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Phytotoxicity of Phenolic Acids From Cereals

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1. Introduction

Allelopathic interactions between individuals of different plant species or those of the same species are caused by plant-produced allelochemicals. Once released into the environment, passively or actively they can influence germination, growth and development of neighboring plant either negatively or positively [1, 2]. Most allelochemicals are characterized by multi-functional phytotoxicity and are often also important for general defense. Generally stressed donor plants increased allelopathic activity due to increased production of allelochemical. Factors such as placement of residues, nutrient status, environmental conditions and microbial activity also affect allelopathy [3-5].

In recent years there has been an increasing focus on the prospects of exploiting allelopathy for controlling weeds but also insects and diseases. Allelopathy plays an important role in the agro ecosystem leading to a wide array of interactions between crop-crop, crop-weed and tree-crop. Generally, these interactions are harmful to the receiver plants but provide a selective benefit to the donor. Several members of crops exhibit allelopathic interactions that play a significant role in the complex environment of the agro ecosystem.

The allelochemicals are released largely by plant residues that are left in the fields after the harvest of a crop or through use of cover crops. Research on allelopathic interactions has been focused in agricultural crops as an option in the development of integrated weed management strategies, reducing environmental effects and the cost of crop protection [6-10]. Secondary metabolites with allelopathic properties are thought to protect plants against competing plants. For instance, allelopathic activity of decomposing wheat (*Triticum aestivum* L.), and oat (*Avena sativa* L.) straw on some crop species has been reported [11]. Allelopathic potential of rye [12-14] and rice [6, 15, 16] has been extensively studied. In cereals such as, maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.), barley (*Hordeum vulgare* L.), rice (*Oryza*

sativa L.) and sorghum (*Sorghum bicolor*) a variety of allelochemicals have been identified including hydroxamic acids, coumarins, alkaloids, flavonoids and phenolic acids. The allelopathic activity of these cereals may arise from one or the combined action of a group of allelochemicals. For example, hydroxamic acids appear to be responsible for the allelopathic effect of wheat, maize and rye [13, 17-19], indole alkaloids in allelopathic effect of barley [20, 21] and phenolic acid in the allelopathic effect of rice and sorghum [15, 22-25]. Rye is an example of a plant which provides excellent weed suppression through allelopathic mechanism. Rye and its residues which strongly inhibit germination and seedling growth of several dicot and monocotyledonous plants species. Several studies have demonstrated the allelopathic characteristic of rye residues are in agreement with the contents of hydroxamic acids in the plants and their degradation products [17, 26-29]. The allelopathy of an allelochemicals depends on the target species, dose, structure and their physicochemical properties. Both biotic and abiotic factor can trigger the allelopathic potential of a plant [3, 30-33]. The effectiveness of allelochemicals is therefore considered to be highly dynamic.

A basic step to understanding the allelopathic properties of a compound is to evaluate its phytotoxic properties. Phytotoxicity in plants may have some of the following effects: toxicity to the radicle growth of monocots and dicots; inhibitory effect on the energy metabolism of chloroplasts and mitochondria and modification of the binding affinity of the receptor sites of membranes. Parameters such as chemical stability, lipophilia, and acid-base or electrophilic-nucleophile interactions can be involved in the molecular mechanism of action or the dynamic in the environment.

Lipophilia is an essential parameter to establish the quantitative structure-activity relationship of phytotoxicity.

The n-octanol/water partition coefficient (K_{ow}) provides direct information on lipophilicity that describes the tendency of distribution of a solute from the aqueous phase into organic constituents of environmental compartments and even into biological membranes [34,35]. This has made it one of the most commonly reported physico-chemical properties of drugs, pesticides and other chemicals [36-38].

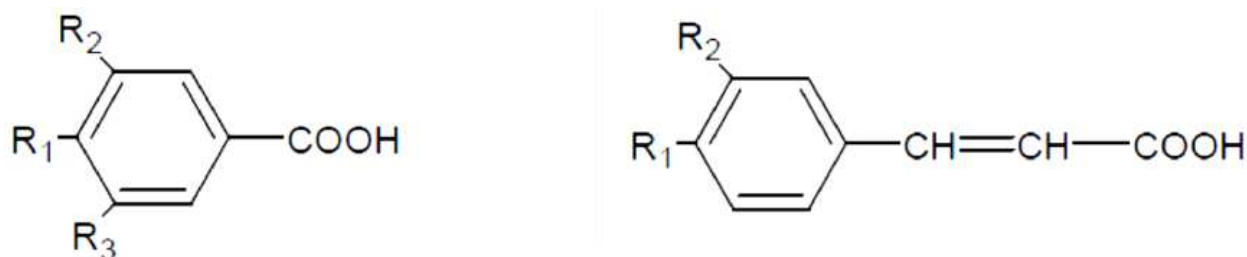
Numerous researchers have realized that there is a close parallel between the retention of compounds in reverse phase high performance liquid chromatographic columns (RP-HPLC) and octanol-water partition coefficients (K_{ow}). This technique is rapid and has the advantages that small samples suffice, the substances need not be pure and the exact volume of the phases need not be known.

Studies have tried to link this correlation to biological activity [39-45]. However, lack of success may be due to insufficient homogeneity or chemical diversity in the data set.

To gain a deeper understanding of the potential allelopathic properties of phenolic acids, we compared the phytotoxic activity of a series of phenolic acids, some of which are present in cereal cultivars (Figure 1).

The structural effect and role of molecular lipophyilia determined from RP-HPLC method were analyzed from the standpoint of a structure-phytotoxicity relationship using lettuce seeds

(*Lactuca sativa*) and the alga *Chlorella vulgaris*, one of the most commonly used species in microalgae toxicity bioassay testing [46].



Phenolic acids:

Benzoic:	$R_1=R_2=R_3=H$	Cinnamic:	$R_1=R_2=H$
p-hydroxy-benzoic:	$R_1=OH; R_2=R_3=H$	Caffeic:	$R_1=R_2=OH$
p-bromo-benzoic:	$R_1=Br; R_2=R_3=H$		
p-cyano-benzoic:	$R_1=CN; R_2=R_3=H$		
p-chloro-benzoic:	$R_1=Cl; R_2=R_3=H$		
Vanillic:	$R_1=OH; R_2=CH_3O; R_3=H$		
Gallic:	$R_1=R_2=R_3=OH$		

Figure 1. Structure of phenolic acids.

2. Experimental

Chemicals: Carboxylic acids were obtained from a commercial source (Aldrich Chemical Co.)

Log_{P_{HPLC}} values: The capacity factor (k') for the compounds used in this study were determined from $k' = (t_R - t_M) / t_M$, where t_R is the retention time of the compound and t_M is the retention time of the non-retained compound (thiourea). RP-HPLC was carried out in a C₁₈ column with mobile phase water (pH:3 phosphoric acid)/ acetonitrile 60:40 v/v [47]. The relation between k' and n-octanol-water partition coefficients were established by linear regression of $\log k'$ and $\log k_{ow}$ values obtained from the literature. Log_{P_{HPLC}} values were derived from the relationship:

$$\log P_{HPLC} = 1.08 \log k' + 1.72$$

Germination assays: 45 lettuce seeds were uniformly placed on Petri dishes covered with cotton film. In order to maintain individual compound concentrations, each plate was watered with 8 mL of an aqueous solution of 100 or 250 µg/mL of each compound. The plates were sealed and incubated at $25 \pm 2^\circ C$ in an 8:16 h light: dark cycle for 6 d. Controls were incubated only with water. Each assay was replicated three times Germination inhibition was expressed as percentage of the control.

Antialgal test: Test compounds were dissolved in nutrient growth medium (Gibco) with the aid of either ultrasound or gentle heating. *Chlorella vulgaris* (Laboratory of Microbiology, Faculty of Science, University of Chile) was grown in nutrient growth medium. Samples were incubated at 25° C for 10 d in test tubes containing 4.0 x10⁴ colony forming units (CFU) under continuous cold white fluorescent light with an intensity of 200 ft. c. The growth of *C. vulgaris* was assessed by turbidity measured by the spectrophotometric method at 600 nm.

Percentage inhibition was obtained as 100 (Ts – Tc)/(100 – Tc), where Ts is the sample transmittance and Tc the control transmittance.

3. Results and discussion

Table 1 shows the germination inhibition activity of phenolic acids (Figure 1). In the concentration range studied (100 – 250 µgmL⁻¹), cinnamic, p-bromo, p-chloro and benzoic acids showed the highest levels of germination inhibition of lettuce seeds. The effect of water-soluble inhibitor compounds associated with allelopathic plants is often more pronounced on the growth of an indicator than on its germination, and depends on the dose and the receptor plant. In fact, previous studies demonstrated that when phenolic acids are exuded from wheat, barley, wild oat and cucumber plants [24, 25], they did not inhibit germination but did inhibit the growth of *Brassica kaber* at a lower concentration than those used in our assays.

Phenolic acid	Germination inhibition (%)		antialgal activity (%)		logk´	log P _{HPLC}
	100 (µgmL ⁻¹)	250 (µgmL ⁻¹)	100 (µgmL ⁻¹)	250 (µgmL ⁻¹)		
Cinnamic	77.0	98.7	5.1	11.3	0.30	2.08
Benzoic	2.6	86.3	0.0	0.0	0.23	1.97
p-hydroxy- benzoic	0.0	5.3	0.0	0.0	- 0.41	1.28
Vanillic	2.1	4.2	0.0	0.0	- 0.37	1.32
Caffeic	0.0	0.0	100	100	- 0.51	1.17
Gallic	0.0	0.0	57.6	81.1	- 1.15	0.48
p-bromo benzoic	16.3	83.1	47.7	48.3	0.98	2.78
p-cyano benzoic	35.6	53.4	11.3	17.7	- 0.1	1.61
p-chloro benzoic	100	100	ND	ND	0.83	2.62

Table 1. Inhibitory effect (%) on germination of lettuce seeds (*L. sativa*), antialgal activity (*C. vulgaris*) and values of logk´ and log P_{HPLC} of phenolic acids.

Each value corresponds to the mean of three samples; replicate values showed errors below 5% in all cases. ND: not determined. The data were analyzed by one-way ANOVA.

In addition, we observed that caffeic acid did not inhibit germination but it stimulated seedling growth, particularly the root elongation of lettuce, in the concentration range 50-500 $\mu\text{g mL}^{-1}$.

Since measuring seedling length is often complicated due to curling and other morphological alterations, seedling fresh weight may be a better bioindicator to evaluate stimulation activity. Figure 2 shows the relation of the percentage of fresh biomass increase with respect to caffeic acid concentration; the greatest increase was observed at 250 $\mu\text{g mL}^{-1}$. An increase in plant growth produced by a chemical structure stimulator may be related to the stimulation of auxin-induced growth or to an improvement of the growth substrate because of organic matter enrichment. Further studies are necessary to establish the cause of the stimulation observed.

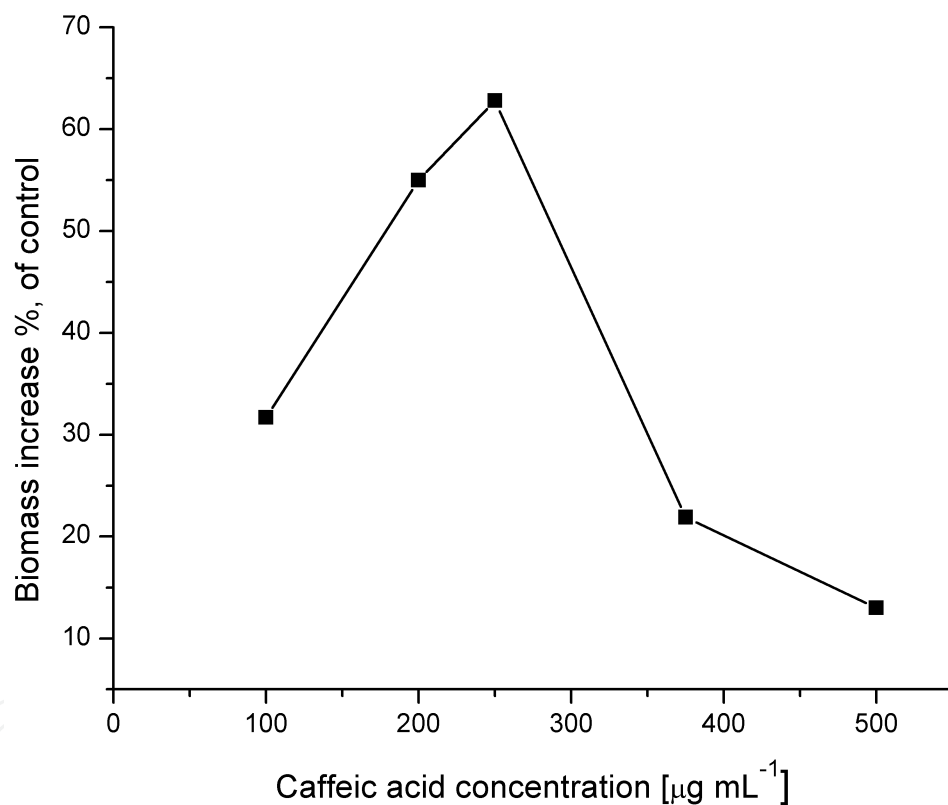


Figure 2. Effect of caffeic acid on fresh biomass increase of lettuce seedlings with respect to control (values correspond to the mean of three samples, replicate values had errors below 5% in all cases).

The phytotoxicity of the compounds involved in allelopathic effects depends upon the target species. Microalgae responded rapidly to environmental changes owing to their short germination time. Green microalgae such as *Chlorella* are taxonomically classified as plants bearing some similarity to higher plants. For this reason, a microalgae test may be used to evaluate herbicidal activity against higher plants [46]. The phytotoxicity of carboxylic acids was also

examined against the fresh water green alga *Chlorella vulgaris*. The percentages of *in vitro* growth inhibition are given in Table 1: gallic, caffeic and p-bromo benzoic acids showed the highest antialgal activity.

The chemical mechanism of the phytotoxicity of phenolic acids is not well understood. However, carboxylic acids and phenols are known to induce uncoupling of oxidative phosphorylation and photophosphorylation in mitochondria and chloroplasts. The uncoupling activity depends upon carboxylic function ionization. Pka values of the series studied were in the small range 4.0-4.5; therefore the substantial differences between the observed activities may not depend upon the ionization of the O-H bond of carboxylic function.

Hansch [48], proposed a theory to rationalize the relationship between the chemical structure and biological activity of auxins. The hypothesis assumes that auxins with an aromatic ring and side chain react with a plant substrate via two points, one on the side chain and another on the aromatic ring. A critical step should be the movement of the compounds from solution to the action sites. The lipophilia parameter is essential in the penetration rate and dynamics in the physiological system. This hypothesis must be considered to understand the mechanism of action the phenolic acids.

Although cinnamic and caffeic acids have identical side chains, they showed opposite phytotoxicities. At the concentration of $250\ \mu\text{gL}^{-1}$, cinnamic acid had the highest germination inhibition activity and low antialgal activity, while caffeic acid stimulated the growth of lettuce seedlings and showed the highest antialgal activity. This suggests that the difference of activity may arise from the structure of the aromatic ring. Compounds with carboxylic function bonding at the aromatic ring and with hydroxyl group substituents such as p-hydroxy benzoic acid, vanillic, caffeic and gallic acids did not show germination inhibition effects but the p-bromo, p-chloro and p-cyano derivatives showed significant activity.

These results suggest that part of the phytotoxicity may be related at the step of interaction of the aromatic ring with the substrate plant and the lipophilic-hydrophilic balance may have a fundamental role.

As mentioned above, the k_{ow} determined from the traditional shake-flash method is generally accepted as being a useful parameter in structure-activity relationship studies of correlation with biological activity of compounds and the dynamic processes of a chemical in the environment. It is now almost equally accepted that the correlation between the capacity factor (k') obtained from RP-HPLC method and k_{ow} to be used for simple and rapid estimation of partition coefficients.

With this in mind, we established a RP-HPLC method to evaluate the lipophilia of phenolic acids, as shown in figure 3.

A linear regression between the capacity factor (k') and $\log k_{ow}$ from literature sources was obtained ($R^2 = 0.99$). From this regression $\log P_{\text{HPLC}}$ values were derived by a single equation (see experimental). Table 1 gives the $\log k'$ and $\log P_{\text{HPLC}}$ values

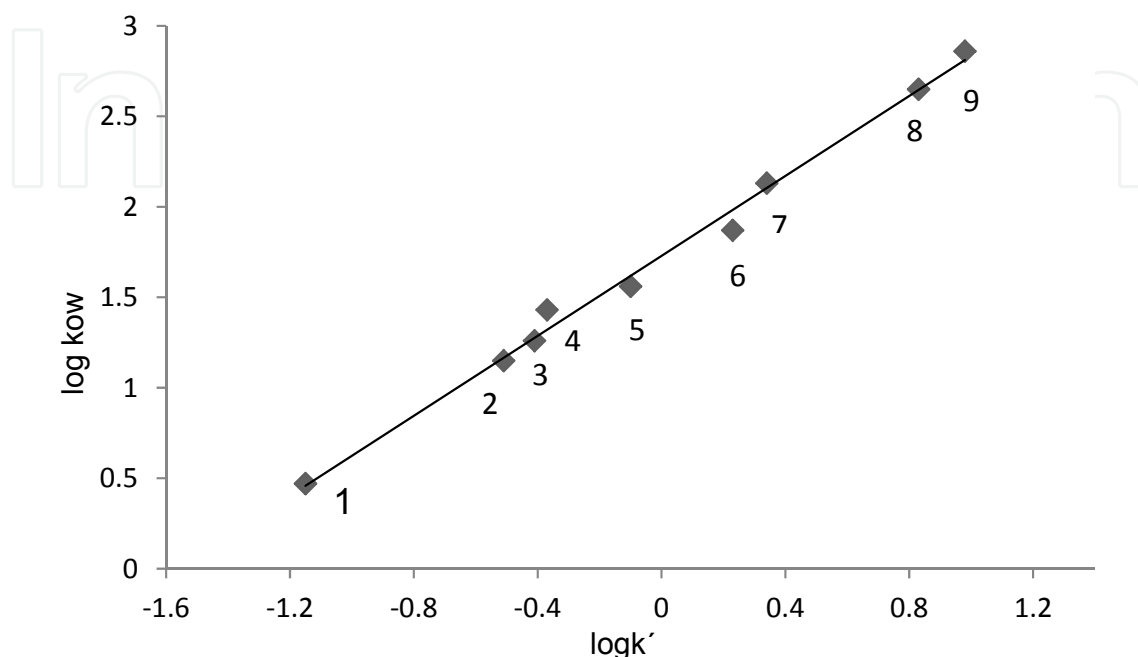


Figure 3. Relationships between the logk_{ow} (literature data) and determined log k' values. (Phenolic acids: 1 = gallic; 2 = caffeic; 3 = p-hydroxy benzoic; 4 = vanillic; 5 = p-cyanobenzoic; 6 = benzoic; 7 = cinnamic; 8 = p-chlorobenzoic; 9 = p-bromobenzoic).

Even on the basis of the limited number of compounds studied, the correlation obtained shows a promise as a simple; direct and rapid method for the estimation of lipophilicity parameters of new phenolic acids and or those for which this data does not exist.

Positive and negative log k' values were obtained, which may arise from the lipophilic character of the aromatic substituents. Lipophilia values can be obtained from the parameter π (π) as a measure of the lipophilic properties of the aromatic substituents [49]. Compounds with negative log k' have substituents with negative π values ($\pi_{\text{CH}_3} = -0.04$; $\pi_{\text{OH}} = -0.62$; $\pi_{\text{CN}} = -0.31$) and compounds with positive log k' have zero or positive π values ($\pi_{\text{H}} = 0$; $\pi_{\text{Br}^-} = 1.02$; $\pi_{\text{Cl}^-} = 0.7$).

More clarity about the lipophilic effect of the substituents on the capacity factor is obtained from the relation between log k' values for p-substituent compounds and the π values. Figure 4 illustrates that log k' increased linearly with the lipophilic character of the substituents.

These results suggest strongly that part of the lipophilic properties of phenolic acids arise from the different lipophilic characters of the aromatic ring substituents.

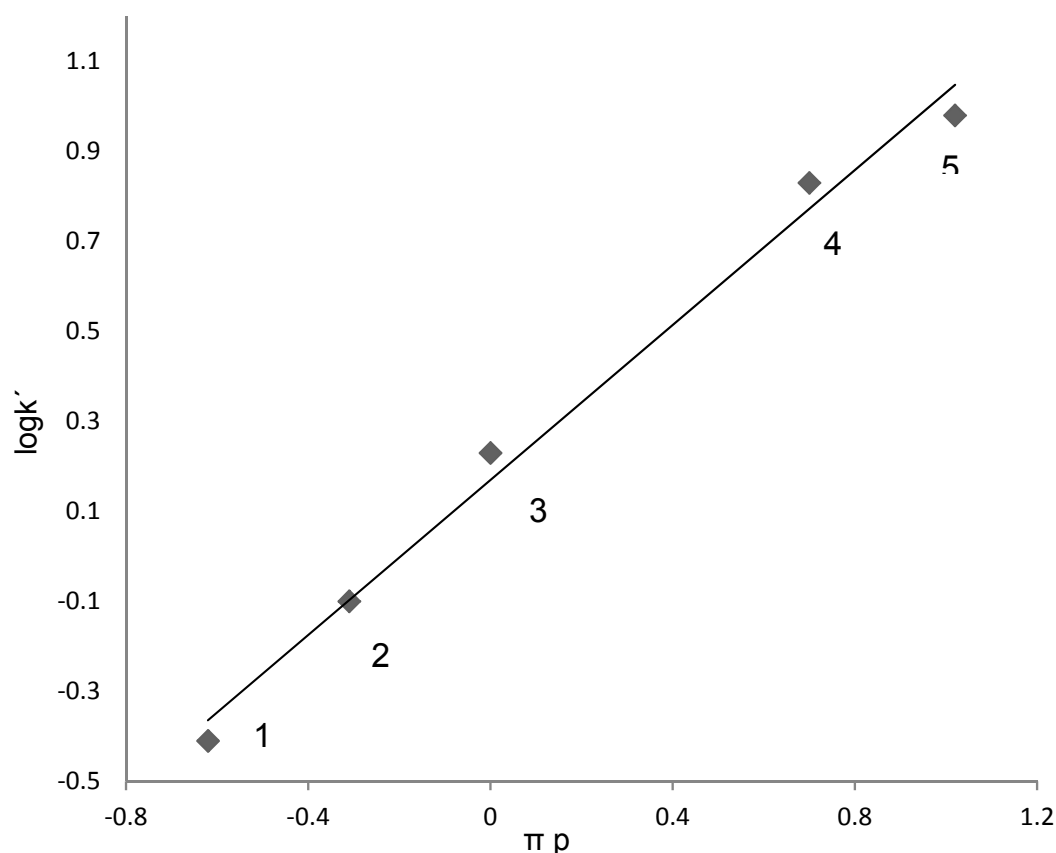


Figure 4. Relationship between $\log k'$ values and πp values for p-substituted phenolic acids (1 = OH; 2 = CN; 3 = H; 4 = Cl; 5 = Br).

The role of lipophilia on the phytotoxicity of the series studied can be analyzed from the $\log k'$ or $\log P_{\text{HPLC}}$ values

In general, the compounds with positive $\log k'$ or $\log P_{\text{HPLC}}$ values ≥ 2.0 showed more germination inhibition activity and the compounds with negative values did not show significant activity. Specifically, $\log k'$ values of caffeic and cinnamic acids suggest that the different activity observed may arise from the different lipophilic character of these compounds. A clearer relationship was obtained from a regression between percentage of germination inhibition activity at the $250 \mu\text{g mL}^{-1}$ concentration and the $\log P_{\text{HPLC}}$ values. Figure 5 shows that germination inhibition activity increased with the lipophilic character of the molecules until the range of 2.0-2.6 values where the higher activity was observed. With the limited data at hand, it is not possible to generalize this observation to other series of compounds at this time, but there are reasons to believe that the parameters $\log k'$ or $\log P_{\text{HPLC}}$ may be appropriate indicators to infer the lipophilic requirement for the germination inhibition activity of phenolic acids.

Antialgal activity cannot be rationalized in terms of the $\log k'$ or $\log P_{\text{HPLC}}$ parameters because only the caffeic, gallic and p-bromobenzoic acids displayed significant activity, and they showed positive and negative $\log k'$ values. It is important to emphasize that caffeic acid

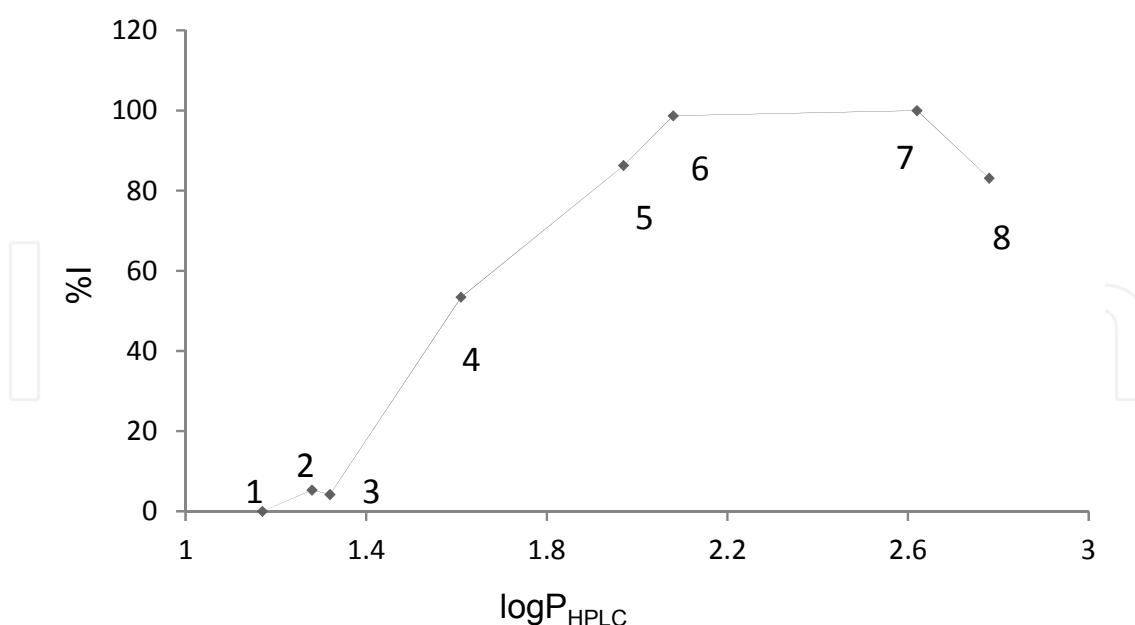


Figure 5. Relationships between $\log P_{\text{HPLC}}$ values and percentage of inhibition germination activity (%I) for phenolic acids (1 = caffeic; 2 = p-hydroxybenzoic; 3 = vanillic; 4 = p-cyanobenzoic; 5 = benzoic; 6 = cinnamic; 7 = p-chlorobenzoic; 8 = p-bromobenzoic)

($\log k' = -0.51$) displayed the highest antialgal activity and the opposite effect on the germination and growth of the lettuce plants.

These results show that the phytotoxic effect depends of receptor species, it is necessary to understand other molecular aspects of phytotoxic interaction to clarify these results.

4. Conclusions

- The series of phenolic acids studied shown varied phytotoxic activity against the germination of lettuce seeds and growth of microalga *C. vulgaris*.
- Caffeic acids showed a particular behavior against the target species stimulated the growth of lettuce plants and inhibited the growth of *C. vulgaris*.
- Part of phytotoxic activity against seed germination is related to the lipophilic character of phenolic acids and it can be inferred from $\log k'$ or $\log P_{\text{HPLC}}$ parameters.
- Although the concentrations used are probably greater than in the field, these results contribute to the knowledge of the relative role of the allelopathy of the naturally occurring phenolic acids of plants with agricultural importance.

The necessity of understanding other aspects such as the chemical stability and persistence in the soil are essential prerequisites if the application of allelochemicals is to become an alternative in the control of weeds.

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