



Article

Phytotoxic Effects and Potential Allelochemicals from Water Extracts of *Paulownia tomentosa* Flower Litter

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Abstract: Flowers of *Paulownia* spp. wither and fall on a large scale after blooming in spring and have potential allelopathic effects on surrounding plants, including crops and weeds. In this study, the phytotoxic effects of water extracts of *Paulownia tomentosa* flower litter (EPF) on wheat (*Triticum aestivum* L.), lettuce (*Lactuca sativa* L.), green bristlegrass (*Setaria viridis* L.) and purslane (*Portulaca oleracea* L.) were evaluated in the laboratory. The mode of action of the phytotoxicity of EPF on lettuce seedlings was studied and the secondary metabolites in EPF were analyzed by liquid chromatography high-resolution mass spectrometry (LC-HRMS). The results show that EPF significantly inhibited the seed germination and seedling growth of four target plants in a concentration-dependent manner. In addition, EPF could induce the excessive accumulation of reactive oxygen species (ROS) flowing with oxidative damage of the lipid bilayer of the biofilm, resulting in reduced cell viability and even apoptosis in lettuce. There were 66 secondary metabolites identified by LC-HRMS in *P. tomentosa* flowers. Among them, 10 compounds, including salicylic acid, caffeic acid, parthenolide, 7-hydroxycoumarin and abscisic acid (ABA), were all known allelochemicals. In summary, *P. tomentosa* flower litter displayed significant allelopathic effects, which were related to the accumulation of ROS in target plants. Phenolic acids, flavonoids as well as ABA are probably the main allelochemicals of *P. tomentosa* flowers.

Keywords: *Paulownia tomentosa*; flower litter; phytotoxicity; allelochemical; mode of action



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1. Introduction

Allelopathy is a negative or positive effect of plants on other plants through chemical interaction in the environment [1]. Litter, such as fallen leaves and flowers, is one of the most direct and effective ways to release plant allelochemicals. During decomposition, the secondary metabolites released from litter can affect the growth and development of surrounding plants [2]. Many tree species demonstrate allelopathic effects, especially the trees belonging to the families of Myrtaceae, Lauraceae, Juglandaceae and Taxaceae [3]. In terrestrial ecosystems, the decomposition of plant litter is crucial for maintaining and restoring soil fertility, as well as playing a significant role in the material cycle. Furthermore, the allelopathy of plant litter has a direct and/or indirect impact on the growth and development of a plant itself and neighboring plants, thereby playing a significant ecological role in community competition and population regulation within plant ecosystems [4]. Therefore, elucidating the relationship between plant litter and allelopathy will help to understand

the role of litter in maintaining soil fertility and community competition and population regulation, and it is critical for the maintenance and utilization of natural resources as well as the sustainable development of agricultural production. In recent years, a number of studies on the isolation and identification of allelochemicals, the effects on growth and development of test plants and the mechanism of the allelopathic effects of tree litter have been carried out [5,6]. However, the allelopathy of litter of many tree species has not yet been thoroughly explored.

Paulownia spp. are the only woody plants in the Scrophulariaceae family. They are native to China and have a cultivation history of more than 2000 years [7]. Now, *Paulownia* spp. have been successfully introduced in Japan, Europe, Australia, America, Africa and other countries [8]. Due to their fast growth, tall tree shape, dense leaves, beautiful flowers and high ornamental value, *Paulownia* spp. are often chosen as landscaping tree species for walkways and courtyards. In addition, *Paulownia* spp. are also widely planted next to farmlands and lawns to conserve soil and water [9].

Paulownia spp. have also been used as traditional Chinese herbal medicines, with a long history. The flowers, leaves, fruits and barks of *Paulownia* spp. all have medicinal value and play important roles in anti-inflammation, cough-relieving, diuresis and blood pressure reduction [10]. The medicinal effects of *Paulownia* spp. have been documented in ancient times. For example, “Compendium of Materia Medica” records that “Leaves of *Paulownia* spp. are used to treat malignant sores and the barks are responsible for hair growth and bruises recovery”. Modern experimental studies have shown that *Paulownia* spp. have a variety of activities, such as antioxidant, antibacterial, anti-inflammatory, anti-tumor and insecticidal effects [11–13]. Therefore, as a traditional Chinese medicine, *Paulownia* spp. have a variety of pharmacological activities and clinical application value.

The pharmacological activities of *Paulownia* spp. are dependent on their active secondary metabolites. Kazi et al. first isolated glycoside compounds from the bark and leaves of *Paulownia* spp. In the 1930s [14]. Then, various compounds mainly including iridoid glycosides, phenylpropanoids, flavonoids, sesquiterpenes and triterpenes were isolated and identified from *Paulownia* spp. by methods of modern spectroscopy and separation [15–18]. Many of these compounds displayed antibacterial, anti-inflammatory, anti-aging and anti-tumor biological activities [19–21].

The abundant active secondary metabolites in *Paulownia* spp. also provide an effective source of allelochemicals. Previous studies showed that *Paulownia* spp. displayed allelopathic potential on other plants. Water extracts of *P. fortunei* leaf litter showed phytotoxic effects on corn (*Zea mays* L.) seed germination and seedling growth in a concentration-dependent manner [22]. Zhao et al. showed that water extract of *P. tomentosa* leaf displayed significant inhibitory effects on the seed germination rate of soybean (*Glycine max* (Linn.) Merr.), wheat and corn at 50 mg/mL [23]. The ethyl acetate extract of *P. tomentosa* flowers showed inhibitory activities on radical growth of redroot pigweed (*Amaranthus retroflexus* L.), lettuce and cucumber (*Cucumis sativus* L.) at 0.5 mg/mL, and p-ethoxybenzaldehyde was obtained from ethyl acetate extract with a phytotoxic effect [24].

These findings indicate that *Paulownia* spp. contain abundant secondary metabolites and have allelopathic potential. The aim of the present study was to evaluate the allelopathic effects of *P. tomentosa* flower litter on selected crops and weeds, determine the composition of its water extract and find potential allelochemicals. The results from this study will provide valuable insights to understand the allelopathy of *P. tomentosa* on surrounding plants and support further application of the allelochemicals from this plant as valuable products in the natural herbicidal industry.

2. Materials and Methods

2.1. Plant Materials

After falling, *P. tomentosa* flowers' litter was immediately collected on the campus of Nanyang Normal University on 8 May 2022. About 1000 g flower litter was dried in darkness at room temperature and then stored at 4 °C before use. Seeds of wheat

(*T. aestivum*), lettuce (*L. sativa*), green bristlegrass (*S. viridis*) and purslane (*P. oleracea*) were purchased from Henan Fengtian Seed Industry CO., LTD (Zhengzhou, China).

2.2. Water Extract of *P. tomentosa* Flower Litter

The dried flower litter of *P. tomentosa* was crushed into fine powder. Then, 1 L distilled water was added to 100 g of the powder and extracted with shaking at 25 °C for 48 h. The extracts were filtered with filter paper and sterilized by a 0.22 µm water-based filter membrane to obtain a water extract with a concentration of 100 g/L, which was diluted with sterile water to 50, 25, 12.5 and 6.25 g/L, respectively. Water extracts of *P. tomentosa* flower litter (EPF) were stored at 4 °C before use.

2.3. Seed Germination

Seeds of wheat, lettuce, green bristlegrass and purslane with uniform size were surface-sterilized with 2% NaClO and then washed 5 times with sterile distilled H₂O. The sterilized seeds were transferred to a Petri dish ($r = 4.5$ cm) containing filter paper with 50 seeds in each dish. EPF (3 mL) with different concentrations was added to the Petri dish, and sterile distilled H₂O was used as a control. Seeds were cultured in darkness at 25 °C, and the number of the germinated seeds was recorded every 24 h. The germination potential and germination rate of seeds were calculated according to the following formula.

Germination potential = (number of germinated seeds in the germination peak period / total number of the subjects' seeds) $\times$ 100%

Germination rate = (final number of germinated seeds / total number of the subjects' seeds) $\times$ 100%

2.4. Seedling Growth

The sterilized seeds were transferred to filter paper soaked with 3 mL sterile distilled H₂O in a Petri dish ($r = 4.5$ cm) and germinated at 25 ± 1 °C in darkness. After germination, at least 6 seedlings of similar size were transferred to every well of a 6-well plate (NUNC, Shanghai, China), which contained various concentrations of EPF and sterile distilled H₂O as a control. After incubating at 25 ± 1 °C in darkness for 48 h, the root and shoot length of the seedlings were measured using a ruler, and the fresh weight was measured by an analytical balance.

2.5. Cell Viability

The root tip cell viability was detected using propidium iodide (PI) and fluorescein diacetate (FDA) double staining method according to Pan et al. with modifications [25]. Lettuce roots were exposed to a mixture of FDA (12.5 µg/mL) and PI (5.0 µg/mL) for 10 min. The stained roots were rinsed with distilled water to remove excess dye on the root surface and then observed under a fluorescence microscope (Leica DMI4000B, Wetzlar, Germany) with excitation 488 nm and emission >510 nm. At least 15 samples were observed for each treatment group.

Cell viability was also evaluated by staining with Evans blue based on its ability to pass through ruptured membrane and stain dead cells [26]. After treatment, root segments (1 cm length from the tip) of lettuce seedlings were stained with 0.25% (w/v) Evans blue (Solarbio, Beijing, China) for 1 h at room temperature, then washed with distilled H₂O for 15 min. N,N-dimethylformamide was used to extract the Evans blue that was taken into the root segments, and then the absorbance of the solution was measured with a spectrophotometer (UV-1750, Shimadzu Corp., Kyoto, Japan) at 600 nm. The relative Evans blue uptake was calculated as the ratio between the optical density (OD) values of the treated group and the control.

2.6. Reactive Oxygen Species (ROS)

Dihydroethidium (DHE) dyeing method was used under room temperature and dark condition [27]. Lettuce roots were gently shaken in the dye solution (DHE, 10 mM; acetone,

0.01%; CaCl_2 , 100 mM; pH 4.75). After soaking for 10 min and washing with 100 mM CaCl_2 for 20 min, the roots were analyzed using a fluorescence microscope (Leica DMI4000B, Wetzlar, Germany) with excitation 488 nm and emission >510 nm. At least 15 samples were observed for each treatment group.

H_2O_2 content detection referred to Frew's method [28]. Lettuce samples about 100 mg for each group were homogenized in 0.1 M phosphate buffer (PB, pH 6.9). The extracted samples were centrifuged at $8000 \times g$ for 10 min at 4°C . Then, 4 mL of the supernatant was added to 0.3 mL of reagent solution (phenol 2.34 g/L, 4-aminoantipyrine 1 g/L, PB 1 mM, pH 6.9). The absorbance value of the solution was measured using a spectrophotometer at 505 nm (UV-1750, Shimadzu Corp., Kyoto, Japan). The content of H_2O_2 in lettuce roots was compared with a standard graph and expressed in $\mu\text{M/g}$ FW.

2.7. Malondialdehyde (MDA)

The content of MDA was measured to evaluate the lipid peroxidation [29]. Lettuce roots (about 100 mg) were homogenized in 5 mL trichloroacetic acid (TCA, 10%, *w/v*) and then centrifuged at $4000 \times g$ for 10 min. The supernatant (1.0 mL) was added to 2.0 mL of thiobarbituric acid (TBA, 0.6%, *w/v*) in 10% TCA. The tube containing the mixture was incubated in boiling water for 15 min, followed by ice bath for 10 min. After centrifugation at $9000 \times g$ for 5 min, the absorbance of the supernatant at 440, 532 and 600 nm was measured, and the levels of MDA were calculated in the following manner:

$$[\text{MDA}] = [6.452 (A_{532} - A_{600}) - 0.56 A_{450}] \cdot VT / (V \cdot W)$$

[MDA] represents the concentration of MDA expressed in $\mu\text{M g}^{-1}$. A_{450} , A_{532} and A_{600} are the absorbance values at 450 nm, 532 nm and 600 nm, respectively. VT and V represent total volume of the extracting solution and the volume used in measurement, respectively. W is the fresh weight of the lettuce roots used.

2.8. Free Proline

Free proline content in lettuce was measured according to Bates et al. [30]. Lettuce root sample about 200 mg was homogenized in 3 mL of sulfosalicylic acid (3%, *w/v*) and centrifuged at $3000 \times g$ for 10 min. The supernatant of 1.0 mL was mixed with ninhydrin and acetic acid at a ratio of 1:1:1. After incubation in boiling water for 1 h, the mixture was quickly transferred to an ice bath for 10 min. Toluene (4.0 mL) was added to the mixture with gentle shaking. After separating of the organic and inorganic phases, the organic phase was spectrophotometrically monitored at 520 nm. Proline content was read from a standard curve constructed with pure proline (Alfa Aesar, Shanghai, China).

2.9. LC-HRMS

HPLC analysis was performed on ACQUITY UPLC HSS T3 column (100 mm $\times$ 2.1 mm, 1.8 μm , Waters Company, Milford, MA, USA). The mobile phase consisted of two solvents: solvent (A) deionized water with 1% formic acid and solvent (B) acetonitrile with 1% formic acid.

Gradient elution was performed at a flow rate of 0.3 mL/min at room temperature. Elution profile was isocratic from 0 to 10 min (100% (A), 0% (B)), from 10 to 25 min (70% (A), 30% (B)), from 25 to 30 min (60% (A), 40% (B)), from 30 to 40 min, isocratic (50% (A), 50% (B)), from 40 to 45 min (30% (A), 70% (B)), from 45 to 60 min (0% (A), 100% (B)), from 60 to 70 min (100% (A), 0% (B)).

Mass spectrometric conditions: the high-resolution mass spectrometry Q Exactive Orbitrap (Thermo Fisher Scientific, Waltham, MA, USA) was used to collect mass spectrometry data. The detection mode was Full MS-ddMS², and the positive and negative ion modes were scanned separately, with the scanning range of m/z 100–1200. The resolution of MS¹ was 70,000, and MS² was 17,500. The ion source voltage was 3.2 kV. The Capillary temp was 320°C , and the Aux gas heater temp was 350°C . The Sheath gas flow rate was 40 L/min, and the Aux gas flow rate 15 L/min. The AGC Target was 10^{-6} , and the TopN

was 5. The collision energy that triggers MS² scanning was the stepped fragmentation voltage NCE with 30, 40 and 50, respectively.

2.10. Statistical Analysis

The laboratory phytotoxic assays were conducted with three replicates for each sample. ANOVA and Duncan tests were performed with SPSS 22.0 (SPSS Inc., Chicago, IL, USA). The means were also evaluated by one-way ANOVA with the Least Significant Differences test at the 0.05 significance level. The concentrations required for 50% inhibition in the assay (defined as IC₅₀) of EPF seed germination and seedling growth of target plants were calculated using SPSS 22.0 software and expressed in g/L.

3. Results

3.1. Effects of EPF on Seed Germination

As shown in Figure 1A, the seed germination potential of four receptive plants was affected by EPF. At 6.25 g/L and above, the seed germination potential of wheat, lettuce and green bristlegrass was significantly reduced. Meanwhile, the seed germination potential of purslane was significantly affected by EPF at 25 g/L and higher. With increased concentration, the germination potential of the seeds was further reduced. The influence of EPF on seed germination potential showed a good concentration-dependent nature. The IC₅₀ values of EPF on the seed germination potential of wheat, lettuce, purslane and green bristlegrass were 22.7, 8.9, 34.6 and 9.2 g/L, respectively (Table 1). After treatment with FPE, the seed germination rate of lettuce was significantly reduced at concentrations of 6.25 g/L and higher, while the wheat and green bristlegrass seed germination rates were significantly affected by EPF at 12.5 g/L and above. However, there was no significant inhibitory effect on the seed germination rate of purslane by EPF at all the treated concentrations (Figure 1B). EPF showed strong inhibitory effects on the seed germination rates of lettuce and green bristlegrass with IC₅₀ values of 17.5 and 18.2 g/L, respectively (Table 1).

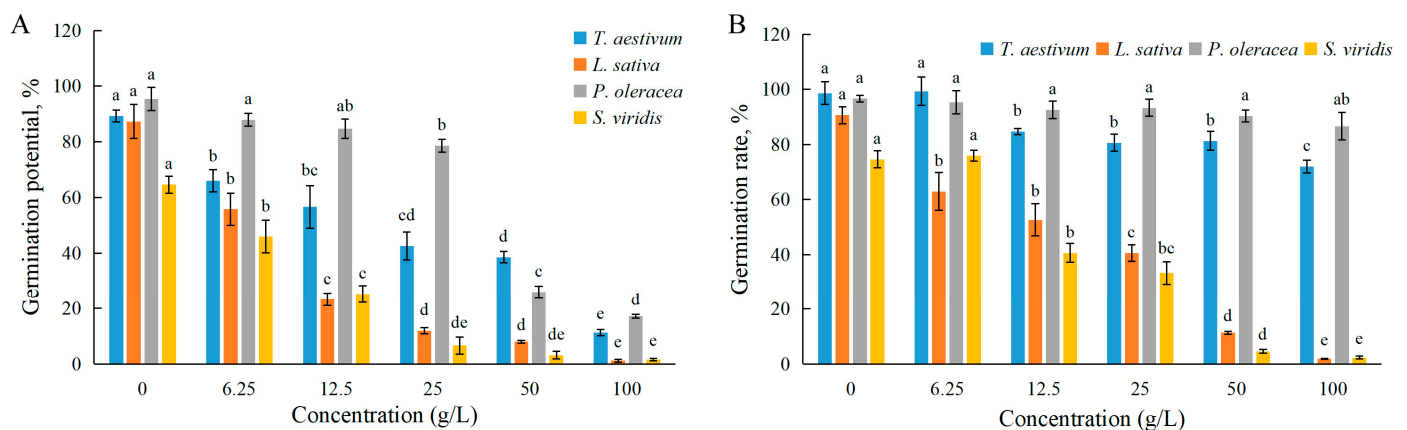


Figure 1. Germination potential (A) and germination rate (B) of four plant seeds under treatment with EPF. The results presented are mean of three replicates $\pm$ SE; different letters denote significant differences at $p < 0.05$ according to one-way ANOVA with an LSD test.

Table 1. IC₅₀ values (g/L) of EPF on seed germination and seedling growth of target plants.

	Germination Potential	Germination Rate	Root Length	Stem Length	Fresh Weight
Wheat	22.7	>100	25.6	53.1	>100
Lettuce	8.9	17.5	23.4	89.0	91.2
Purslane	34.6	>100	21.5	>100	95.7
Green bristlegrass	9.2	18.2	47.8	77.4	93.9

3.2. Effects of EPF on Seedlings' Growth

EPF has inhibitory effects on the root growth of plant seedlings, and the inhibitory effects strengthened with the concentration increasing (Figure 2A). Compared with the control group, EPF showed a significant inhibitory effect on the root lengths of wheat, lettuce and purslane at 12.5 g/L and higher, while it demonstrated a significant inhibitory effect on green bristlegrass root growth at 25 g/L and above. Among the four plants, lettuce root growth is most sensitive to FPE. Under treatment with 100 g/L, the lettuce root length was 2.22 mm, which was only 10.2% of the controls. The IC_{50} values of EPF on the root lengths of wheat, lettuce, purslane and green bristlegrass were 25.6, 23.4, 21.5 and 47.8 g/L, respectively (Table 1). The inhibitory effect of EPF on the growth of the seedling roots was positively correlated with the treated concentration, showing a good dose–effect relationship.

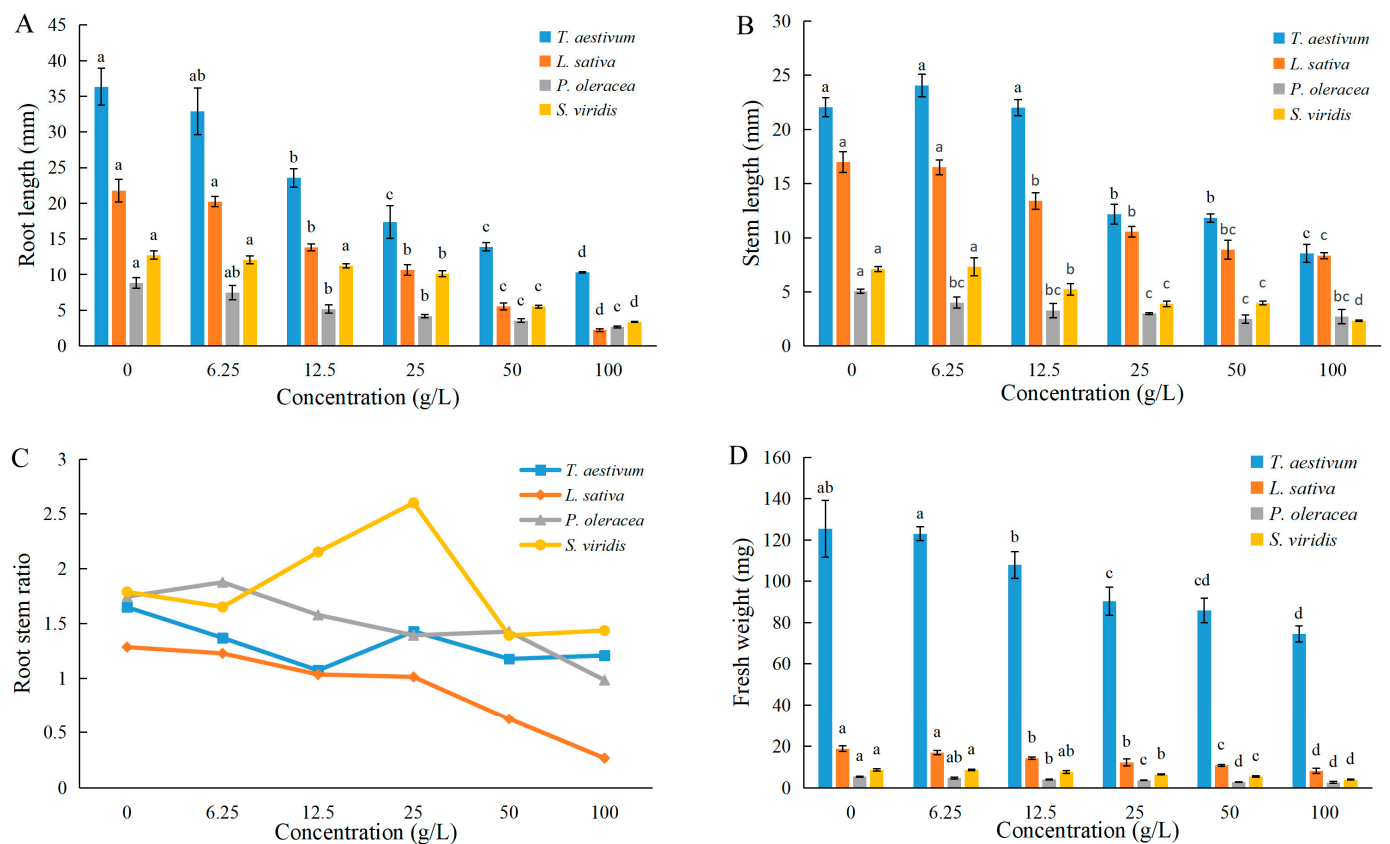


Figure 2. Seedling growth of four plants under treatment with EPF. (A), Root length; (B), Stem length; (C), Root stem ratio; (D), Fresh weight. The results presented are mean of three replicates $\pm$ SE; different letters denote significant differences at $p < 0.05$ according to one-way ANOVA with an LSD test.

As shown in Figure 2B, overall, EPF has inhibitory effects on the seedling stem growth of target plants, although it showed slight promoting effects on the stem length of two monocotyledonous plants, wheat and green bristlegrass at 6.25 g/L. EPF significantly affected the stem length of wheat and green bristlegrass when the concentrations reached 12.5 and 25 g/L, respectively. Meanwhile, the stem lengths of all four plant seedlings were largely reduced by EPF at 50 and 100 μ g/L. EPF showed relatively strong inhibitory effects on the stem growth of wheat, with an IC_{50} value of 53.1 g/L (Table 1).

The root–stem ratio of the plants after treatment represents the degree of influence of EPF on the growth of roots and stems. As shown in Figure 2C, the root–stem ratio of two dicotyledonous plants, lettuce and purslane, showed a downward trend with the increase in treatment concentration. The results indicate that the growth of lettuce and purslane

roots was more sensitive to EPF than that of stems. However, the root–stem ratio of two monocotyledonous plants, wheat and green bristlegrass, did not show regular change characteristics with the increase in the concentration of FPE.

The fresh weight of the four seedlings was gradually decreased with increased concentration of EPF (Figure 2D). The seedlings of dicotyledonous plants lettuce and purslane were more sensitive to EPF, with a significant reduction in biomass under 12.5 g/L. Meanwhile, EPF showed significant inhibitory effects on monocotyledonous plants wheat and green bristlegrass when the concentration reached 25 g/L. Under the high concentrations of 50 and 100 μ M, the fresh weight of all the seedlings was largely reduced. The IC_{50} values of EPF on the fresh weight of four target plants were all more than 90 g/L (Table 1). Overall, EPF at low concentration had no obvious effect on the seedling growth of wheat, lettuce, green bristlegrass and purslane, but, with the increase in the concentration of EPF, it showed an inhibitory effect, and the degree of inhibition was positively correlated with the concentration of EPF, showing a good dose–effect relationship and typical allelopathy characteristics.

3.3. Cell Viability in Root Tips of Lettuce Seedlings after EPF Treatment

As shown in Figure 3, red fluorescence of PI, which represents dead cells, was induced by EPF in lettuce root tips under the concentration of 12.5 g/L. Then, with the increase in EPF concentration, the red fluorescence in the root tips was rapidly enhanced, indicating that the number of dead cells was largely increased.

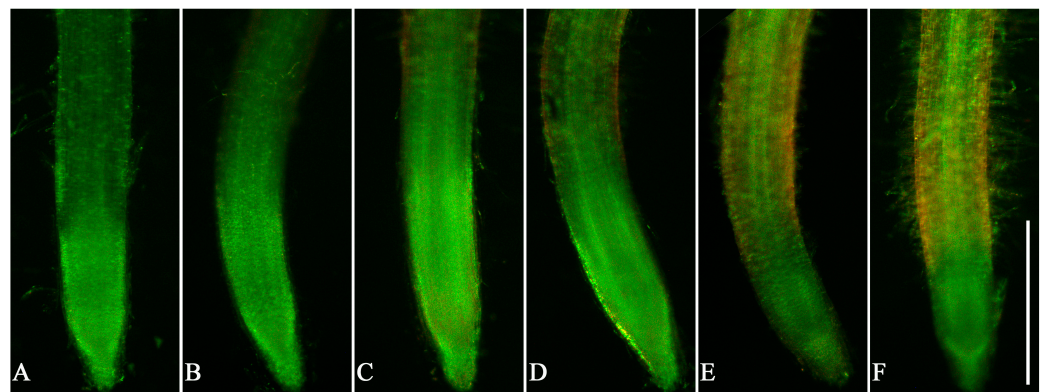


Figure 3. Cell viability in lettuce root tips after treatment with EPF. Lettuce seedlings were treated with EPF at concentrations of (A) 0, (B) 6.25, (C) 12.5, (D) 25, (E) 50 and (F) 100 g/L for 48 h. Roots were stained with FDA/PI. Green fluorescence and red fluorescence indicate viable and dead cells in root tips, respectively. Bar = 500 μ m.

The ratios of dead cells in lettuce root were further quantified by determination of Evans blue uptake. The results show that the relative uptake of Evans blue by lettuce roots at 6.25 g/L of EPF was similar with that of the controls. However, when the concentration reached 12.5 g/L and higher, the relative uptake of Evans blue was significantly higher than that of the controls (Figure 4). The results are in accord with the fluorescence staining experiment.

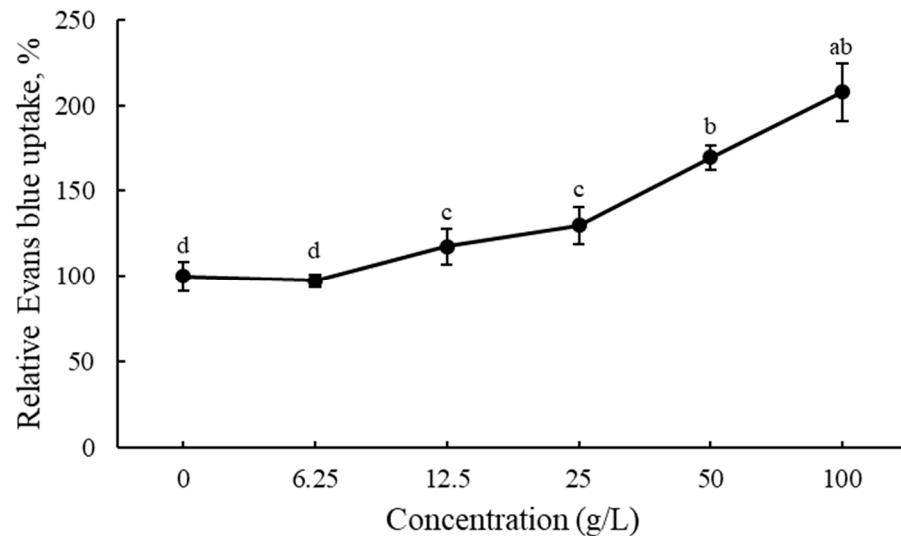


Figure 4. Relative Evans blue uptake of lettuce seedlings after treatment with EPF. The results presented are mean of three replicates $\pm$ SE; different letters denote significant differences at $p < 0.05$ according to one-way ANOVA with an LSD test.

3.4. ROS Production and Lipid Peroxidation in Lettuce Seedlings after Treatment with EPF

After staining with DHE, the bright fluorescence, which mainly represents $O_2^{\cdot-}$, was induced at 6.25 g/L of FPE and enhanced at 12.5 g/L. Then, with the increase in EPF concentration, the fluorescence intensity in lettuce roots was rapidly enhanced. At the highest treated concentration of 100 g/L, there was very strong fluorescence in the treated roots (Figure 5).

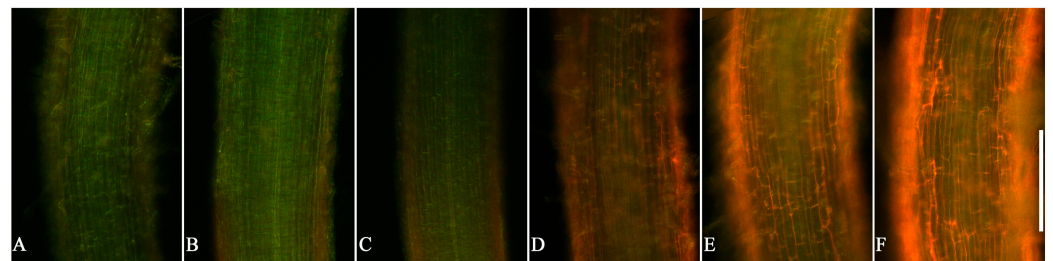


Figure 5. ROS production in lettuce roots after treatment with acacetin. Lettuce seedlings were treated with EPF at concentrations of (A) 0, (B) 6.25, (C) 12.5, (D) 25, (E) 50 and (F) 100 g/L for 48 h. Roots were stained with DHE. Bright fluorescence shows ROS production (presumably $O_2^{\cdot-}$). Bar = 200 μ m.

H_2O_2 is a common ROS that is closely related to oxidative stress in plants. After treatment with EPF, the H_2O_2 content in lettuce was gradually raised with the increased concentration (Figure 6). When the concentration of EPF reached 25 g/L, the content of H_2O_2 was significantly higher than that of the controls.

MDA is the product of lipid peroxidation. The content of MDA in lettuce seedlings under 6.25 g/L of EPF did not increase significantly compared with the control group but reached a significant level at 12.5 g/L and higher. At the highest treated concentration of 100 g/L, the content of MDA increased by 100.92% compared with the control group (Figure 7).

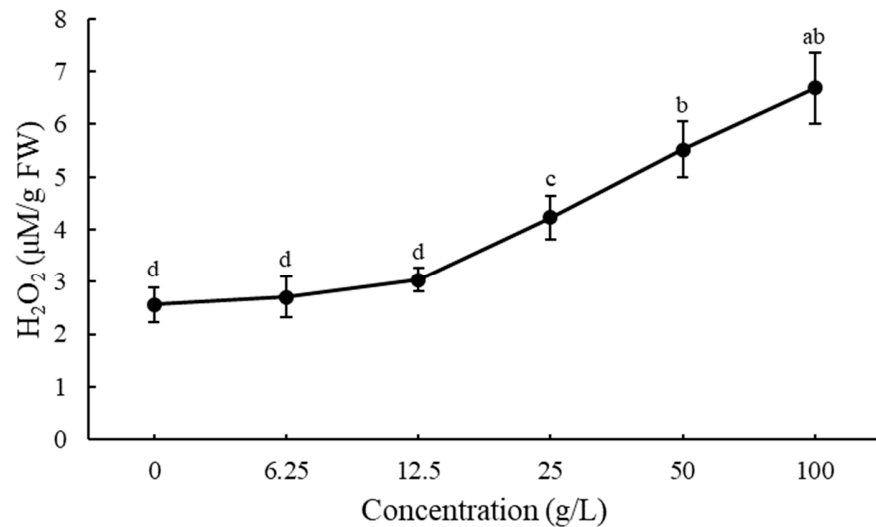


Figure 6. H₂O₂ content in roots of lettuce seedlings after treatment with EPF. The results presented are mean of three replicates $\pm$ SE; different letters denote significant differences at $p < 0.05$ according to one-way ANOVA with an LSD test.

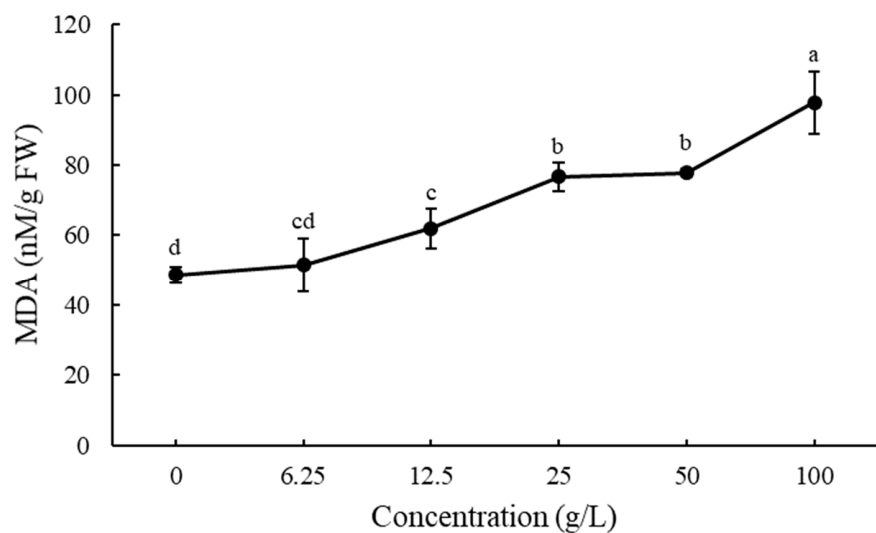


Figure 7. MDA content in roots of lettuce seedlings after treatment with EPF. The results presented are mean of three replicates $\pm$ SE; different letters denote significant differences at $p < 0.05$ according to one-way ANOVA with an LSD test.

3.5. Effect of EPF on Free Proline Accumulation in Lettuce Seedling Roots

The content of free proline is related to the degree of stress suffered by plants. The results show that the content of free proline in lettuce increased significantly at all treatment concentrations in a concentration-dependent manner (Figure 8). These results indicate that exogenous application of EPF had an obvious stress effect on the growth of lettuce seedlings.

3.6. Analysis of Active Compounds in EPF

According to LC-HRMS analysis, 66 secondary metabolites were detected from EPF, including phenolic acids, flavonoids, organic acids and so on (Table 2 and Table S1). There were 24 compounds with the relative amount $\geq 0.5\%$ (Table 2). Among them, nicotinic acid, 7-hydroxycoumarin, citric acid, harpagide, caffeic acid, abscisic acid and apigenin were the most abundant compounds, with more than 5% relative amount of all secondary metabolites in EPF.

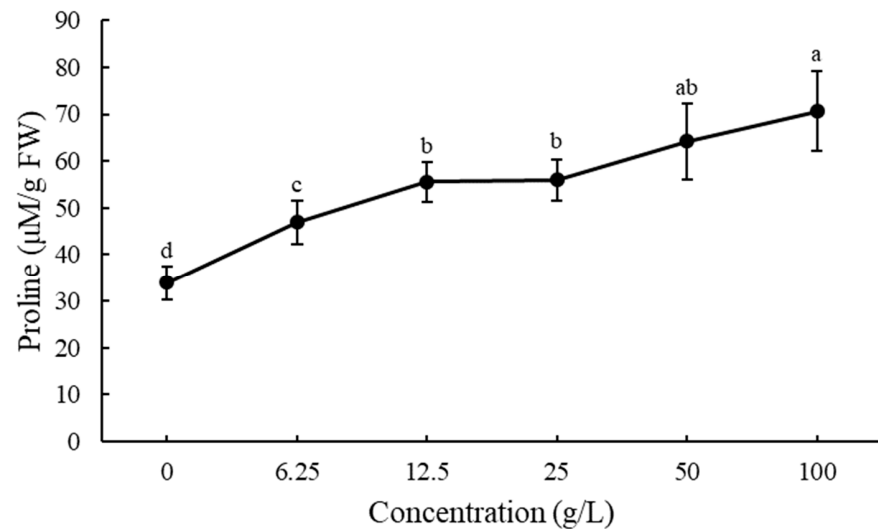


Figure 8. Proline content in roots of lettuce seedlings after treatment with EPF. The results presented are mean of three replicates $\pm$ SE; different letters denote significant differences at $p < 0.05$ according to one-way ANOVA with an LSD test.

Table 2. LC-HRMS identification of main secondary metabolites from EPF.

No.	Proposed Compounds	Molecular Formula	MW	Mass Error (ppm)	Main Fragment MS2	RT (min)	Peak Area (%)
1	2-Pyrrolidinecarboxylic acid	C ₅ H ₉ NO ₂	115.06315	−1.55	70.0652, 68.0496, 98.0599	2.21	2.57
2	Nicotinic acid	C ₆ H ₅ NO ₂	123.0319	−1.04	80.0494, 96.0344, 124.0393	2.23	5.31
3	Quinic acid	C ₇ H ₁₂ O ₆	192.06316	−1.21	173.0088, 111.0086, 191.0195, 85.0293	2.23	0.56
4	Maleic acid	C ₄ H ₄ O ₄	116.01076	−1.7	115.0035, 113.0244, 71.0137, 87.0086	2.26	4.01
5	7-Hydroxycoumarin	C ₉ H ₆ O ₃	162.03147	−1.41	149.0450, 145.0282, 107.0490, 135.0440, 95.0490, 79.0542, 53.0390, 173.0090, 154.9982, 129.0193,	2.32	6.45
6	Citric acid	C ₆ H ₈ O ₇	192.0268	−1.03	111.0086, 87.0086, 85.0293, 67.0188, 57.0344	2.38	14.84
7	Fumaric acid	C ₄ H ₄ O ₄	116.01077	−1.66	71.0138	4.72	1.07
8	Harpagide	C ₁₅ H ₂₄ O ₁₀	364.13657	−1.04	183.0660, 89.0243, 201.0771, 165.0555, 157.0504	21.15	6.82
9	Paeonol	C ₉ H ₁₀ O ₃	166.06284	−0.94	149.0596, 91.0543	21.15	1.65
10	Caffeic acid	C ₉ H ₈ O ₄	180.04207	−1.07	135.0451, 133.0296, 117.0344, 107.0501, 89.0396	23.55	5.16
11	Protocatechuic acid	C ₇ H ₆ O ₄	154.02643	−1.18	109.0293, 91.0188, 80.9650, 65.0396, 81.0344	23.72	1.88
12	Rosarin	C ₂₀ H ₂₈ O ₁₀	428.16767	−1.34	110.0242	25.24	2.30
13	Cinnamic acid	C ₉ H ₈ O ₂	148.0522	−1.54	121.0647, 93.0698, 81.0696, 65.0389, 107.0853	25.56	2.08
14	4-Methoxysalicylic acid	C ₈ H ₈ O ₄	168.04203	−1.38	167.0347, 108.0215	26.29	0.50
15	Azelaic acid	C ₉ H ₁₆ O ₄	188.10461	−1.32	125.0970, 123.0815, 97.0658, 83.0502, 57.0345	26.84	1.11
16	Parthenolide	C ₁₅ H ₂₀ O ₃	248.14088	−1.47	249.1481, 231.1378, 213.1273, 203.1431, 185.1325, 145.1011, 119.0855, 105.0698	26.92	2.46
17	Salicylic acid	C ₇ H ₆ O ₃	138.03152	−1.24	108.0214, 94.0377, 93.0344, 65.0395	27.37	2.70
18	Methylparaben	C ₈ H ₈ O ₃	152.04717	−1.17	153.0546, 125.0597	27.54	0.71
19	Abscissic acid	C ₁₅ H ₂₀ O ₄	264.13569	−1.78	219.1388, 204.1153, 51.0763, 97.0291, 241.0502, 217.0501, 134.0327, 152.0068, 267.0289, 257.0456, 213.0561, 151.0035, 197.0608, 283.0315, 175.0398, 133.0293	28.77	15.64
20	Luteolin	C ₁₅ H ₁₀ O ₆	286.04745	−1	269.0456	29.21	3.99
21	Apigenin	C ₁₅ H ₁₀ O ₅	270.05238	−1.62	299.0558, 284.0323	31.14	9.87
22	Diosmetin	C ₁₆ H ₁₂ O ₆	300.06299	−1.32	300.0271, 271.0242, 163.0025, 151.0035	31.58	0.87
23	Isorhamnetin	C ₁₆ H ₁₂ O ₇	316.058	−0.96	121.1011	31.88	1.04
24	Clareolide	C ₁₆ H ₂₆ O ₂	250.19287	−1.63		33.94	0.76

4. Discussion

Allelopathy is a common phenomenon in both natural and artificial ecosystems, stemming from long-term natural selection of plants and serving as a crucial means for enhancing their viability. It plays an important role in the formation of dominant species, community succession and vegetation restoration [31]. Plant litter is one of the main sources of allelochemicals. When the allelochemicals generated by litter decomposition enter the soil, they will alter the physical and chemical properties of the soil, as well as the composition and structure of microbial communities. These changes can significantly impact the micro-ecological environment, influencing the growth and natural regeneration of surrounding plants. In extreme cases, this process may ultimately lead to community degradation [32]. Consistent with the report that extracts of *Paulownia* spp. leaves and flowers show phytotoxic effects on several target plants [22–24], our results show that EPF could display phytotoxic effects on several target plant species with a good dose–effect relationship, indicating that flower litter of *P. tomentosa* had allelopathy potential on surrounding plants.

Once released into the environment, allelochemicals could inhibit or promote the growth and development of target plants at all stages [33]. The intensity of plant allelopathy is related to the concentration of extract, the types of allelochemicals and the species of tested plants [34]. Seed germination is crucial for species renewal as it ensures the continuation of the population. Any reduction in seed germination rate can substantially undermine the competitiveness of plants and impact their abundance in natural communities, resulting in alterations in community structure and even ecosystem degradation [35,36]. In this study, compared with the germination rate, the germination potential of four test plant seeds was more sensitive to EPF, especially at low concentrations, suggesting that the allelochemicals from EPF showed more significant inhibition on the germination speed of target seeds than the final germination rate. Moreover, EPF displayed stronger inhibitory effects on the seed germination of lettuce and green bristlegrass than wheat and purslane at the same treated concentrations, indicating that the seed germination of the tested plants showed different sensitivity to EPF.

The seedling stage is a crucial phase in plant growth as it represents the period during which plants actively absorb nutrients from the environment to support survival, reproduction and biomass accumulation. The degree of plants being affected by allelochemicals can be evaluated by the morphological changes in both aboveground and underground parts of target plants. Previous studies showed that the water extract of *P. fortunei* leaf litter inhibited the root biomass of wheat seedlings, and the organic solvent extracts of *P. tomentosa* flowers inhibited plant radical growth [22,24]. Our results show that EPF significantly affected the biomass accumulation and inhibited the root and stem growth of test plant seedlings in an obvious concentration-dependent manner. After treatment with EPF, the root–stem ratio of plants' seedlings and the IC_{50} values indicated that the root growth of target plants is more sensitive to EPF compared to stem growth, particularly in the case of dicotyledonous plants lettuce and purslane. These findings indicate that allelochemicals from *P. tomentosa* flowers affected the absorption of nutrients by plants from the environment, thus affecting the seedling growth of the tested plant seedlings. In bioassays, the root system of a target plant is in direct contact with the extracts, so it is inhibited by allelochemicals for a longer time. The leaves transport nutrients by the roots, and, only when the root system is under allelopathic stress to a certain degree, the leaves will show an obvious response [37,38]. The bioassay results indicate that the allelopathy of EPF on four receptor crops was species-selective and concentration-dependent.

Generally, there is a dynamic balance between the production and removal of ROS in plants. Excessive ROS will be produced in plants under stresses, and the activities of protective enzymes will be enhanced or maintained at a high level so that ROS can be removed and maintained at a low level to avoid the damage of free radicals to the structure and function of biofilm [39]. MDA is one of the products resulting from lipid peroxidation within the biofilm system, and its concentration serves as an indicator of both

the membrane lipid peroxidation intensity and the extent of damage to the membrane system [40]. This study showed that the levels of O_2^- and H_2O_2 in lettuce increased under the stress of EPF, indicating that EPF could induce the accumulation of ROS in lettuce. At the same time, the MDA content of lettuce after treatment was higher than that of the control group, suggesting that membrane lipid peroxidation occurred in recipient plants and plant cells were seriously damaged. In addition, the activity of lettuce root tip cells decreased significantly after treatment with EPF, and the number of dead cells gradually increased with the increase in treated concentration. The content of free proline in lettuce increased significantly after EPF treatment, which indicated that exogenous application of EPF had obvious stress effects on the growth of lettuce seedlings. The results are consistent with the effects of allelochemicals such as artemisinin, umbelliferone, daphnoretin, liquiritin and acacetin on lettuce cells [41–44]. Therefore, the allelochemicals from flowers' litter of *P. tomentosa* could induce the accumulation of ROS in target plants, and excessive ROS can lead to cell peroxidation and cell membrane structure damage, which will lead to a decline in cell vitality and even apoptosis, and finally affect the growth and development of target plants.

Up to now, many kinds of allelochemicals have been isolated and identified from various plant species. Previous studies showed that the water and ethanol extracts of *Paulownia* spp. leaf and flower displayed obvious allelopathic activities [22–24]. However, the allelochemicals of *Paulownia* spp. are not yet clear. In this study, 66 compounds were identified from EPF by LC-HRMS, and many of them have been reported to have allelopathic activities. Phenolic compounds are a class of the most important and widespread allelochemicals from many plant species. Phenolic allelochemicals have been observed in both natural and managed ecosystems, where they are related to a range of ecological and economic problems, such as reduced crop yields, regeneration failure in natural forests and replanting problems [45]. In *P. tomentosa* flower litter, phenolic acids salicylic acid, caffeic acid and cinnamic acid were all known allelochemicals with relatively high content and showed significant phytotoxic activities [45]. In addition, citric acid inhibited the seed germination and bud growth of Chinese cabbage and increased the cell membrane permeability of recipient plants [46]. Protocatechuic acid showed phytotoxic effects on seedling growth of *Lepidium sativum* L. [47]. Moreover, salicylic acid, caffeic acid and cinnamic acid could induce the accumulation of ROS in lettuce seedlings, which was related to the phytotoxicity of these compounds [48,49]. The relative concentrations of these phenolic acids were high, suggesting that they may play an important role for the phytotoxic effects of EPF.

Flavonoids are also common allelochemicals in many plant species. Parthenolide reduced seed germination and inhibited seedling growth of wheat, lettuce, radish (*Raphanus sativus* L.) and onion (*Allium cepa* L.) [50]. Pan et al. [51] revealed that 7-hydroxycoumarin had significant inhibition on seedling growth of purslane and lettuce. Purified apigenin inhibited the seed germination of *Arabidopsis thaliana* [52]. Nicotinic acid showed marked inhibitory effects on the colony formation of lettuce cells derived from the protoplasts, indicating that nicotinic acid is toxic in high concentrations for cell division of plant cells [53]. Luteolin displayed inhibitory effects on seed germination and seedling growth regarding wheat [54]. These flavonoids were all found in *Paulownia* spp. flower with high relative concentrations and probably contributed to the phytotoxicity of EPF.

Abscissic acid (ABA), an important phytohormone, has an essential role in multiple physiological processes of plants, including leaf senescence, seed germination and growth inhibition [55]. Up to now, ABA has been well studied in phytophysiology and molecular biology as a phytohormone. In addition, ABA has also been reported as an allelochemical of tall fescue grass (*Festuca arundinacea*) [56]. Under certain conditions, like those in this study, the following flowers of *P. tomentosa* contain high levels of ABA, which could be released to the environment. ABA acts as an allelochemical that could influence the growth of surrounding plants. In addition to phenolic acids, flavonoids and ABA, there may be other compounds in EPF with phytotoxicity, which requires further study.

5. Conclusions

EPF significantly inhibited the seed germination of four test plants. The root and stem growth and biomass of the seedlings were also affected by EPF in a concentration-dependent manner. Especially, the root growth of recipient plants is more sensitive to EPF than stems. Therefore, EPF displayed strong phytotoxic effects on test plants, which were species-selective and concentration-dependent. The EPF treatment led to increases in O_2^{2-} , H_2O_2 , MDA and free proline in lettuce and a decrease in cell viability, indicating that the inhibitory effect of EPF on lettuce growth was related to the accumulation of ROS and the flowing peroxide stress. According to the LC-HRMS results, phenolic acids and flavonoids with high relative concentrations and strong phytotoxic effects are probably the main allelochemicals of *P. tomentosa* flowers. Overall, the results suggest that the flower litter of *P. tomentosa* was an effective source to release allelochemicals, which may play a potential role in the ecological competition between *P. tomentosa* and surrounding plants.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/agronomy14020367/s1>, Table S1: LC-HRMS identification of secondary metabolites with relative amount < 0.5% from EPF.

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