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This is a postprint (final draft post-refereeing) of an article published in

Allelopathy Journal

ISSN: 0971-1693 (print version)

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The definitive version is available in
http://www.allelopathyjournal.org/

DOI: 0971-4693/94

Pérez-Corona M.E., de las Heras P., Vázquez de Aldana B.R.
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Allelopathy Journal 32: 101-112 (2013)

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Running title: *Allelopathic potential of Ulmus pumila litter*

Allelopathic potential of invasive *Ulmus pumila* on understory plant species

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ABSTRACT

In Petriplate bioassay and Pot culture, we studied the allelopathic potential of leaf litter of Siberian elm (*Ulmus pumila* L.) invasive tree species on the germination and growth of three herbaceous plant native species (*Dactylis glomerata* L., *Trifolium repens* L. and *Chenopodium album* L.) commonly found in riparian understory communities. Two assays were done with leaf litter of *U. pumila* from riverine ecosystems (i). the effects of aqueous litter extracts in Petri dish assays and (ii). the effect of litter residues in pot experiments. The leaf litter extracts inhibited the radicle growth of *D. glomerata* and *T. repens* but did not effect the germination. However in pots, leaf litter inhibited the germination speed of *C. album* and growth of *D. glomerata* and *T. repens.* Our results showed the allelopathic potential of *U. pumila* litter, which reduced the growth of understory species (*T. repens* and *D. glomerata*). In the litter of *U. pumila* many phenolic compounds (hidroxibenzoic, ferulic, coumaric, protocatechuic, vanillic and rosmarinic acids, and quercetin) were detected.

Keywords: *Chenopodium album*, *Dactylis glomerata,* germination, litter extract, litter residue, phenolic compounds, root growth, Siberian elm, *Trifolium repens, Ulmus pumila.*

INTRODUCTION

Invasive plant species affects the structure of native plant communities through changes in the diversity or abundance of native species (9,33) and the biotype growth forms (19,21). Allelopathy has been considered as one of the main mechanisms for the remarkable success of invader plants, because native species may be devoid of the chemicals produced by the invasive species (3,14). Allelochemicals are released into the soil either as exudates from living plant tissues or by decomposition and leaching from plant residues (i.e. litter) (4,26). The production of allelochemical compounds is a constitutive mechanism of plant species in which the presence of a specific plant competitor is not required. However, once metabolites have been released into the soil they may affect multiple components of ecosystem (2,3,13,14). The release of allelochemicals from litter decomposition could inhibit the establishment of seedlings not only of native tree species but also of herbaceous vegetation. Germination and seedling establishment are the most critical stages for plant population dynamics and success in the plant community.

Riparian forest ecosystems are particularly sensitive to plant invasion due to their favorable environmental conditions for plant growth and their capacity to act as very effective ecological corridors for plant dispersion. Along the riverbanks of Spain, several invasive or exotic tree species such as the black locust (*Robinia pseudoacacia* L.), the tree of heaven (*Ailanthus altissima* (Mill.) Swingle) and the Siberian elm (*Ulmus pumilla*) are often detected. These exotic species compete and coexist with the native tree species [white poplar (*Populus alba* L.), field elm (*Ulmus minor* Mill.) or narrow-leafed ash (*Fraxinus angustifolia* Vahl)] and in early growth stages with understory herbaceous species

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(20). Allelopathic effects are well documented for the invaders Ailanthus (12,15,31), the black locust (16,22) and elms such as *Ulmus americana* (17,32). However, to the best of our knowledge no report about the allelochemical effect of *Ulmus pumila* has been addressed in the current scientific literature.

Siberian elm (*Ulmus pumila*) is a fast-growing tree native to northern China, eastern Siberia, Manchuria and Korea. It invades the contrasting habitats such as riverbanks or dry mesic prairies and is the hardest of all elms and does well even in areas with cold winters and long periods of summer droughts. Elms tolerate a variety of conditions such as poor soils and low soil moisture and they are fairly wind-tolerant. They are found along roadsides, in pastures and grasslands and they also grow easily in moist soils along streams. In the USA and EU elms have been recognized as invasive plant species of dry mesic prairies (5,23). The Siberian elm has been used for the restoration of riparian areas in the USA, causing remarkable invasion problems. *U. pumila* is becoming frequent as an exotic species in riverine areas in Spain (11).

This study aimed to determine the allelopathic potential of leaf litter of invasive tree species *U. pumila* on the germination and growth of three native herbaceous plant species: orchardgrass (*Dactylis glomerata*), white clover (*Trifolium repens*) and fat-hen (*Chenopodium album*) commonly found in understory riparian communities. We tested (i) the effects of aqueous extracts of leaf litter of *Ulmus* in Petri dishes and (ii) the effects of leaf litter residues in pot experiments.

MATERIALS AND METHODS

I. Plant material and preparation of aqueous extracts

The study was done at Soto de El Encín (40°32' N; 3°16' O, Annual rainfall: 470 mm, 595 m above sea level) on the right riverbank of the Henares River in the municipality of Alcala de Henares (Madrid, Spain) (19). The climate is continental-Mediterranean, mitigated by the local conditions of a riparian zone. The area has a well-conserved riparian forest with reedbeds (*Phragmites australis, Typha spp*), willow woods (*Salix spp*), poplar groves (*Populus spp*), and elm groves (*Ulmus minor*), although the main formations are *Populus alba* groves (20). In some restricted areas, the presence of exotic tree species, such as *A. altissima, U. pumilla* or *R. pseudoacacia* had been detected (personal observation). In this area, *U. pumilla* tree produces 38.1 g m^{-2} $year^{-1}$ senescent leaves and litter (9). Litter was collected from 10 trees during October-November 2009. The leaves were dried at room temperature and then at 65 °C in a forced-air stove until constant weight was reached. Part of this plant material was stored at -60 °C until used in pot experiments.

Aqueous extracts were prepared by soaking 1 g of dried leaf litter in 100 mL of distilled water (i.e. 0.810 g /100 mL conc) for 24 h in an orbital shaker at 80 rpm. The resulting solution was filtered first with conventional filter paper and then through a sterile filter (Millipore Express-Plus, 0.22 µm). The filtrate was defined as 100% extract and was further diluted with distilled water at 1:2 (50%) and 1:5 (20%). These three doses (20%, 50%, 100%,) resembled the average amount of aqueous extract released by *U. pumila* trees during the year, calculated from the litter and rainfall ratios in the study area (Soto de El Encín) (Table 1).

As target species, we selected three common herbaceous species in study area (20): the perennials *Dactylis glomerata* (*Gramineae*) and *Trifolium repens* (*Leguminosae)* and the annual *Chenopodium album* (*Chenopodiaceae*). Seeds of the three herbaceous species were obtained commercially (Semillas Silvestres S.L.) and kept at 4 °C until used for the experiments.

II. Petriplate Bioassays

We examined the effects of aqueous leaf litter extracts on the germination and radicle growth of 3- target understorey species. The germination and radicle growth of the target species were assessed in separate assays.

Seed germination: Seeds of the target species were soaked in water for 24 h to break their dormancy. The experiment was conducted using a one-way factorial arrangement considering the dose factor with four levels (control, 20%, 50% and 100%), within a randomized design with three replicates. The experimental unit was a Petri dish (9 cm dia). Twenty seeds from the target species were placed on paper germination disks (filters from ANOIA S.A.) on Petri dishes, each containing 4 mL of the corresponding extract doses or controls. Distilled water was used as a control. The Petri dishes were placed randomly in a germination chamber in dark at 24 °C for 30 days. The germinated seeds were counted daily to calculate: (i). Germination (%) and (ii). Germination speed, calculated using the index of Einhellig *et al.* (6):

$$S = (N_1 \cdot 1) + \frac{N_2 - N_1}{2} + \frac{N_3 - N_2}{3} + \ldots + \frac{N_n - N_{n-1}}{n},$$

Where, N_1, N_2, N_3, N_n represent the proportion of germinated seeds on day *1, 2, 3…n* after the start of the experiment. The germination speed S varied from 0 (if no seeds germinate by the end of the experiment) to 100 (if all seeds germinate on the first day).

Radicle growth: To evaluate the effect of aqueous extracts of leaf litter on radicle growth, a separate bioassay was done with pre-germinated seeds from each target species (*D. glomerata, T. repens, C. album*). After breaking their dormancy as previously indicated, seeds were placed on trays with filter paper over a layer of sterile perlite soaked in sterile distilled water. The duration of this pre-treatment to obtain pre-germinated seeds was 36 h for *T. repens*, 72 h for *D. glomerata* and 96 h for *C. album.* Twenty uniformly germinated seeds (1 mm radicle length) were selected and placed on a Petri dish on germination paper (filters from ANOIA S.A.) soaked in 4 ml of plant extract. The experimental design was that used in the germination assay, considering the dose factor with four levels (control, 20%, 50% and 100%). For *C. album*, the 20% and 50% doses could not be prepared owing to lack of germinated seeds with radicle of 1 mm or more (the germination speed and germination rate for this species were low). All Petri dishes were incubated for 48 h at 24 °C in dark. Radicle length was then recorded by scanning the seedlings with a desk-scanner. Length measurements were obtained from digital images using Photoshop CS4 (Adobe).

TABLE 1 here

III. Pot experiments

Pot experiments were done to evaluate the effects of leaf litter residue on the seed germination and plant growth of 3-target species (*D. glomerata, T. repens* and *C. album*). The assay consisted of treatment with the leaf litter of *U. pumila* and treatment without litter as a control. For each target species and litter treatment, four replicates were considered (total 24 pots). The amount of litter added was estimated considering the average amount of litterfall in the riverine ecosystem (10) (Table 1).

Pots with a square surface (64 cm^2) were partially filled with a mixture of perlite (Agroperlita F-13; Semillas Diago S.L., Valencia, Spain) and sand (2:1 v/v). In each pot, 0.48 g (Equivalent to 750 Kg/ha) of freeze-dried litter was crushed by hand and uniformly mixed with extra perlite-sand mixture (75 cm^3). Pots were topped up with the mixture. Ten seeds of each target species were sown per pot, which were placed at random in a glasshouse [24 °C/15 °C (day/night), 60 % relative humidity and the natural light conditions of spring]. The pots were irrigated with tap water every two days. Two weeks after sowing, a commercial fertilizer (Platinum, Productos Flower S.A. Lleida, Spain) was added once a week using the dose recommended by the manufacturer. Seedling emergence was controlled daily and two weeks after sowing five seedlings were left in each pot. Four weeks after sowing, all plants were harvested, the roots

were separated from the shoots, and the number of leaves was counted. Root length was recorded by scanning plants with a scanner (HP ScanJEt 5530). All plant material was dried at 60 °C over 48 h and then weighed. Length measurements were obtained from digital images using WinRhizo (Régent Instruments Canada Inc.).

IV. Chemical analyses

Preparation of extracts: Litter extracts for chemical analysis were prepared as per Reynaud *et al.* (27) as under. An aliquot of 100 mg of dried litter sample was extracted with 80% EtOH (5 mL) for 60 min. After the extraction, the samples were filtered (Filter-lab 1244) and the residue was washed with solvent (5 mL) and filtered. The two sample extracts were combined in a total extract that was evaporated to dryness in a rotary vacuum evaporator at 50 °C. The residue was dissolved in 80% EtOH (1 mL), filtered through a nylon membrane (13 mm; 0.45 μm) and stored at -20 °C until chemical analysis.

Determination of total phenolic contents: The concentration of total phenolic compounds was determined by the colorimetric method using the Folin-Ciocalteau reagent (30). A 150-μL aliquot of each sample was mixed with 3 ml of distilled water followed by the addition of 250 μL of Folin-Ciocalteu reagent (Scharlab Chemie S.A.). After 6 min, 750 μL of a 7% Na_2CO_3 solution was added to the sample. The mixture was shaken and then brought up to 5.0 mL with distilled water. Absorbance was measured after 120 min at 760 nm in a UV-visible Cary 50 Probe Spectrophotometer, using gallic acid (Acrós Organics) as a reference standard for quantification.

Characterization of phenolic compounds by HPLC: The concentrations of main phenolic compounds were determined in the extracts with a HPLC system comprising a Waters 2695 Separations module systems with a Symmetry C18 Waters column (5μm, 3.9 × 150 mm), and a photodiode array detector (Waters 996).

To analyse the phenolic acids (benzoic and cinnamic acid derivatives) the gradient consisted of solvent *A* (methanol supragradient HPLC grade) and solvent *B* (2% acetic acid) applied at a flow rate of 0.6 mL/min as follows: 5% *A* for 5 min; from 5% *A* at 5 min to 30% *A* at 20 min in a linear gradient; from 30% *A* at 28 min to 60% *A* at 37 min in a linear gradient; from 60% *A* at 37 min to 100% *A* at 42 min in a linear gradient. The signal was monitored at 260 nm and 330 nm and phenolic compounds were identified by comparing their retention times and UV spectra with those of standards and were quantified using external standard calibration curves. The phenolic acid standards for caffeic, p-coumaric, p-hydroxibenzoic, ferulic, gallic, protocatechuic, sinapic, syringic, vanillic and chlorogenic acids were purchased from Acrós-Organics.

To analyse the flavonoids, the gradient consisted of solvent *A* (methanol supragradient HPLC grade) and solvent *B* (0.5% H_3PO_4) was applied at a flow rate of 1.2 mL/min as follows: 2% *A* for 6 min; from 2% *A* at 6 min to 45% *A* at 12 min in a linear gradient, 45% *A* for 13 min. The signal was monitored at 270 nm and 330 nm. The standards, such as quercetin and ellagic and rosmarinic acids were purchased from Sigma-Aldrich.

V. Statistical analysis

The effects of litter extract concentration on the germination (%), germination speed and radicle growth were studied by one-way ANOVA. The homocedasticity assumption was verified in all cases using Bartlett´s test ($p > 0.05$), so no variable transformation was required. Least significant difference (LSD) was used for comparison of treatments. The effects of litter residue were tested in one-way ANOVA. All statistical analyses were done using the STATISTICA 6.0 statistical program package for Windows (Statsoft). For each parameter, % inhibition (or stimulation) was calculated from the difference between treatments (T) and control (C) using the following equation: $I(\%) = [(C-T)/C] \cdot 100$. Thus a positive value represented % of inhibition and a negative value % of stimulation.

RESULTS AND DISCUSSION

Leaf litter extracts

Aqueous litter extracts of *U. pumila* did not effect the germination (%) and germination speed of any target species (Table 1). The germination rate of *T. repens* was highest and germination (%) was lowest in *C. album*.

The extracts inhibited the radicle growth of *T. repens* and *D. glomerata* but not of *C. album* (Table 1). The inhibition of radicle growth in *T. repens* increased with increase in the extract concentration up to 100% dose (Figure 1A). The inhibition in radicle growth of *D. glomerata* was maximum at 50% dose and there were no significant differences between the 50% and 100% doses. For *C. album* only 100% dose was tested (for the reasons explained in Materials and Methods) and did not have any significant effect on radicle growth.

The inhibitory effect of aqueous extracts of *U. pumila* on the radicle growth of *T. repens* and *D. glomerata* was proportional to the extract concentration used i.e. was concentration dependent (Figure 1A). The highest inhibitory effect was obtained with the 50% dose in *D. glomerata* and with highest extract concentration (100%) in *T. repens*. This showed that *D. glomerata* was more sensitive to extracts, since a lower concentration was more effective.

TABLE 1 here

FIGURE 1

Litter residue effects

The addition of leaf litter to pots had no significant effect on the emergence (%) of any target species. However the emergence speed was slowed down in *C. album* (Table 2).

The leaf litter reduced the growth of *T. repens* and *D. glomerata* in pots. The litter of *U. pumila* reduced the root biomass of *D. glometara* by 36% and that of *T. pratense* by 54% (Table 2) and root length by 22% and 46% in *D. glomerata* and *T. repens* respectively (Figure 1B). The leaf litter inhibited the shoot growth only in *T. repens* due to decrease in number of leaves per plants and biomass production per leaf (Table 2). The litter of *U. pumila* did not affect the growth of *C. album*. The observed inhibitory effects suggested that phytotoxic chemicals could be released from the litter during the experimental period (30 days), inhibiting the root growth of *T. repens* and *D. glomerata.*

TABLE 2 here

Comparing the effects of extracts and residues

In both assays, root growth was more sensitive indicator of growth inhibition than seed germination or emergence (percentage or speed). This is in agreement with the results of several authors (24,28,29) because more processes were affected during the seedling growth than in germination. These results suggested that the effect of litter becomes evident as plant growth depends on external resources and the germination remained unaffected.

The results of germination (or emergence) and radicle growth in both assays were similar, except for the germination speed in *C. album*. The only significant effect detected in *C. album* was the emergence speed in the residue assay. The lack of effect with extracts, even at the highest concentration, could be due to differences in the time-course between both assays and to the characteristics of this species. The seeds of *C. album* used in these experiments had low germination (%) and slow germination speed, and the differences in the time-course between both assays could lead to differences in the results. Thus, the longer duration of residue assay would allow higher germination (%) of control seeds (40% in the residue assay *versus* 20% in the extract assay) and the detection of a significant effect of litter on the germination speed. Moreover, *C. album* is an annual, fast-growing species and early growth was not affected by external conditions.

In *D. glomerata*, radicle length was inhibited in the extract assays and the results from the residue assay showed that root growth biomass production was affected and not the length (Table 2). The standard errors of root length in the residue assay were very high and the differences were not statistically significant. These high error values could be due to very fine roots of this species and it is possible that part of thin roots could have been lost in sampling. However, the root growth (biomass) of *D. glomerata* was significantly inhibited and would be less affected by sampling error. Similarly, high standard errors were found in root length of *T. repens* but the effect was significant.

The amount of litter added to pots (0.48 g/pot) was mixed with about 100 cm^3 of mixture (perlite and sand) i.e. equivalent to 50% dose of litter extracts. Since in both assays the results were similar, it could be suggested that phytotoxic chemicals could have been released from the litter during the experimental period (30 days). The assay in pots was designed to estimate the effect of leaf litter residues, considering the suggestions of Politycka and Lipinska (25) such as the use of unpowdered plant material (leaf litter) and the spreading of plant material on the soil surface (2 cm). During litter decomposition, the development of microbiological metabolites or intermediate products of mineralization processes showing a higher allelopathic activity than their precursor plants is possible (25). We obtained similar results in both assays (extracts and residue), suggesting that the effect of litter residue could be mainly due to allelopathic activity (toxicity of residues) and not to the effect of soil microorganisms.

Phenolic composition

The concentration of total phenolic compounds in *U. pumila* litter was 8.46 g/kg dry matter. With HPLC we identified the benzoic acid derivatives (hydroxybenzoic, protocatechuic and vanillic acids), the cinnamic acid derivatives (coumaric and ferulic acids) and the flavonoids quercetin and rosmarinic acid (Table 3). Other peaks appeared in the chromatograms but their identity could not be determined. Most of these compounds have been described as allelopathic agents (7,18,32).

Understory species inhibition

We found a differential response of understory species to *U. pumila* litter. *C. album* was least affected species. *T. repens* was most affected (both shoot and root growth were inhibited), while only the root growth of *D. glomerata* was affected. However, *D. glomerata* was more sensitive to lower concentrations of extract. Several observations report a differential response of dicots and monocots to the allelopathic effect of forest trees. Legumes (dicots) seem to be more sensitive than grasses (monocots) to the allelopathic effects of *Eucalyptus* and *Acacia* (8) and to *Pinus* and *Quercus* (1) and also to other species such as the nodding thistle (*Carduus nutans* L.) (34). The influence of *Ulmus* was stronger in *D. glomerata* and *T. repens* than in forb species. Grasses and legumes are the most abundant botanical families in the grassland and herbaceous understory communities such that changes that affect these families may lead to clear effects on understory structure and function.

Our results showed the allelopathic potential of *U. pumila* that may limit the establishment of species such as *T. repens* and *D. glometara*. Similarly, Lodhi and Johnson (17) reported that variations in the understory vegetation under several tree species such as the American elm (*Ulmus americana*) were not correlated with changes in soil parameters, suggesting evidence of an allelopathic effect. Those authors concluded that the organic substances released in the immediate environment of dominant trees and their litter influence soils and associated herb growth.

To our knowledge, this is the first report demonstrating the allelopathic effect of *Ulmus pumila* leaf litter. We found that extracts and residues from the litter of the exotic *U. pumila* had a significant effect on the seedling growth of the understory species *T. repens* and *D. glomerata* and on the germination speed of *C. album.* Our results supported the idea that allelopathy could be an important mechanism for the successful invasion of *U. pumila* in riparian ecosystems.

ACKNOWLEDGEMENTS

This research was funded by project POII10-0179-4700 of the Junta de Comunidades de Castilla La Mancha. Thanks are due to Pablo Catalán, Alberto Fernández-Seral and Amador Alvarez for technical help.

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Table 1. Effects of litter extracts of *Ulmus pumila* on germination (percentage and speed) and root length of three target species (*D. glomerata, T. repens and C. album*) in Petriplate bioassay.

Extract concentration (%)	Germination		Seedling growth
	Per cent	Speed	Root length (cm)
	Dactylis glomerata		
0	71.6	25.3	0.869
20	60.0 (-16.3)	15.1 (-40.3)	0.507 (-41.7)
50	51.7 (-27.9)	14.7 (-41.7)	0.343 (-60.5)
100	55.0 (-23.3)	13.5 (-46.6)	0.327 (-62.4)
Mean	59.6	17.1	0.512
P	0.505	0.065	0.000
LSD 0.01			0.086
	Trifolium repens		
0	88.3	48.6	0.820
20	83.3 (-5.7)	46.5 (-4.2)	0.653 (-20.4)
50	73.3 (-16.9)	37.2 (-23.4)	0.471 (-42.6)
100	73.3 (-16.9)	32.8 (-32.5)	0.275 (-66.5)
Mean	79.6	41.3	0.554
P	0.116	0.243	0.007
LSD 0.01			0.172
	Chenopodium album		
0	21.7	18.3	0.714
20	25.0 (+15.4)	20.6 (+12.1)	0.840 (+17.6)
50	41.7 (+92.3)	28.2 (+53.6)	
100	35.0 (+61.5)	18.6 (+1.5)	
Mean	30.8	21.4	0.777
P	0.098	0.062	0.294

Data in parenthesis is indicated the percentage of inhibition (-) or stimulation (+) over control. *P* = level of significance from ANOVA. When significant ($P<0.05$), the value of LSD 0.01 is indicated

Table 2. Effects of leaf litter of *Ulmus pumila* on emergence and growth parameters of three target species (*D. glomerata, T. repens* and *C. album*) in Pot culture.

Parameter	Control	Litter	P- value	Inhibition (%)
		Dactylis glomerata		
Emergence (%)	56.8	47.5	0.628	-16.4
Emergence speed	7.32	5.82	0.323	-20.5
Shoot biomass (mg DM/plant)	26.2	20.82	0.168	-20.6
Root biomass (mg DM/plant)	8.09	4.66	0.032	-36.6
Root length (cm/plant)	77.1	59.73	0.270	-22.5
Number of leaves (per plant)	4.26	4.32	0.513	+1.4
Leaf biomass (mg DM)	6.15	4.82	0.196	-21.6
		Trifolium repens		
Emergence (%)	75.0	72.5	0.730	-3.3
Emergence speed	9.8	9.5	0.731	-3.1
Shoot biomass (mg DM/plant)	12.92	5.18	0.007	-59.9
Root biomass (mg DM/plant)	4.17	1.94	0.015	-53.5
Root length (cm/plant)	18.3	9.86	0.045	-46.1
Number of leaves (per plant)	1.95	1.25	0.000	-35.9
Leaf biomass (mg DM)	6.56	4.12	0.030	-37.2
		Chenopodium album		
Emergence (%)	41.7	30.0	0.178	-28.1
Emergence speed	7.46	4.52	0.050	-39.4
Shoot biomass (mg DM/plant)	28.06	28.60	0.961	+2.02
Root biomass (mg DM/plant)	5.00	4.23	0.578	-15.3
Root length (cm/plant)	99.15	81.47	0.641	-17.8
Number of leaves (per plant)	5.5	4.97	0.491	-9.6
Leaf biomass (mg DM)	5.10	5.75	0.812	+12.7

The P –value indicates the significance level of the ANOVA for the respective pairs of means. Percentage of inhibition (-) or stimulation (+) over control

Table 3. Concentration of phenolic compounds detected in litter of *Ulmus pumila.*

Chemical class	Compound	Concentration (mg/Kg)
Benzoic acid derivatives	Hydroxibenzoic	8.03
	Vanillic	21.4
	Protocatechuic	122.4
Cinnamic acid derivatives	Coumaric	54.9
	Ferulic	41.6
Flavonoids	Quercetin	856.1
	Rosmarinic	324.3
Total phenolics (colorimetry)		$8.46\ 10^{3}$

Figure 1. Inhibitory effects of *Ulmus pumila* litter on germination and growth of three target species in Petri plate assays with aqueous extracts at different concentrations and in pot culture with leaf litter.

PETRI PLATE ASSAY **POT CULTURE**