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Relation between lipophilicity of alkyl gallates and antifungal activity against yeasts and filamentous fungi

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ABSTRACT

The antifungal activity of a complete series of 15 *n*-alkyl gallates and six analogues acting against a representative panel of opportunistic pathogenic fungi was studied in order to analyze their role in: the importance of the fungi tested, the importance of the hydroxyls, the influence of the chain length and the hydrophobicity of the compounds. It was demonstrated that dermatophytes were the most susceptible species and that hydroxyls appear to be necessary but not sufficient for the activity. When the log *P* of each gallate was calculated and related to the different values of MIC against *Microsporum gypseum* it was observed that hexyl, heptyl, octyl and nonyl gallates exhibit a significant positive deviation from the curve corresponding to a polynomial equation obtained for the other gallates. This suggests that these compounds have a further mode of action besides their hydrophobicity, possibly the inhibition of some enzyme involved in ergosterol biosynthesis.

Gallic acid and its *n*-alkyl esters, particularly propyl (C_3), octyl (C_8) and dodecyl (C_{12}), are used as food additives due to their antioxidant activity.[1]

Considering that foods can carry contaminating fungi from natural sources as a consequence of handling and processing, methyl- (C_1) and hexyl to dodecyl (C_{6-12}) gallates have previously been investigated for antifungal activity against the industrially important fungus *Saccharomyces cerevisiae*.[2] Kubo reported that the maximum activity was found for decyl gallate (minimum inhibitory concentration MIC = 12.5 μg/mL), and that when the alkylic chain was longer (undecyl or dodecyl) the ester compounds were inactive. This is known as the cut-off phenomenon.[2] Nonyl, octyl, heptyl and hexyl gallates displayed lower fungistatic activity with MIC values of 25, 100, 400 and 800 μg/mL, respectively, whereas gallic acid and its methyl ester did not show any activity up to 3200 μg/mL.[1]

In a second study by Kubo, only propyl, octyl and dodecyl gallates were tested for antifungal activity against the fungi *S. cerevisiae, Zygosaccharomyces bailii, Candida albicans* and *Aspergillus niger*.[3] Of these, only octyl gallate was found to be active against *S. cerevisiae* (MIC = 25 μg/mL) and also against the other fungi tested, with MIC values between 25 and 50 μg/mL. Propyl and dodecyl esters were inactive.

In a third paper, Kubo et al.[1] found that nonyl gallate showed the best activity against *S. cerevisiae* with an MIC value of 6.25 μg/mL, better than the value for decyl gallate of 12.5 μg/mL.

The antifungal activity of alkyl gallates appeared, in these previous studies, to be dependent on the presence of a catechol moiety, along with a hydrophobic alkyl chain (similarly to that of alkanols).[3] The mechanism of antifungal action was proposed to involve their ability to disrupt the native membrane, as in the case of nonionic surfactants.

From these suggestions, it was clear that the lipophilicity of each gallate could play an important role in its activity. In addition, the activity appeared to be dependent on the microorganism tested.[1,3]

A study of the antifungal activity of a complete series of *n*-alkyl gallates, including gallic acid and other derivatives, against a representative panel of opportunistic pathogenic fungi, was carried out in order to analyze the role of the four factors mentioned above, on the antifungal activity of alkyl gallates: (a) the importance of the fungi tested; (b) the influence of the chain length; (c) the importance of free hydroxyls; and (d) the hydrophobicity of the compounds.

To this aim, we investigated the antifungal activity of 20 compounds: 15 gallates (methyl to octadecyl esters) and six analogues, including pyrogallol, 3,4,5-methyl gallic acid and 3,4,5-acetyl benzoic acids, which were tested against four yeasts (*C. albicans, Candida tropicalis, S. cerevisiae, Cryptococcus neoformans*), three hialohyphomycetes (*Aspergillus fumigatus, Aspergillus flavus, A. niger*) and four dermatophytes (*Microsporum gypseum, Epidermophyton floccosum, Trichophyton rubrum and Trichophyton mentagrophytes*).

The log *P* of each alkyl gallate was calculated and related to the different MIC values in order to establish the relationship between lipophilicity and antifungal activity (LAR).

The compounds were prepared as previously described.[4] And for the antifungal evaluation, standardized strains from the American

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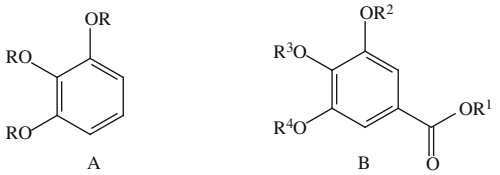
Table 1
Antifungal activity of compounds



Compound	N	Type	R^1	R^2	R^3	R^4	Ca	Ct	Sc	Cn	Afu	Afl	An	Mg	Ef	Tr	Tm
Pirogallol	**1**	A	—	H	H	H	100	100	100	62.5	100	>250	>250	50	12.5	25	50
Gallic acid	**2**	B	H	H	H	H	>250	>250	>250	>250	>250	>250	>250	>250	100	100	125
3,4,5-Triacetylbenzoic acid	**3**	B	H	Acetyl	Acetyl	Acetyl	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
3,4,5-Trimethoxybenzoic acid	**4**	B	H	Me	Me	Me	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
Ethyl trimethoxybenzoate	**5**	B	CH_2CH_3	Me	Me	Me	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
Methyl gallate	**6**	B	CH_3	H	H	H	>250	>250	>250	>250	>250	>250	>250	100	100	100	100
Ethyl gallate	**7**	B	CH_2CH_3	H	H	H	>250	>250	>250	>250	>250	>250	>250	100	100	100	100
Propyl gallate	**8**	B	$(CH2)_8CH_3$	H	H	H	>250	>250	>250	>250	>250	>250	>250	75	100	50	100
Butyl gallate	**9**	B	$(CH2)_3CH_3$	H	H	H	>250	>250	>250	>250	>250	>250	>250	50	100	50	100
Pentyl gallate	**10**	B	$(CH2)_4CH_3$	H	H	H	>250	>250	>250	>250	>250	>250	>250	50	100	50	50
Hexyl gallate	**11**	B	$(CH2)_5CH_3$	H	H	H	>250	>250	>250	>250	>250	>250	>250	25	25	25	25
Heptyl gallate	**12**	B	$(CH2)_6CH_3$	H	H	H	100	100	100	62.5	100	100	100	6.25	6.25	6.25	12.5
Octyl gallate	**13**	B	$(CH2)_7CH_3$	H	H	H	62.5	62.5	50	50	62.5	62.5	62.5	6.25	6.25	6.25	12.5
Nonyl gallate	**14**	B	$(CH2)_8CH_3$	H	H	H	8	8	8	8	62.5	62.5	125	8	25	8	8
Decyl gallate	**15**	B	$(CH2)_9CH_3$	H	H	H	100	100	100	50	100	>250	>250	50	25	12.5	50
Undecyl gallate	**16**	B	$(CH2)_{10}CH_3$	H	H	H	250	200	200	100	>250	>250	>250	125	n.t.	125	100
Dodecyl gallate	**17**	B	$(CH2)_{11}CH_3$	H	H	H	>250	>250	>250	>250	>250	>250	>250	100	n.t.	50	50
Tetradecyl gallate	**18**	B	$(CH2)_{13}CH_3$	H	H	H	>250	>250	>250	250	>250	>250	>250	100	>250	50	50
Hexadecyl gallate	**19**	B	$(CH2)_{15}CH_3$	H	H	H	>250	>250	>250	>250	>250	>250	>250	250	n.t.	250	125
Octadecyl gallate	**20**	B	$(CH2)_{17}CH_3$	H	H	H	>250	>250	>250	>250	>250	>250	>250	>250	250	250	>250
Amphotericin B							0.78	0.5	0.50	0.25	0.50	0.50	0.50				
Ketoconazole							1.56	0.125	3.12	0.39	0.78	0.78	1.56				
Terbinafine														0.04	0.004	0.01	0.04

The activity of compounds against yeasts, hialohyphomycetes and dermatophytes[*] (μM). The gray shaded column shows the data used to build the Figures 1 and 2.
[*]**Ca** (*Candida albicans*); **Ct** (*Candida tropicalis*); **Sc** (*Saccharomyces cerevisiae*); **Cn** (*Cryptococcus neoformans*); **Afu** (*Aspergillus fumigatus*); **Afl** (*Aspergillus flavus*); **An** (*Aspergillus niger*); **Mg** (*Microsporum gypseum*); **Ef** (*Epidermophyton floccosum*); **Tr** (*Trichophyton rubrum*); **Tm** (*Trichophyton mentagrophytes*).

Type Culture Collection (ATCC), Rockville, MD, USA, and CEREMIC (C), Centro de Referencia Micológica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Suipacha 531-(2000)-Rosario, Argentina, were used in the first screening: *C. albicans* ATCC 10231, *C. tropicalis* C131, *S. cerevisiae* ATCC 9763, *C. neoformans* ATCC 32264, *A. flavus* ATCC 9170, *A. fumigatus* ATTC 26934, *A. niger* ATCC 9029, *T. rubrum* C 110, *T. mentagrophytes* ATCC 9972, and *M. gypseum* C 115, *E. floccosum* C 114.

Strains were grown on Sabouraud-chloramphenicol agar slants for 48 h at 30 °C, maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid) and subcultured every 15 days to prevent pleomorphic transformations. Inocula of cell or spore suspensions were obtained according to reported procedures and adjusted to $1–5 \times 10^3$ cells/spores with colony forming units (CFU)/mL.[5]

The minimum inhibitory concentration (MIC) of each compound was determined using broth microdilution techniques according to the guidelines of the CLSI (formerly NCCLS) for yeasts (M27-A2) and for filamentous fungi (M 38 A).[5]

MIC values were determined in RPMI-1640 (Sigma, St. Louis, MO, USA) buffered to pH 7.0 with MOPS. Microtiter trays were incubated at 35 °C for yeasts and hialohyphomycetes and at 28–30 °C for dermatophyte strains in a moist, dark chamber, and MICs were visually recorded after 48 h for yeasts, and at a time according to the control fungus growth, for the rest of the fungi.

For the assay, stock solutions of pure compounds were diluted (2:1) with RPMI from 250 to 0.98 μg/mL (final volume = 100 μL) and a final DMSO concentration of ⩽1% was obtained. A volume of 100 μL of inoculum suspension was added to each well with the exception of the sterility control where sterile water was added to the well instead. Ketoconazole, terbinafine, amphotericin B, were used as positive controls.

To carry out the antifungal evaluation, gallates and analogues **1**–**20**, up to a concentration = 250 μg/mL, were incorporated into growth media according to CLSI (formerly NCCLS) guidelines.[5]

Table 1 summarizes the minimum concentration that completely inhibited the growth (MIC) of the 11 strains of the panel. Results demonstrated that the 15 alkyl gallates tested (**6**–**20**) showed antifungal activity (MIC ⩽ 250 μg/mL) against one or more strains tested. Regarding the role of the different fungi [factor (a), see above] dermatophytes were the most susceptible species, since almost all gallates inhibited their growth with MIC values between 8 and 250 μg/mL.

A series of 15 gallates esterified with alcohols of C_1 to C_{18}, along with gallic acid and other analogues, were tested for antifungal properties against a panel of human opportunistic pathogenic fungi. Results showed that the activity can vary with the fungi tested, mainly between groups of fungi (yeasts, hialohyphomycetes or dermatophytes). Regarding the structure-activity relationships, the three free OH appeared to be necessary but not sufficient for activity, and most of the active compounds possess an alkyl chain of 6–9 carbon atoms, nonyl gallate being the most active compound.

The log *P* value of each gallate was compared to its MIC, revealing that the most active compounds possess log *P* values of 2.5–4.5, with 3.7 being the log *P* of the most active compound, nonyl gallate.

The lack of activity of 3,4,5-trimethoxybenzoic and 3,4,5-triacetylbenzoic acids as well as the comparison of activities of ethyl trimethoxybenzoate (MICs > 250 μg/mL) and ethylgallate (MICs between 100 and (>250) μg/mL) suggest that the three free OH groups could play a role in the antifungal activity [factor (c)]. However, although their presence appears to be necessary, it is not sufficient for gallates to inhibit fungal growth since: (i) pyrogallol but not gallic acid displayed antifungal activity; (ii) not all alkyl gallates inhibited the fungal growth or, in the case of activity, they showed important variations.

From the point of view of the chain length [factor (b)] and its contribution to the hydrophobicity [factor (d)], it is known that

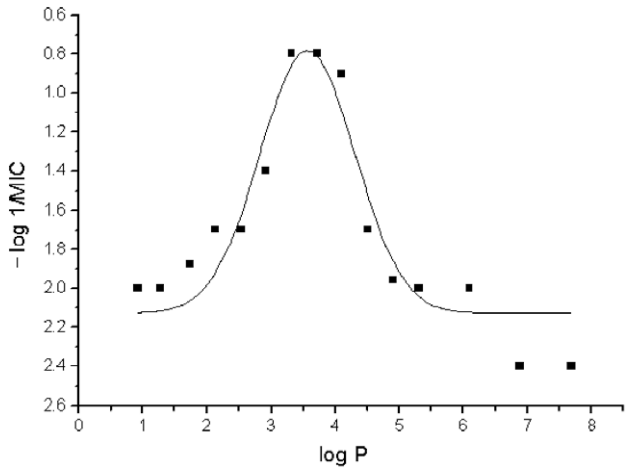


Figure 1. Lipophilicity-antifungal activity for *M. gypseum.* log 1/MIC (of each gallate) against log *P* for *Microsporum gypseum*.

log *P* (the logarithm of the partition coefficient in a biphasic system, e.g., *n*-octanol/water) describes the macroscopic hydrophobicity of a molecule, which is a factor that determines its ability to penetrate fungal cell membranes and to reach the interacting sites, thus influencing the antifungal activity of compounds.[6]

In Figure 1 the log 1/MIC (of each gallate) is drawn against the log *P* for *M. gypseum*, a sensitive fungi with a behavior similar to that of the other fungi tested. From Figure 1, it is clear that molecules having log *P* values between 2.5 and 4.5 have a positive deviation of the curve, showing a very high activity in this series (gallates with alkyl chains of 6–9). Octyl gallate, which has the lowest MIC values in the case of *M. gypseum*, showed the optimum value for log *P* (3.7). In contrast, compounds with log *P* values lower than 2.5 and higher than 4.5 were active, having a polynomial relation with log *P*. Thus, if we do not consider the log *P* values corresponding to hexyl, heptyl, octyl and nonyl gallates and we make a polynomial regression, we obtain a correlation coefficient of $R = 0.93$, with the polynomial equation $Y = -0.0399X^2 + 0.26472X + 2.1863$ (Fig. 2).

These results suggest that the activity of gallates possessing alkyl chains from 6 to 9 are not only related to the hydrophobicity. In addition, these results do not show a cut off with any of the fungi. Heptyl, octyl, nonyl and decyl esters were the most active compounds and had a broader spectrum of action, inhibiting yeasts, dermatophytes and hialohyphomycetes. Of these, nonyl ester showed the best activity with a MIC value of 8 μg/mL for the four yeasts and 8–25 μg/mL for dermatophytes.

The significant positive deviation of the curve of log 1/MIC *versus* log *P* of the compounds, with alkyl chains lengths of 6–9, indicates that they must have a specific effect on some target molecule.

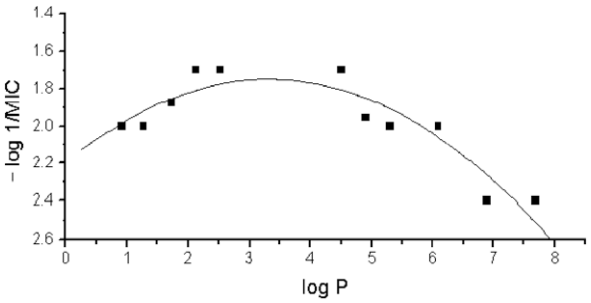


Figure 2. Lipophilicity-antifungal activity corrected for *M. gypseum* log 1/MIC (without considering hexyl, heptyl, octyl and nonyl gallates) against log *P* for *Microsporum gypseum*.

This deviation of the curve was also observed in the case of *S. cerevisiae* for the MIC values obtained by Kubo et al.[2,7]

Abe et al.[4,8] have demonstrated that octyl, dodecyl and cetyl gallates along with some phenyl alkyl gallates, such as phenylbutyl, phenylhexyl, phenyloctyl and phenyldecyl gallates, have an inhibitory effect on vertebrate squalene epoxidase (SE), a non-metal flavoprotein monoxygenase that catalyses the conversion of squalene to 2,3-oxidosqualene, a rate-limiting step to cholesterol biosynthesis. Thus, it is possible to assume that the alkyl gallates (alkyl chain 6–9), considering the similarity between enzymes of vertebrates and fungi, could inhibit the SE of fungi and consequently the biosynthesis of ergosterol. Another possibility is that these compounds inhibit another enzyme related to the synthesis of ergosterol.[2]

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