, a rational basis for this approach is still in an embryonic stage. Hence, in this study, sorbic acid (**2**), a currently used food preservative, and anethole (**3**) were individually combined with polygodial to determine enhanced activity against *Z. bailii*.



1 2 3

Figure 1. Chemical structures of polygodial (**1**), sorbic acid (**2**), and anethole (**3**).

MATERIALS AND METHODS

Chemicals. Polygodial and anethole were available from our previous studies (*8*). Sorbic acid and octyl gallate were purchased from Sigma Chemical Co. (St. Louis, MO). Octanol was obtained from Aldrich Chemical Co. (Milwaukee, WI). Because the most commonly used sorbates are sorbic acid and its potassium salt (*10*), sorbic acid was used as a primary antifungal compound in combination experiments

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to study antifungal activity against *S. cerevisiae*. For the experiment, all of the compounds were first dissolved in *N*,*N*-dimethyl formamide (DMF) purchased from EM Science (Gibbstown, NJ).

Test Strains. *Z. bailii* ATCC 60483 and *S. cerevisiae* ATCC 7754 were purchased from the American Type Culture Collection (Manassas, VA).

Medium. *Z. bailii* and *S. cerevisiae* were maintained at −80 °C in yeast nitrogen broth (Difco Lab, Detroit, MI) containing 25% glycerol and subcultured at 30 °C in Sabouraud's dextrose agar medium (1% bactopeptone, 4% dextrose, and 1.8% bacto-agar). A fresh culture was preincubated with shaking for 5 h at 30 °C in YPD (1% yeast extract, 2% bactopeptone, and 2% dextrose) broth.

Antifungal Assay. The final concentration of DMF in each medium was 1%. The highest concentration tested was 1600 μg/mL, unless otherwise specified. Broth macrodilution MICs were determined as previously described (*9*). Briefly, serial 2-fold dilutions of the test compounds were made in DMF, and 30 μL of 100 × concentrated solution was added to 3 mL of YPD broth. These were inoculated with 30 μL of seed culture to give the final inoculum of 10^5 colony forming units (CFU)/mL. The assay tubes were incubated without shaking at 30 °C for 48 h. The MIC is the lowest concentration of test compound that demonstrated no visible growth. The minimum fungicidal concentrations (MFCs) were examined as follows. After determining the MIC, a 30 μL of aliquot was taken from each clear tube and added into 3 mL of drug free YPD medium. After 48 h of incubation, the MFC was determined as the lowest concentration of the test compounds in which no recovery of microorganisms was observed. Time−kill studies were performed to examine the effects of combinations of compounds in more detail. The culture tubes were prepared as described above and incubated at 30 °C for 5 h. A 30 μL aliquot of the culture was inoculated into 3 mL of YPD broth containing appropriate concentrations of the test compounds. Samples were taken at selected times during 48 h of exposure, and serial dilutions were made in sterile saline before the samples were plated onto YPD agar plates. The plates were incubated at 30 °C for 48 h before the number of CFUs was counted.

Combination studies were performed by a broth checkerboard method (*11*). A series of 2-fold dilutions of one compound were tested in combination with 2-fold dilutions of the other compounds. The assays were performed in triplicate on separate occasions. The fraction inhibitory concentration (FIC) indices were calculated from checkerboard data. The FICs for these combinations were calculated as (MICa combination/MICa alone) + (MICb combination/MICb alone), where a and b were two compounds tested. The FICs or fractional fungicidal concentrations (FFC) presented are significant values obtained from the checkerboard matrix. FIC and FFC indices were used to define the interaction of combined compounds: synergistic (×0.5), additive (1× > 0.5), indifferent (4× > 1), or antagonistic (× > 4).

Plasma Membrane Fluidity. The plasma membrane fluidity was detected by pyrene excimer fluorescence (*12*, *13*). The exponentially growing *S. cerevisiae* ATCC 7754 cells in YPD broth were washed with phosphate-buffered saline (PBS) and then incubated with shaking in PBS at 30 °C for 30 min. The cells were rewashed and then incubated in 20 mM potassium phosphate buffer (pH 7.4) containing 1.2 M D-sorbitol (lysis buffer). Zymolyase 20T (Seikagaku Corp., Japan) was added to the suspension to lyse the yeast cell wall. The spheroplast cells obtained were washed and then suspended in the lysis buffer. Three milliliters of cell suspension (1×10^7 CFU/mL) was preincubated in the lysis buffer with 10 mM pyrene at 30 °C for 1 min. After polygodial, octyl gallate and *n*-octanol were added to the suspension, and the suspension was incubated for another 3 min. The intensities of monomer fluorescence of pyrene at 386 nm (I_M) and of excimer fluorescence at 480 nm (I_E) were collected with excitation at 340 nm, respectively. The ratio of intensities, I_E/I_M, is increased by pyrene collision related to the fluidity of plasma membrane. Therefore, the membrane fluidity was evaluated by I_E/I_M. Values are means ± standard deviations (n = 3).

RESULTS

The compounds selected mainly from our previous studies against *S. cerevisiae* were tested against *Z. bailii* using a broth dilution method and compared with those against *S. cerevisiae*. The results are listed in **Table 1**. As expected, the compounds characterized as antifungal agents against *S. cerevisiae* also exhibited similar activities against *Z. bailii*, although to a lesser extent than against *S. cerevisiae*. Among the compounds tested, polygodial was the most effective against *Z. bailii*. In contrast to a potent antifungal activity against *S. cerevisiae*, polygodial exhibited a moderate activity against *Z. bailii* with the MFC of 50 μg/mL (0.17 mM). This MFC is 16-fold more than that against *S. cerevisiae*, which was obtained with malt extract medium. As the growth rate of *Z. bailii* in malt extract medium is quite slow, YPD medium was used as a culture medium for *Z. bailii* in this report. The moderate activity may be explained by the following two possibilities. First, the possibility is that *Z. bailii* is more resistant to polygodial. Second, polygodial is probably inactivated in YPD medium by its reactivity on primary amino groups (*14*). In fact, the MIC of polygodial against *S. cerevisiae* was reported to be 25 μg/mL in YPD medium (*15*). The latter possibility seemed to be reasonable. No differences in the MIC and MFC of polygodial against *Z. bailii* were noticed, suggesting no residual fungistatic activity. The activity of individual compounds may not be potent enough for practical use. While polygodial was first isolated as the principal pungent agent from the sprouts of *P. hydropiper*, it should be noted that it exhibited antifungal activity against *Z. bailii* at a level below the taste threshold.

Although sorbic acid and benzoic acid are known to be more inhibitory at lower pH values due to a higher rario of undissociated molecules, their fungicidal activity (MFC) against *Z. bailii* was not influenced by pH values (**Table 2**), indicating that there seems to be resistance to acid media. The undissociated form of sorbic acid [(2*E*,4*E*)-hexadiennoic acid] is very likely to penetrate the plasma membrane lipid bilayer at a pH lower than 5. In these conditions, large amounts of the undissociated form of sorbic acid enter into the cytoplasm of *Z. bailii* supporting potentiation of fungistatic activity (MIC) of sorbic acid (**Table 2**). *Z. bailii* is known for its capacity to survive stress and, in particular, in acid media such as mayonnaise, salad dressing, and wine. *Z. bailii* utilizes benzoate and

Table 1. Antifungal Activity of Selected Compounds against *Z. bailii*[a]

compounds tested	antifungal activity (μg/mL) MFC[b]	MIC[c]
polygodial	50	50
anethole	200	400
sorbic acid	800	1600
(2*E*)-hexenoic acid	800	1600
(2*Z*)-hexenoic acid	800	1600
hexanoic acid	800	3200
methyl sorbate	>3200	>3200
miconazole	12.5	12.5

[a] The pH value of the YPD medium used for the antifungal assay was 6.2. [b] MFC, minimum fungicidal concentration. [c] MIC, minimum growth inhibitory concentration.

Table 2. pH Effect of Sorbic Acid against *Z. bailii*[a]

pH	antifungal activity (μg/mL) MIC[c]	MFC[d]	undissociated form (%)[b]
3	100	1600	98.0
5	400	1600	37.0
7	800	1600	0.6
9	1600	1600	

[a] The antifungal assay was done using YPD medium. Prior to the MIC assay, YPD medium was adjusted to pH 3–9 as indicated. A blank cell indicates that it was not tested. [b] Data extracted from ref *10*. [c] MIC, minimum growth inhibitory concentration. [d] MFC, minimum fungicidal concentration.

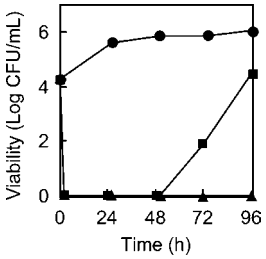

Figure 2. Effect of polygodial on the growth of *Z. bailii*. The exponentially growing cells of *Z. bailii* ATCC 60483 were incubated in YPD broth at 30 °C. Polygodial was added to the culture at 0 (circle), 50 (square), and 100 (triangle) μg/mL.

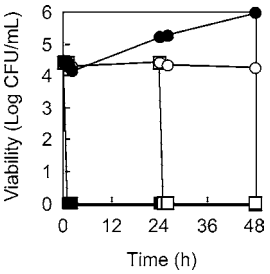

Figure 3. Effect of polygodial on the cycloheximide-treated cells of *Z. bailii*. The exponentially growing cells of *Z. bailii* ATCC 60483 were incubated in YPD broth with (open) or without (closed) 50 mg/mL of cycloheximide. Polygodial was added to the culture at 0 (closed square) and 24 (open square) h.

sorbate as a sole carbon source thereby promoting growth (*16*). Therefore, extremely high concentrations of these acids are needed for fungicidal action against *Z. bailii*. (2*Z*)-Hexenoic acid and hexanoic acid—analogues of sorbic acid—showed weak antifungal activities against *Z. bailii* as did sorbic acid (**Table 1**). However, methyl sorbate showed no activity up to 3200 μg/mL. This indicates that dissociated groups are needed for antifungal action.

The fungicidal effect of polygodial against *Z. bailii* was confirmed by the time−kill curve method. The cultures of *Z. bailii*, with a cell density of 1×10^4 CFU/mL, were exposed to two different concentrations of polygodial, the MFC and 2 × MFC. The results are illustrated in **Figure 2**. The number of viable cells was determined following different periods of incubation with polygodial. The result verifies that 2 × MFC was needed for a complete lethality. Thus, MFC quickly reduced viability, but the final cell count was not significantly different to controls. Additionally, lethality occurs within the first 1 h following the addition of polygodial. A membrane-disrupting fungicide, amphotericin B, causes prompt cell death (*17*). Rapid lethality induced by polygodial against *Z. bailii* also may be in part associated with membrane disruption.

Amphotericin B expresses fungicidal effects in nongrowing cells as well as those growing exponentially (*17*). The fungicidal effect of polygodial on the cycloheximide-treated cells of *Z. bailii* was confirmed (**Figure 3**). Cycloheximide at 50 μg/mL completely inhibited vegetative cell growth but showed no fungicidal effect throughout incubation. After 24 h of incubation with cycloheximide, polygodial at MFC was added to the culture and showed rapid lethality. A fungistatic cycloheximide inhibits macromolecular biosyntheses such as cytoplasmic protein synthesis, which are needed for cell division. It is thus not likely that the reduced fungal viability induced by polygodial is due to interaction with the synthesis of macromolecules such as DNA, RNA, and proteins.

Subsequently, we investigated the effect of growth phase on the fungicidal ability of polygodial against *Z. bailii*. After 24, 48, and 72 h of incubation, 2 × MFC of polygodial was added to *Z. bailii* cultures. It rapidly reduced cell viability after 1 h of each addition (**Figure 4**). At 48 h, the cell growth was arrested representing entry to the stationary phase. This indicates that polygodial acts regardless of growth phase, supporting fungicidal action against nongrowing cells as amphotericin B does.

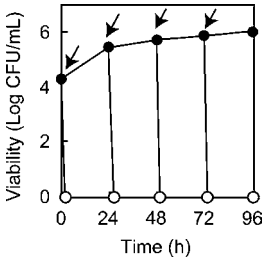

Figure 4. Effect of growth phase on the fungicidal activity of polygodial against *Z. bailii*. The exponentially growing cells of *Z. bailii* ATCC 60483 were incubated with (open) or without (closed) polygodial in YPD broth. Arrows indicate the time when polygodial (100 μg/mL) was added to the culture.

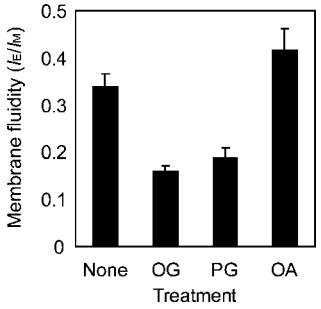

Figure 5. Change in plasma membrane fluidity. Plasma membrane fluidity was determined using the spheroplast cells of *S. cerevisiae* ATCC 7754. The spheroplast cell suspension (10^7 CFU/mL) was treated in the lysis buffer containing 1% DMF added with no drug (none), OG (100 μg/mL of octyl gallate), PG (25 μg/mL of polygodial), or OA (100 μg/mL of *n*-octanol) at 30 °C for 3 min prior to measurement of the fluidity. Each drug concentration tested was MFC against *S. cerevisiae* ATCC 7754 in the antifungal assay using YPD medium.

Membrane-disrupting fungicides such as octyl and nonyl gallates reduced plasma membrane fluidity physically disrupting the plasma membrane, followed by cytoplasm leakage in *S. cerevisiae* (*13, 18*). On the other hand, *n*-alkanols increased the fluidity of plasma membrane affecting (Ca^{2+}/Mg^{2+}) ATPase activity in nerve cells (*19*). *n*-Octanol did not induce the leakage of cytoplasm in *S. cerevisiae* (data not shown). The effect of polygodial on plasma membrane fluidity was investigated. The fungal cell wall needs to be removed for the measurement of plasma membrane fluidity (*13*). As little of the composition and structure of the cell wall of *Z. bailii* is known (*20*), it was difficult to prepare the spheroplast cells of *Z. bailii*; we instead used *S. cerevisiae* ATCC 7754. Polygodial, as well as octyl gallate, reduced the fluidity of spheroplast cells in *S. cerevisiae* as shown in **Figure 5**. In addition, polygodial was reported to leak the intracellular materials of *S. cerevisiae* cells (*21*) as well as alkyl gallate. Our results were reflected in those reported. Meanwhile, our observation that *n*-octanol increased the fluidity (**Figure 5**) also agreed with that given in the case of the nerve cells.

The combination effects are first discussed based on the MIC data obtained by the checkerboard method (*11*). However, the MIC, which is obtained by turbidity measurement after a 48 h incubation, did not fully characterize the antifungal activity of the combination. For example, it is not clear if the combination

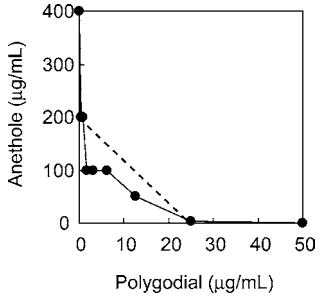

Figure 6. Resulting isobologram of the minimal fungicidal concentrations obtained with combinations of polygodial and anethole against *Z. bailii.* Data are indicated as MFCs and belong to the area left under the dotted line, which shows synergy on the MFC.

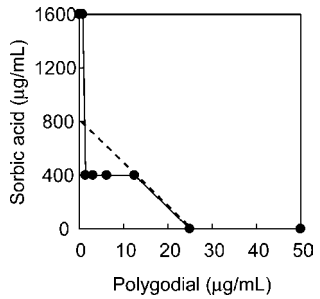

Figure 7. Resulting isobologram of the minimal fungicidal concentrations obtained with combinations of polygodial and sorbic acid against *Z. bailii.* Data are indicated as MFCs and belong to the area left under the dotted line, which shows synergy on the MFC.

has fungicidal or fungistatic activity. Polygodial and several other compounds, sorbic acid, benzoic acid, anethole, eugenol, and methyleugenol, against *Z. bailii* were investigated in combination (data not listed). These combinations synergistically seemed to retard the growth rate of *Z. bailii*. However, we could not confirm synergism from the data obtained because of slow growth rate of *Z. bailii*.

The combination of polygodial with anethole converted the former compound's activity to fungicidal against *Z. bailii*. The MFC of polygodial was lowered from 50 to 0.39 μg/mL when 200 μg/mL of anethole was combined (**Figure 6**). As a result, the activity of polygodial was increased 128-fold. Interestingly, the fungicidal activity of anethole was also enhanced 128-fold by polygodial. The MFC of anethole (400 μg/mL) against *Z. bailii* was lowered from 400 to 3.13 μg/mL in combination with 25 μg/mL of polygodial.

Sorbic acid was also combined with polygodial to see if the combination has the same enhancing activity. The complete killing action of sorbic acid was observed at 100 μg/mL within 12 h when it was combined with 1/2 MFC of polygodial (data not shown). The combination of sorbic acid and polygodial on the fungicidal action against *Z. bailii* was objectively proven to be highly synergistic due to the concave nature of their isobologram (**Figure 7**) and FFC index of 0.25. The fungicidal activity of sorbic acid was increased 511-fold in combination with 1/2 MFC of polygodial. The MFC of sorbic acid was lowered from 1600 to 3.13 μg/mL when 25 μg/mL of polygodial was combined. Sorbic acid or polygodial each exhibited complete killing action within 12 h at 1600 or 6.25 μg/mL, respectively. For example, the combination of polygodial (1/2 MFC) and 100 μg/mL of sorbic acid, 16-fold lower than its MFC, rapidly killed *Z. bailii*.

DISCUSSION

On the basis of the above, an attempt was made to clarify the enhancing activity of polygodial on a molecular basis. The addition of glucose to an unbuffered suspension of cells results in the extrusion of acid. The change in external pH upon the addition of glucose is characteristic of yeast cells. This extruded acid may be due to the action of the plasma membrane H^+-ATPase (*22*). The activation of the H^+-ATPase by glucose is not yet fully understood at the molecular level, but the maintenance of internal pH homeostasis is essential for the cell to survive since intracellular pH is important for the activity of a number of enzymes with narrow pH optima (*23*, *24*). This glucose-induced medium acidification process was inhibited by polygodial in *S. cerevisiae* (*25*), presumably by inhibition of the H^+-ATPase. In support of this, polygodial predictably inhibits the isolated H^+-ATPase of *Z. bailii* as well as of *S. cerevisiae*. Therefore, it is possible that the antifungal activity of polygodial is, at least, due in part to its inhibition of the plasma membrane H^+-ATPase.

Similar to other weak acid preservatives, the antifungal activity of sorbic acid against *S. cerevisiae* increases with a lowering of the pH in the suspending medium in which sorbic acid should be mostly in the undissociated form (*10*). This undissociated form is soluble in the membrane phospholipid and is thought to enter the cell by passive diffusion across the plasma membrane (*26*). Therefore, the undissociated form is a toxic form, similar to octanoic and decanoic acids (*27*). It is believed that the primary mode of action of weak acid preservatives is to reduce the internal pH below the normal physiological range, leading to growth arrest (*28*, *29*). Yeast cells are known to adapt to weak acid stress by (i) the restoration of internal pH via the export of protons by the plasma membrane H^+-ATPase in an energy-depending process and (ii) the generation of sufficient ATP to drive this process while still allowing growth to occur. This adaptation is not due to metabolic removal or exhaustion of the sorbic acid (*30*). The major effect of weak acids on *Z. bailii* seems to be a decrease in intracellular pH, which in turn inactivates key enzymes such as phosphofructokinase and to a lesser extent hexokinase (*31*, *32*). As mentioned above, sorbic acid appeared to stimulate plasma membrane H^+-ATPase activity and the protons were pumped out to the external medium. Polygodial inhibits this acid-mediated acidification by inhibiting the plasma membrane H^+-ATPase. In other words, polygodial inhibits the adaptation of yeast to sorbic acid stress by inhibiting the plasma membrane H^+-ATPase and, as a result, enhances the fungicidal activity of sorbic acid. If this is so, the synergistic activity of polygodial should not be specific to sorbic acid. Therefore, the other most commonly used weak acid preservative, benzoic acid, was also tested in combination with polygodial (data not shown). As expected, the MFC of benzoic acid was enhanced 400-fold by combining it with 1/2 MFC of polygodial.

The antifungal activity of the three analogues of sorbic acids, (2*Z*)-hexenoic acid, hexanoic acid (also known as caproic acid), and methylsorbate, was also tested. The results are listed in **Table 1**. It appears that the antifungal activity is not specific to sorbic acid and that the double bond does not seem to be essential to elicit the activity. For example, a more stable and common hexanoic acid showed slightly weaker activity as compared to sorbic acid (*33*). In general, the antifungal activity of fatty acids is not potent but they are considered to possess little or no mammalian toxicity. In addition to this weak activity, their strong odor has limited their use, but this can be solved by reducing the amount in combination with a small amount of polygodial. Therefore, further combination study, especially with H^+-ATAase inhibitors, should be conducted. On the other hand, anethole also showed synergistic antifungal activities combined with polygodial against *Z. bailii* (**Figure 6**) in addition to *S. cerevisiae* and *C. albicnas*. The antifungal activity of anethole

is potentiated in fermentatively growing cells of *S. cerevisiae* (*34*). In our antifungal assay, incubation was done without shaking. Therefore, yeast cells grow anaerobically and large amounts of carbonate are then intracellularly produced. For the above reason, the antifungal activity of anethole might be enhanced through a decrease in intracellular pH by a blockage of proton exhaust triggered by polygodial.

Safety is a prime consideration for fungicidal agents, especially those used in food products, which may be utilized in unregulated quantities on a regular basis. The fungicidal agents isolated from plants being used as spices and/or characterized as flavor substances in many edible plants from old times should be superior as compared to nonnatural ones.

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