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Antifungal efficacy of octylgallate and 4-isopropyl-3-methylphenol for control of *Aspergillus*

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Abstract

Background: Control of fungi is problematic, since effective methods for intervening fungal infection or contamination, especially for those resistant to drugs/fungicides, are often very limited. Generally regarded as safe (GRAS) agents, such as natural compounds or their structural analogs could be potential sources of sustainable antifungal agents. As a proof of concept, antifungal efficacy of twenty-one compounds (mostly GRAS) and six conventional preservatives was investigated against *Aspergillus brasiliensis*, one of the challenge microbes for preservative evaluation. In particular, efficacy of octylgallate (OG) and 4-isopropyl-3-methylphenol (4I3M) was evaluated for synergism as well as for overcoming fludioxonil (phenylpyrrole fungicide) tolerance of *Aspergillus* mutants.

Findings: Out of twenty-one compounds examined (Clinical Laboratory Standards Institute Protocol), five compounds showed the highest antifungal activity, viz., OG > benzaldehyde > nonylgallate > 2-hydroxy-4-methylbenzaldehyde > carvacrol (higher to lower activity), where OG and benzaldehyde exhibited fungicidality. For formulation purpose, synergism was further investigated between OG (showing the highest activity) and conventional preservatives. 4I3M was the most responsive preservative to OG, where antifungal efficacy of 4I3M was augmented four-fold with OG + 4I3M combination. Model yeast bioassay showed vacuolar and antioxidant mutants were highly susceptible to 4I3M. 4I3M further overcame fludioxonil tolerance of oxidative signaling mutants of *Aspergillus*. Of note, while 4I3M and its natural derivatives (thymol, carvacrol) were compatible with two out of three commercial surfactants explored, the remaining surfactant antagonized antifungal activity of 4I3M and its derivatives.

Conclusions: OG (GRAS agent) and 4I3M (conventional preservative), alone or in combination, effectively prevented fungal growth, where vacuolar detoxification and/or antioxidant system were important for fungal survival against 4I3M. While 4I3M overcame fludioxonil tolerance of *Aspergillus* mutants, types of surfactants co-applied were crucial for achieving optimum activity of antifungal compounds. Accordingly, comprehensive characterization of compound-compound or compound-surfactant specificity would be required further when developing sustainable antifungals.

Keywords: Antifungal, *Aspergillus*, fludioxonil resistance, 4-Isopropyl-3-methylphenol, octylgallate, surfactant compatibility, sustainability, yeast

Background

Filamentous fungi in the genus *Aspergillus* are opportunistic pathogens and/or producers of hepato-carcinogenic mycotoxins, which cause serious human health risks [1,2]. Among those, *Aspergillus brasiliensis*, a member of the black aspergilli, has recently been identified as a causative agent of keratitis [3]. *A. brasiliensis* is one of the challenge microorganisms for evaluating preservative efficacy in industrial products, by tracking decreases in microbial populations [4].

There are increasing demands for safe, sustainable pre-

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servatives, which present no significant human health or environmental side effects, and therefore, could avoid negative consumer perception. Many natural products are "generally regarded as safe (GRAS)" compounds, and are potential sources of antifungal agents, either in their intact structure or as lead compounds for more potent analogs [5].

Despite their utility, preservatives can be deactivated by certain components, such as surfactants, in the formula. Surfactants are widely used in household products and industrial applications [6 and references therein]. Although possible toxicities of surfactants to living organisms have been investigated [6], the mechanism of preservative deactivation by certain surfactants is vastly undetermined.

In this study, twenty-one compounds and six conventional preservatives were investigated for the search of sustainable alternatives to toxic, allergic preservatives. Candidate compounds, such as octylgallate (OG) or 4-isopropyl-3-methylphenol (4I3M), effectively prevented fungal growth and/or overcame fungicide tolerance with compound-compound or compound-surfactant specificity.

Findings

Antifungal synergism between OG and conventional preservatives: Compound-compound specificity

Initially, antifungal efficacy of twenty-one compounds was evaluated (up to 1 mM cut off) against *A. brasiliensis*, where OG (GRAS agent) exhibited the highest activity (See **Additional File 1**; **Supplementary Table S1** for details).

For formulation purpose, levels of synergism between OG and eight compounds, *viz*., six conventional preservatives (**Table 1**) and two natural analogs of 4I3M (thymol, carvacrol), were investigated *via* checkerboard bioassay (**Additional File 1: Methods**).

For MICs, co-application of OG with six agents (four preservatives, thymol, carvacrol) resulted in augmentation of antifungal efficacy (both OG and six agents) [Fractional Inhibitory Concentration Indices (FICIs)=0.8 to 1.0; **Table 1**], while that with phenoxyethanol or 2,3,4-trihydroxybenzaldehyde showed indifference (FICI=2.0). Of note, lowest MICs (preservatives) were achieved with 4I3M (and thymol, carvacrol) (MIC=0.4 mM; **Table 1**), while that with other preservatives ranged 0.8 to 12.8 mM (*viz*., less effective). 4I3M was the most responsive preservative to

Table 1. Antifungal efficacy of combined application of OG (mM) with six preservatives or natural analogs of 4I3M (thymol, carvacrol; mM) tested against *A. brasiliensis*.*

Compounds	MIC alone	MIC combined	FICI	MFC alone	MFC combined	FFCI
OG	0.1	0.1	2.0	0.2	0.2	2.0
Phenoxyethanol	12.8†	12.8		12.8†	12.8	
OG	0.1	0.1	2.0	0.2	0.2	2.0
2,3,4-Trihydroxy-benzaldehyde	12.8†	12.8		12.8†	12.8	
OG	0.1	0.025	0.8	0.2	0.2	2.0
Caprylyl glycol	6.4	3.2		12.8†	12.8	
OG	0.1	0.025	0.8	0.2	0.2	2.0
Caprylhydroxamic acid	1.6	0.8		12.8†	12.8	
OG	0.1	0.05	1.0	0.2	0.2	2.0
4'-Hydroxyacetophenone	12.8†	6.4		12.8†	12.8	
OG	0.1	0.05	0.8	0.2	0.2	2.0
4-Isopropyl-3-methylphenol	1.6	0.4		6.4	6.4	
OG	0.1	0.05	1.0	0.2‡	0.2	2.0
Thymol	0.8	0.4		1.6	1.6	
OG	0.1	0.05	1.0	0.2	0.2	2.0
Carvacrol	0.8	0.4		6.4	6.4	
Mean:						
OG	0.1	0.06	1.4	0.2	0.2	2.0
Preservatives & analogs	6.2	4.7		9.8	9.8	
t-Test§:						
OG	--	$p = 0.001$	--	--	$p = 1.0$	--
Preservatives & analogs	--	$p = 0.588$	--	--	$p = 1.0$	--

* MIC: Minimum inhibitory concentration; MFC: Minimum fungicidal concentration; FICI: Fractional inhibitory concentration indices; FFCI: Fractional fungicidal concentration indices (See **Additional File 1: Methods** for calculations).
†Compound was tested up to 6.4 mM. For calculation purpose, 12.8 mM (doubling of 6.4 mM) was used.
‡99.8% fungal death.
§Student's t-test for paired data, namely, mean MIC or MFC of each compound (combined) vs. mean MIC or MFC of each compound (alone), was determined in OG and eight compounds [viz., six preservatives and two natural analogs of 4I3M (thymol, carvacrol)].

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OG, showing four-fold enhancement in antifungal efficacy when combined with OG. For MFCs, co-application of OG with all eight compounds (six preservatives, thymol, carvacrol) resulted in indifference [Fractional Fungicidal Concentration Indices (FFCIs)=2.0; **Table 1**].

Therefore, we concluded that the effect of "OG+ preservative" co-application was chiefly enhancing compounds' growth inhibitory efficacy, but not heightening their fungicidality. 4I3M was the most interactive preservative to OG.

4I3M and cellular ion/redox homeostasis

4I3M (4-isopropyl-3-methylphenol) is a synthetic analog of the natural products thymol (2-isopropyl-5-methylphenol) and carvacrol (5-isopropyl-2-methylphenol). As with thymol and carvacrol, 4I3M has been developed as an antimicrobial preservative [**7**,**8**], where the color/odor-neutral characteristic of 4I3M is more appealing to consumer perception. However, comparing to its natural analogs, molecular targets of 4I3M-were largely uncharacterized.

By using vacuolar (*vph2*Δ, *vma1*Δ) and antioxidant (*sod2*Δ, *sod 1*Δ, *glr1*Δ, *yap1*Δ) mutants of the model fungus *Saccharomyces cerevisiae,* we previously observed thymol was involved in cellular ion and redox homeostasis in fungi [**9**]. Preliminary results confirmed that the synthetic compound had the same effect as that of the natural compounds, where vacuolar (*vph2*Δ, *vma1*Δ) and cytosolic superoxide dismutase (*sod1*Δ) mutants were especially more susceptible to 4I3M compared to other yeasts tested (Figure data not shown).

Therefore, we speculated that, like the natural analog thymol, 4I3M might also be involved in cellular ion (which links to xenobiotic detoxification [**10**]) and redox homeostasis. Phenolics function as deleterious redox cyclers against microbial cells, and thus prevent microbial growth by disrupting cellular redox homeostasis [**11**,**12**]. Consequently, fungi having deficiency in vacuolar/antioxidant systems, *viz*., abnormality in xenobiotic detoxification and antioxidation, would be more susceptible to 4I3M.

Overcoming fludioxonil tolerance of *Aspergillus* mutants by 4I3M

The usefulness of 4I3M for overcoming fungicide tolerance was further investigated. The phenylpyrrole fungicide fludioxonil inhibits fungal growth by triggering excessive stimulation of signaling pathway, such as mitogen-activated protein kinase (MAPK) pathway [**13**]. This MAPK pathway is responsive to oxidative cues, thus protects fungi from environmental oxidative stressors. However, fungi having mutations in MAPK genes escape fludioxonil toxicity, resulting in development of fludioxonil tolerance [**13**].

We included the WT and two oxidative signaling MAPK mutants (*sakA*Δ, *mpkC*Δ) of *Aspergillus fumigatus* for this study, considering the mechanisms of MAPK signaling were well characterized in this species [**14**,**15**]. As shown in **Figure 1**, the growth of *A. fumigatus* WT was completely inhibited by 50 μM fludioxonil, while MAPK mutants exhibited tolerance to fludioxonil (**Additional file 1: Methods**). However, 4I3M effectively overcame fungal fludoxonil tolerance, where co-application of sub-inhibitory concentration of 4I3M (0.6 mM) with fludioxonil completely inhibited the escape of MAPK mutants from fludioxonil toxicity.

Noteworthy is that, with independent treatment of 4I3M, *sakA*Δ and *mpkC*Δ antioxidant mutants developed less radial growth (*viz*., more sensitive to 4I3M) compared to WT (**Figure 1**). Thus, in accordance with yeast bioassay (See above), results further indicated 4I3M negatively affected fungal redox homeostasis, where antioxidant MAPK mutants having deficiency in oxidative stress defense were more susceptible to 4I3M. We further surmised that higher susceptibility to 4I3M could be one contributing mechanism of overcoming fludioxonil tolerance of MAPK mutants.

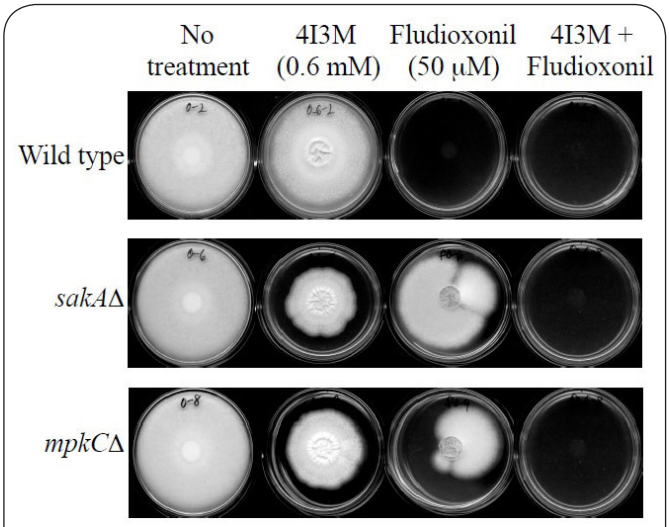

Figure 1. Overcoming fludioxonil tolerance of *A. fumigatus* MAPK mutants (*sakA*Δ, *mpkC*Δ) by 4I3M.

Compatibility of 4I3M and its natural derivatives with commercial surfactants: Compound-surfactant specificity

Ingredients' compatibility is essential for the functionality of antifungal agents in preservative formulation. For example, surfactants (Surface Active Agents) are amphiphilic organic agents, which lower surface tension of liquids in many industrial products, thus enabling emulsification or compound delivery easier [**16**]. While surfactants do not have to possess antimicrobial activities *per se*, they could adversely affect the functionality of active ingredients depending on types of surfactant formulation. To further assess 4I3M's antifungal efficacy in formulation, compatibility of 4I3M (and its natural derivatives thymol and carvacrol for comparison) with three commercial surfactants was investigated by determining MICs and MFCs (**Additional File 1: Methods**). For MICs, no fungal growth was detected during liquid incubation (*viz*., 10%

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surfactant solution in water; 48 hrs, 168 hrs) even w/o incorporation of 4I3M or derivatives into three surfactant solutions.

However, for MFCs, two different fungal responses were detected depending on types of surfactants. With surfactant 1 [C_{12-14} fatty alcohol polyglycol ether-based; Trade name: Genapol (pH 7.4) (See **Additional File 1: Methods**)], 5 to 6% fungal recovery was initially determined in control plates (*viz*., w/o 4I3M or its derivatives; both 48 and 168 hrs) (**Figure 2**). However, incorporation of 4I3M or derivatives (12.8 to 51.2 mM) into surfactant 1 resulted in notable increase in fungal recovery (*viz*., enhanced fungal growth; **Figure 2**), indicating surfactant 1 negatively affected the antifungal function of 4I3M or its derivatives.

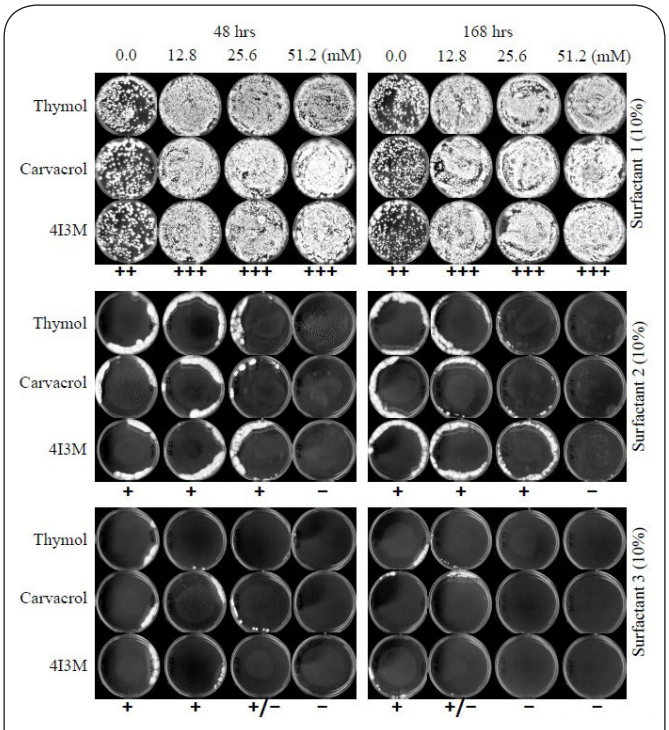

Figure 2. Compatibility of 4I3M, thymol and carvacrol with commercial surfactants. (+), Less than 5% fungal survival; (++), 5 to 6% fungal survival; (+++), Higher than 6% fungal survival; (+/-), Either less than 5% fungal survival or no fungal survival, depending on types of compounds; (-), No fungal survival.

In contrast, the other two surfactants, i.e., surfactant 2 [C_{8-16} fatty alcohol (alkyl polyglucosides)-based; Trade name: Glucopon (pH 7.6)] and surfactant 3 [sodium lauryl sulfate-based; Trade name: Stepanol (pH 7.7)], interacted positively with 4I3M or its derivatives for antifungal activity, where co-application of 4I3M/derivatives (up to 51.2 mM) with surfactant 2 or 3 achieved complete inhibition of fungal growth (both 48 and 168 hrs; **Figure 2**). Of note, surfactant 2 or 3, alone, possessed potent antifungal activity, where only peripheral fungal growth was detected on recovery plates even without incorporation of 4I3M/derivatives (**Figure 2**). Higher concentration (*viz*., 25.6 to 51.2 mM) of 4I3M/derivatives achieved enhanced antifungal efficacy (48 or 168 hrs).

It is noteworthy that, although both surfactants 1 and 2 were fatty alcohol-based products, they exhibited completely opposite specificity to 4I3M or its derivatives (resultantly, opposite antifungal efficacy; See above). While determining precise mechanism of differential specificity is necessary, we speculated additional ingredients that might reside in (commercial) surfactants could also contribute to the differential specificity. To further our understanding of 4I3M/derivatives compatibility with conventional surfactants, comprehensive research by including additional numbers of surfactants (*viz*., four different types: anionic, cationic, nonionic, amphoteric) is currently underway.

Collectively, characteristics of surfactants, including intrinsic antifungal activity, antagonism (shown above), etc., are important parameters, and thus, should be well-elucidated when developing sustainable antifungal formulation.

Conclusions

In this study, antifungal efficacy of GRAS compounds and conventional preservatives was investigated against model yeast and *Aspergillus*. The selected compounds OG and 4I3M, alone or in combination, effectively prevented fungal growth, where 4I3M could also overcome fludioxonil tolerance of *Aspergillus* mutants. Application of adequate surfactants was crucial for achieving optimum antifungal activity of compounds, thus emphasizing the importance of compound-compound or compound-surfactant specificity during the development of sustainable antifungal agents.

Additional files

Additional File 1: Methods

List of abbreviations

4I3M: 4-Isopropyl-3-methylphenol
CFU: Colony Forming Units
CLSI: Clinical Laboratory Standards Institute
FDA: Food and Drug Administration
FFCI: Fractional Fungicidal Concentration Index
FICI: Fractional Inhibitory Concentration Index
GRAS: Generally Regarded As Safe
MAPK: Mitogen-Activated Protein Kinase
MFC: Minimum Fungicidal Concentration
MIC: Minimum Inhibitory Concentration
OG: Octyl gallate
WT: Wild type

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Authors' contributions	JHK	WHC	KLC	LWC	WJO	KJ
Research concept and design	✓	✓	--	--	--	--
Collection and/or assembly of data	✓	--	✓	--	--	--
Data analysis and interpretation	✓	✓	✓	✓	✓	✓
Writing the article	✓	--	--	--	--	--
Critical revision of the article	✓	✓	✓	✓	✓	✓
Final approval of article	--	--	--	✓	--	--
Statistical analysis	✓	--	--	--	--	--

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