

Research Article

Inhibitory Effect of Natural Phenolic Compounds on *Aspergillus parasiticus* Growth

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Considering the impact of *Aspergillus* species on crops, it appears to be highly desirable to apply strategies to prevent their growth, as well as to eliminate or reduce their presence in food products. For this reason, the aims of this investigation were to evaluate the effects of ten natural phenolic compounds on the *Aspergillus parasiticus* growth and to determine which physicochemical properties are involved in the antifungal activity. According to the results of minimum inhibitory concentration (MIC) values of the individual compounds, isoeugenol, carvacrol, and thymol were the most active phenolic components (1.26 mM, 1.47 mM, and 1.50 mM, resp.), followed by eugenol (2.23 mM). On the other hand, creosol, p-cresol, o-cresol, m-cresol, vanillin, and phenol had no effects on fungal development. Logarithm of the octanol/water partition coefficient ($\log P$), refractivity index (RI), and molar volume (MV) were demonstrated to be the descriptors that best explained the antifungal activity correlated to lipophilicity, reactivity of the components, and steric aspect. These findings make an important contribution to the search for new compounds with antifungal activity.

1. Introduction

Crops are susceptible to fungal growth, with the economic impact of this contamination occurring at all levels of agricultural commodities: in the field and during storage at various commercial stages, including both products and by-products. Besides the economic losses due to the deterioration of foods, many of the phytopathogenic fungi produce secondary metabolites, such as mycotoxins that could have harmful effects on the consumers' health. This causes also a reduction in the quality of foods and feeds. In terms of agricultural safety, *Aspergillus* section *flavi* are one of the most significant fungal genera because they contaminate crops during

pre- or postharvest periods. *Aspergillus parasiticus* is one of the most important species of this section since some isolates of this species are able to produce carcinogenic secondary metabolites, aflatoxins, resulting in an accumulation of this mycotoxin in crops [1, 2]. Due to the negative effect of fungal growth, and its metabolites, on agricultural products and on animal and human health, several preservation methods have been used in order to prevent its development and also to extend the shelf-life of foods. The use of synthetic fungicides is not suitable because of their side effects on human and animal health, the environmental pollution, and the development of fungicide resistant strains [3]. Given these undesirable effects and considering the negative impact of

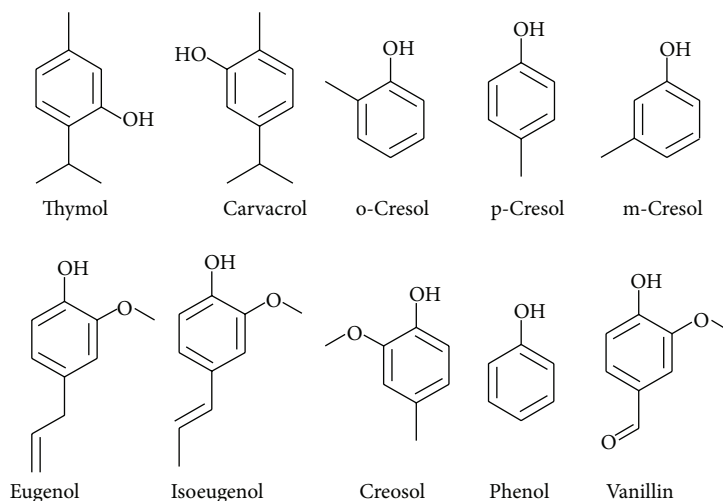


FIGURE 1: Chemical structure of the natural phenolic compounds.

Aspergillus in crops, it is desirable to apply strategies that may prevent its presence in agricultural products. Thus, in order to reduce the use of synthetic fungicides, the development of harmless and biodegradable alternatives has been considered [2, 4]. In recent years, there has been a wide interest in the search of natural sources for controlling fungal growth. Essential oils and plant extracts have been shown to be active against a wide number of microorganisms [5–10]. Numerous publications have reported that essential oils show antimicrobial and antifungal activity; however, the main composition of these natural compounds could differ depending on the growing region, genetic variability, occurrence of abiotic and biotic factors, and the method of essential oil extraction [11, 12]. In addition, natural phenolic compounds have been reported to be the active compounds present in the composition of essential oils such as thymol, carvacrol, and isoeugenol [13–17]. For these reasons, the aims of this investigation were to evaluate the effects of ten natural phenolic compounds on the *Aspergillus parasiticus* growth and to determine which physicochemical properties are involved in the antifungal activity.

2. Material and Methods

2.1. Fungal Isolate. *Aspergillus parasiticus* AF 59 was isolated from soils destined to maize production located in the south of Córdoba province (Argentina). The strain was identified by classic taxonomy according to the methodology proposed by Klich [18]. The strain was maintained in glycerol (15%) at -80°C and kept in the culture collection at the Department of Microbiology and Immunology, National University of Río Cuarto, Córdoba, Argentina.

2.2. Phenolic Compounds. Phenol, 2-methylphenol (ortho-cresol), 3-methylphenol (meta-cresol), 4-methylphenol (para-cresol), 2-methoxy-4-methylphenol (creosol), 5-methyl-2-propan-2-ylphenol (thymol), 2-methyl-5-propan-2-ylphenol (carvacrol), 2-methoxy-4-prop-2-enylphenol (eugenol), 2-methoxy-4-[(E)-prop-1-enyl]phenol (isoeugenol), and

4-hydroxy-3-methoxybenzaldehyde (vanillin) were used and purchased from Fluka-Kahlbaum-Germany (Figure 1). Stock solutions of each phenolic compound were prepared in dimethyl sulfoxide (DMSO), and the appropriate amount to obtain the concentrations of 0.2, 0.3, 0.4, 0.6, 0.8, 1.0, 1.2, 1.6, 2.0, 2.4, and 3 mM was added to the culture medium. In the control treatments the equivalent amount of DMSO was added to the culture medium.

2.3. Medium and Culture Conditions. For the evaluation of antifungal activities, experiments were performed using Czapek-Dox agar in Petri dishes (90 mm). The water activity (a_w) of basic medium was adjusted to 0.980 by the addition of known amounts of nonionic solute, glycerol, according to Marin et al. [19]. The culture medium was autoclaved at 120°C for 15 minutes. Before cooling at 45°C , an appropriated volume of phenolic compounds was added to the media to reach the intended concentrations mentioned above. The a_w of representative samples of each treatment was checked with an AquaLab Series 3 (Decagon Devices, Inc., WA, USA). Additionally, control plates were prepared following the same procedure; however, no phenolic compounds were added to the culture media.

The *A. parasiticus* strain inoculum used was spore suspensions obtained from a 7-day-old plate culture in Czapek-Dox agar (Oxoid). Spores of the fungal strain were suspended in sterile distilled water containing a drop of a wetting agent (Tween 80). The concentration of spores was measured with a Neubauer chamber and adjusted to 10^5 spores mL^{-1} .

Czapek-Dox plates were inoculated centrally with $10\ \mu\text{L}$ of the spore suspension. Petri plates were placed in closed plastic containers together with beakers of glycerol-water solution of the same a_w in order to maintain the correct equilibrium of relative humidity inside the boxes. Containers were incubated at 30°C and the experiment was carried out in four replicates per treatment. All the experiments were repeated twice.

2.4. Growth Assessment. Two perpendicular diameters of the growing colonies were measured daily (mm) until the colony reached the edge of the plate. The radio of the colonies was plotted against time, and a lineal regression was applied in order to obtain the “growth rate” as the slope of the line and the “lag phase” as the time (hours) in which each colony reaches 5 mm of diameter for each treatment [20]. The “minimum inhibitory concentration (MIC)” (the lowest concentration of the phenolic compounds at which fungus did not grow) and lethal doses values (LD_{25} , LD_{50} , and LD_{90}) were calculated by interpolation in the above mentioned lineal regression.

2.5. Statistical Analysis. Multiple linear regression analyses (MLR) were calculated in order to examine the quantitative relationships between linear combinations of the dependent variable (\log_1/LD_{25}) and the predictor variables (structure and molecular properties). Molar concentrations of the LD_{25} values were used for the MLR analyses. In the MLR equations, N is the number of data points, r is the correlation coefficient between observed values of the dependent variable and the values calculated from the equation, and r^2 is the square of the correlation coefficient and represents the goodness of fit. The quantitative structure-activity relationship (QSAR) model was validated with the root mean square prediction error (RMSPE) obtained by the cross validation leave-one-out procedure. Results with P values < 0.05 were considered significant. All statistical analyses were calculated by using the InfoStat software Professional 2010 [21].

3. Results

The effect of phenolic compounds on *A. parasiticus* growth was evaluated in a range of concentrations between 0 and 3.0 mM. The compounds showed different levels of antifungal activity against the assayed strain and the inhibition was widely dependent upon the compound concentration. The most active inhibitors were isoeugenol, carvacrol, and thymol with MIC values of 1.26 mM, 1.47 mM, and 1.50 mM, respectively, followed by eugenol (MIC value: 2.23 mM). Creosol, p-cresol, o-cresol, m-cresol, vanillin, and phenol had no effect on fungal development at the evaluated concentrations in the present study showing MIC values between 6.19 and 11.68 (Table 1).

Table 2 shows the effects of phenolic compounds in different concentrations on lag phase (h) of *A. parasiticus* strain. The lag phase increased significantly as isoeugenol, carvacrol, thymol, and eugenol concentrations increased. Carvacrol, eugenol, and thymol assayed at the highest concentration (3.0 mM) revealed a marked effect on the lag phase from 32.8 to 351.5, 274.4, and 292.3 h, respectively. Furthermore, isoeugenol at the highest concentrations, 2.0, 2.4, and 3.0 mM, showed that *A. parasiticus* was not able to reach the exponential phase. On the other hand, creosol, m-cresol, p-cresol, and o-cresol only showed effects on lag phase at high evaluated concentrations (≥ 2.0 mM). No significant effect on lag phase was observed for phenol and vanillin in the levels assayed.

The effect of phenolic compounds on growth rate is shown in Figure 2. The results revealed that this growth

TABLE 1: Antifungal activity of natural phenolic compounds against *Aspergillus parasiticus* AF54 strain.

Phenolic compounds	MIC ^a	LD_{90}	LD_{50}	LD_{25} ^b
		(mM)		
Carvacrol	1.47	1.26	0.40	0.13
Creosol	6.19	5.55	2.99	1.38
m-Cresol	8.11	7.29	3.99	1.92
p-Cresol	6.47	5.89	3.57	2.13
o-Cresol	6.74	6.10	3.55	1.95
Eugenol	2.23	2.01	1.15	0.62
Isoeugenol	1.26	1.11	0.51	0.14
Phenol	11.68	10.65	6.50	3.90
Thymol	1.50	1.29	0.39	0.18
Vanillin	6.71	6.02	3.27	1.55

^aMinimal inhibitory concentration (MIC).

^bThe lethal doses₂₅ (LD_{25}) values were used in multiple linear regression analyses (MLR).

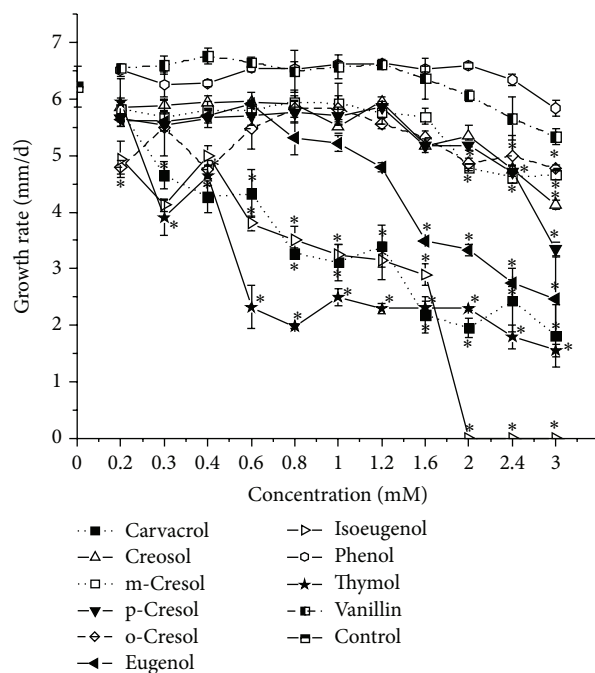


FIGURE 2: Effects of phenolic compounds on the growth rate of *Aspergillus parasiticus* AF54 strain. *Significant difference with the control according to Kruskal-Wallis nonparametric test ($P < 0.05$). All pairwise comparison was used to compare the means among treatments ranges.

parameter was affected differently depending on the compound assayed and its concentration in the medium. The most active inhibitor on fungal growth was isoeugenol, which completely suppressed mycelia growth at concentrations higher than 2.0 mM, followed by thymol, carvacrol, and eugenol.

In order to determine the quantitative relationships between antifungal activity and structural and molecular

TABLE 2: Effects of phenolic compounds on the lag phase of *Aspergillus parasiticus* AF54 strain.

Phenolic compounds	Lag phase (h)												
	Concentration (mM) \pm SD												
	0	0.2	0.3	0.4	0.6	0.8	1.0	1.2	1.6	2.0	2.4	3.0	
Carvacrol	32.8 \pm 3.0	47.1 \pm 2.3	62.4 \pm 1.1*	76.1 \pm 17.6*	69.9 \pm 11.2*	97.4 \pm 6.0*	112.3 \pm 7.5*	142.2 \pm 40.1*	191.3 \pm 26.7*	229.9 \pm 9.3*	289.7 \pm 41.1*	351.5 \pm 74.6*	
Creosol	32.8 \pm 3.0	36.1 \pm 3.3	36.8 \pm 2.5	39.8 \pm 3.0	39.6 \pm 0.4	39.0 \pm 4.2	44.9 \pm 3.5	43.4 \pm 4.2	45.5 \pm 7.8	48.8 \pm 1.1*	52.5 \pm 1.8*	56.2 \pm 4.4*	
m-Cresol	32.8 \pm 3.0	35.4 \pm 6.8	31.5 \pm 2.9	35.0 \pm 4.9	34.1 \pm 4.3	39.6 \pm 5.6	45.4 \pm 5.5	40.7 \pm 4.4	44.1 \pm 0.9	46.4 \pm 3.9	47.0 \pm 4.3	50.7 \pm 4.7*	
p-Cresol	32.8 \pm 3.0	29.7 \pm 2.3	26.1 \pm 2.6	29.9 \pm 5.1	35.4 \pm 3.8	35.4 \pm 4.3	34.5 \pm 4.2	42.3 \pm 1.0	40.4 \pm 1.7	42.5 \pm 3.1	49.5 \pm 1.4*	43.0 \pm 6.6	
o-Cresol	32.8 \pm 3.0	26.9 \pm 6.7	26.6 \pm 14.1	28.5 \pm 5.4	37.1 \pm 1.5	36.8 \pm 5.3	37.8 \pm 5.4	33.3 \pm 4.4	45.7 \pm 3.7	45.0 \pm 5.0	46.2 \pm 4.5	56.0 \pm 2.1*	
Eugenol	32.8 \pm 3.0	28.8 \pm 4.2	29.9 \pm 3.1	37.5 \pm 1.3	42.2 \pm 4.8	54.5 \pm 3.7*	60.2 \pm 0.8*	68.5 \pm 3.4*	123.6 \pm 2.9*	134.1 \pm 5.1*	221.1 \pm 19.4*	274.4 \pm 20.2*	
Isoeugenol	32.8 \pm 3.0	37.5 \pm 17.2	45.6 \pm 10.4	44.9 \pm 4.5	65.3 \pm 5.9*	77.6 \pm 4.6*	87.4 \pm 3.0*	181.2 \pm 3.6*	186.6 \pm 19.8*	ND*	ND*	ND*	
Phenol	32.8 \pm 3.0	29.6 \pm 3.4	22.8 \pm 5.9	23.4 \pm 2.2	30.4 \pm 2.0	30.3 \pm 1.9	32.1 \pm 1.3	32.7 \pm 2.3	30.4 \pm 1.4	32.1 \pm 0.9	34.5 \pm 3.2	34.4 \pm 2.1	
Thymol	32.8 \pm 3.0	47.2 \pm 1.7	47.0 \pm 2.1	52.5 \pm 3.9*	50.4 \pm 18.5	77.7 \pm 25.4*	98.8 \pm 5.2*	165.3 \pm 17.9*	197.6 \pm 31.3*	215.8 \pm 13.0*	235.4 \pm 11.8*	292.3 \pm 30.9*	
Vanillin	32.8 \pm 3.0	30.3 \pm 2.1	31.6 \pm 3.6	35.7 \pm 3.1	33.2 \pm 2.8	29.2 \pm 10.0	35.6 \pm 5.9	34.4 \pm 1.0	36.3 \pm 6.9	40.8 \pm 3.1	41.6 \pm 10.5	44.8 \pm 8.1	

SD: standard deviation.

ND: not determined.

* This indicates significant difference with the control according to Kruskal-Wallis nonparametric test ($P < 0.05$). All pairwise comparison was used to compare the means among treatments ranges.

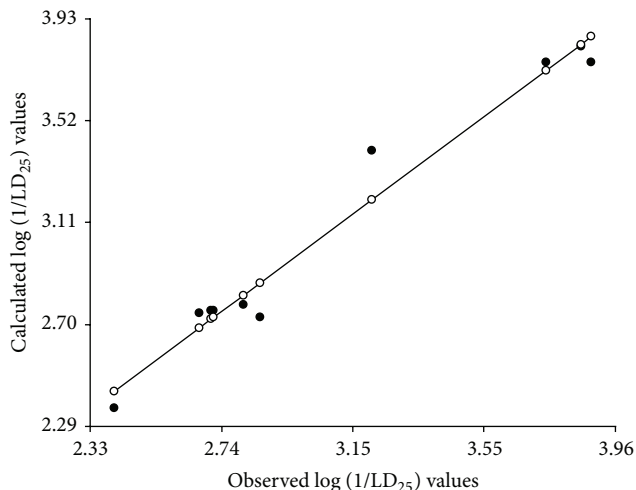


FIGURE 3: Plot of calculated versus experimental $\log 1/LD_{25}$ of the ten phenolic compounds on *Aspergillus parasiticus* AF54 growth. Multiple linear regression analyses (MLR) calculated to examine quantitative relationships between linear combinations of the dependent variable ($\log 1/LD_{25}$) and the physicochemical descriptors. N is the number of data points, r is the correlation coefficient between observed values of the dependent variable and the values calculated from the equation, and r^2 is the square of the correlation coefficient and represents the goodness of fit. The obtained QSAR model was validated with the root mean square prediction error (RMSPE) obtained by cross validation leave-one-out procedure.

properties MLR analyses were carried out. The different physicochemical descriptors selected for the present study were obtained from the data reported by Gutiérrez-Larraínzar et al. [17]. The equation obtained (1) produced a model representing 97.0% of the variance ($R^2 = 0.97$), demonstrating a good correlation between fungal inhibition and physicochemical parameters, and is given by the following expression:

$$\log\left(\frac{1}{LD_{25}}\right) = 10.35(RI) + 0.61(\log P) + 0.01(MV) - 15.58, \quad (1)$$

$$N = 10; \quad r^2 = 0.97; \quad \text{RMSPE} = 6.479\%.$$

In this study, the relation between observed and predicted activity of the phenolic compounds is shown in Figure 3. The obtained model showed a prediction error of 6.479% (RMSPE = 6.479%). The regression model obtained revealed that the antifungal activities of the phenolic compounds analyzed in the present study increase with a rise in refractive index (RI), octanol/water partition coefficient ($\log P$), and molar volume (MV).

4. Discussion

Considering the impact of *Aspergillus* species on crops, the application of strategies to prevent their growth, as well as to eliminate or reduce their presence in food products, is

desirable. Over recent years, the interest in natural compounds for the control of fungal growth as an alternative to chemicals has increased. Several studies have reported that phenolic compounds are mainly responsible for the antimicrobial properties of plant essential oils [4, 17, 22, 23]. In the present study, we determined the effect of ten phenolic compounds on *A. parasiticus* growth. According to the results of MIC values of the individual compounds, isoeugenol, carvacrol, and thymol were the most active phenolic components followed by eugenol, whereas MIC values for cresol, p-cresol, o-cresol, and vanillin were about three times higher than those reported for eugenol. With regard to m-cresol and phenol, they had little effect on *Aspergillus* growth. Similar results were observed by Zabka and Pavela [4], who informed that thymol and carvacrol were the most active phenolic compounds against *Aspergillus flavus* and *Aspergillus fumigatus*. Morcia et al. [24] reported that thymol was the most effective antifungal compound tested against mycotoxigenic plant pathogens. In previous studies, we also reported a marked effect of thymol, carvacrol, and isoeugenol on *Fusarium verticillioides* and *Candida* strain growth [16, 25]. Moreover, the results obtained in the present study showed that the natural phenolic compounds in general had significant inhibitory effects on lag phase and growth rate of *A. parasiticus* strain. The antifungal activity is related to compound concentration; thus when concentration increased growth rate decreased; also lengthened lag phases were observed, except for phenol. Additionally, isoeugenol completely suppressed fungal growth at concentrations ≥ 2.0 mM. The inhibitory effects of phenolic compounds on microbial growth could be related to their molecular structure: a nonpolar part to make possible their passage through the membrane and a hydroxyl group combined with a system of delocalized electrons that confers an acidic character to the molecules [17], resulting in cell membrane destabilization [15, 26]. Thymol and carvacrol have been reported to be the most active phenolic compounds against a wide range of microorganisms; this inhibitory effect could be due to their high hydrophobicity [16, 17, 27], which is correlated to the $\log P$ (the ratio of the concentration of the lipophilic compounds in octanol/water) [14]. However, isoeugenol, a less lipophilic compound than thymol and carvacrol, showed a greater antifungal activity. On the other hand, vanillin the most hydrophilic compound employed in this study was more active against *A. parasiticus* than m-cresol and phenol. Thus, these results suggested that not only the hydrophobic property is associated with the efficiency of the phenolic compounds but also other properties appear to be involved in the inhibitory activity. Accordingly, in order to establish which properties were implicated in the antifungal efficiency, a structure-activity relationship analysis was performed. Antifungal activity data determined as LD_{25} were used as dependent variables in the QSAR study. Based on the QSAR studies, the results revealed that $\log P$ refractivity index (RI) and molar volume (MV) in the equation are positive, which indicated that the antifungal activity of the phenolic compounds is positively correlated to their lipophilicity, reactivity of the components, and steric aspect, respectively. This suggests that the activity of the phenolic compounds first

increases with an increase in hydrophobicity, represented by log *P*. These findings are in agreement with previous studies described above, which indicated the ability of the phenolic compounds to perturb the cell membrane integrity. Thus, the mechanism depends mainly on the ability of the phenols to affect the function of the cellular lipoprotein membranes [26, 28]. Moreover, refractivity index and molar volume are related to the antifungal effect, indicating that specific interactions between the phenolic compounds and the target receptor could be implicated in the inhibitory activity.

Our previous studies on *Fusarium* and *Candida* species also revealed lipophilicity, reactivity, and steric aspect as the molecular properties of the phenolic compounds involved in the antifungal activity [16, 25]. Because the physicochemical properties reported in the present study are related to those mentioned above, this could suggest that the phenolic compounds could act by the same mechanism on different fungal genus, indicating that the compound could inhibit more than one fungal genus simultaneously. This observation is remarkable, since it is known that it is highly probable that fungal species cooccur in crops and/or foods [29, 30].

5. Conclusions

According to the results obtained in this study, we can suggest that isoeugenol, thymol, and carvacrol are the most promising phenolic compounds to control *A. parasiticus* by affecting the lag phase and the growth rate. In addition, the mathematical model obtained from QSAR leads us to predict the antifungal activity from the molecular properties of the phenolic compounds. The characteristic of the antifungal activity identified in the present report may be useful for the control of toxicogenic fungi and constitutes an important contribution in the search of novel active compounds. Further studies will be carried out in order to determine the effect of the evaluated compounds on aflatoxin production.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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