

## Germination-Inhibitory Effect of *Pulsatilla koreana* N. Leaves; Protoanemonin as Active Principle

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**The germination of *Lactuca sativa* seeds was significantly inhibited by the water extract of the fresh leaves of *Pulsatilla koreana* N. including abundant ranunculin. Germination inhibitory activity increased in a dose-dependantly. Protoanemonin, produced from ranunculin by enzymatic action during maceration process of leaves, was proved to be the active principle with inhibitory activity was above 90% at concentration of 1 mg/ml.**

**Key words:** *Pulsatilla koreana* N., *Lactuca sativa*, germination inhibitory activity, ranunculin, protoanemonin

The continuous use of herbicides has caused many serious problems such as the ecosystem destruction, advent of herbicide-resistant plant, and the environmental pollution, although there is no doubt about a chemical weeding method effectively weed controls. Therefore, a sustainable agriculture system is urgently required. Allelopathy, as an important expedient to solving the environmental problems, is presently in the spotlight<sup>1</sup>. It is a strategy for inter-specific as well as intra-specific competition in populations<sup>2</sup>. Some plant species secrete toxic natural substances, interfere with the vital processes of other plants, and inhibit seed germination and seeding growth. Thus, the allelopathic substances could be applied either in the form of a mixture with existing herbicides or as a pure substance to eradicate unwanted plants and to protect the environment from herbicide pollution. Plant species such as rice, Indian millet, alfalfa, cucumber, sweet potato, and bean contain allelopathy materials, which are mainly phenolic compounds, and trace amounts of aldehydes, aromatic acids, flavonoids, alkaloids, terpenoids, and steroids<sup>1,3-5</sup>. However, the weed-removing method using allelopathy effect has not been put to practical use up to now, and related researches are also very restricted to the solvent extracts of plants organ and phenolic compounds and their derivatives. Therefore, demands on finding more effective weed control agents, as well as more effective separation and purification methods of the allelochemicals are increasing.

Therefore, the objective of this study was to evaluate the inhibitory activity of *Pulsatilla koreana* leaves on the germination of *Lactuca sativa* and find the active principle involved in the activity. In Europe, the *Pulsatilla* leaves have

been used for the treatment of neuralgia, migrane, cough, bronchitis, egout, and diuretic, among others<sup>6</sup>.

### Materials and Methods

Ranunculin and protoanemonin were synthesized in our laboratory<sup>7</sup>. The leaves of *P. koreana* were collected from Kuemsan, Chungnam during July, 2002 and refrigerated. *P. koreana* was authenticated by Professor Bae, Chungnam National University, and "The Flora of Korea" of Professor Lee. *L. sativa* seeds (HanKuk JongMyo Inc.) screened to equal sizes, then sterilized with 5% sodium hypochloride solution for 3 min. and rinsed at least three times with distilled water. The solvents used for this investigation were either of first class or special quality. The TLC plate (Kieselgel 60) and the incubator were purchased from Merck (Germany) and Forma Scientific Co., respectively.

Germination inhibitory activities of *n*-hexane, EtOAc, and water extracts obtained from *P. koreana* leaves (Exp-I). The dried and powdered leaves of *P. koreana* (27 g) were extracted with 80% MeOH (300 ml) at room temperature for 6 h, followed by vacuum-filtration. After repeating this procedure twice, the extracts were combined and dried under reduced pressure at 40°C, and a green colored MeOH extract (8 g) was obtained. The MeOH extract was suspended in distilled water (200 ml), then extracted with *n*-hexane (200 ml × 3) and ethyl acetate (200 ml × 3), concentrated, and evaporated to give 1.4 g of yellowish *n*-hexane extract (HX) and 1.7 g of light-brown ethyl acetate extract (EX), respectively. The remaining water layer was dried to yield 4.5 g of water extract (WX) as a dark-brown syrup. Each 1 g solvent extract was diluted in 10 ml of distilled water (Frt A). To observe the concentration dependence, the Frt A was diluted two and ten times with distilled water, giving Frt A × 2 and Frt A × 10, respectively.

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The seeds were put into a filter paper-laid Petri dish (170 mm diameter) and added with 6 ml of the test solution. The dish was then shaken to wet the filter paper evenly. For the control group, 6 ml of distilled water was added to the Petri dish. Twenty-five *L. sativa* seeds were put into each Petri dish containing Frt A, Frt A × 2, and Frt A × 10 samples, and water (control). The Petri dishes were incubated at 25°C, and the germinated embryos were counted every 24 hr for 5 days. The germination percentage of the control (GPC) and the relative germination ratio (RGR) were calculated<sup>8)</sup> by applying the following equations. The experiment was replicated three times.

$$\text{GPC (\%)} = \frac{\text{Number of germinated seed of control}}{\text{Total number of seeds of control}} \times 100$$

$$\text{RGR (\%)} = \frac{\text{Germination percentage of treated group}}{\text{Germination percentage of control}} \times 100$$

Germination inhibitory activity of ranunculin and protoanemonin (Exp-II). The powdered leaves (5 g) were added with 50 ml of water, subjected to Ultrasonic wave shaking for 10 min, and centrifuged for 10 min at 3000 rpm. The resulting water layer was extracted with EtOAc (20 ml × 3), and the remaining water was adjusted to 50 ml with water (WX-1). Subsequently, 25 ml of WX-1 was heated in a microwave heater for approximately 3 minutes to make the plant enzymes inactive and designated as Heat WX-1. Sample A was composed of 4 ml of Wt-1 and 2 ml of water, and sample B was made up of 4 ml of Heat WX-1 and water (Table 2). Sample C is a 6 ml water solution containing 6 mg of ranunculin. Then, 2 ml of

sample C was added into samples A and B to obtain samples D and E, respectively. Finally, to compare the germination inhibitory effects of ranunculin and protoanemonin, sample F was prepared by dissolving 6 mg of protoanemonin in 6 ml of water. Water (6 ml) was used as the control group, and GPC and RGR values were determined using the same calculation method of Exp-I.

## Results and Discussion

Through continuous observation, we observed an allelopathic effect by crushing *P. koreana* leaf tissues against some plant seeds. To obtain more concrete evidence, the germination inhibitory activities of *n*-hexane (HX), ethyl acetate (EX), and water (WX) extracts of *P. koreana* leaves against *L. sativa* seeds were measured (Exp-I). Among of them, WX showed the strongest inhibition of germination, and the rest did not show any activity (Table 1). Moreover, the inhibitory activity of the WX showed concentration-dependency based on the observation that the inhibitory effect disappeared after a tenfold dilution. In addition, the germination inhibitory substance extracted from the *P. koreana* leaves had high polarity and good solubility in water, which strongly suggested a high probability of the presence of glycoside featuring high polarity. Therefore, the leaves of *P. koreana* were examined to confirm the presence of a glycoside, and to determine whether the substance responsible for the inhibitory activity was the secondary substance generated from the enzymatic reaction or the innate substance within the leaves, as well as to pursue the active principle (Exp-II). As described

**Table 1. Relative germination ratio (RGR) of *n*-hexane (HX), ethyl acetate (EX), and water (WX) extracts obtained from *P. koreana* leaves in the Exp-I**

	Group <sup>a</sup>	RGR <sup>c</sup>		
		Frt A <sup>b</sup> (0.1 mg/ml)	Frt A×2 <sup>c</sup> (0.05 mg/ml)	Frt A×10 <sup>d</sup> (0.01 mg/ml)
Exp-I	HX	+++	+++	+++
	EX	+++	+++	+++
	WX	---	+	+++

<sup>a</sup>*n*-Hexane (HX), ethyl acetate (EX), and water (WX) extracts of *P. koreana* leaves was grouped.

<sup>b-d</sup>Each 1 g solvent extract was diluted in 10 ml of distilled water (Frt A). The Frt A was diluted two and ten times with distilled water, giving Frt A×2 and Frt A×10, respectively.

<sup>c</sup>(%): > 90; +++, 90-70; ++, 70-50; +, 50-30; -, 30-10; --, < 10; ---.

**Table 2. Manufacturing conditions and relative germination ratio (RGR) of samples A-H in the Exp-II**

	Sample	Condition	RGR <sup>c</sup>
Exp-II	A	WX-1 <sup>a</sup> 4 ml + water 2 ml	---
	B	Heat WX-1 <sup>b</sup> 4 ml + water 2 ml	+++
	C	Ranunculin 6 mg + water 6 ml (1 mg/ml)	+++
	D	WX-1 2 ml + sample C 2 ml + water 2 ml	--
	E	Heat WX-1 2 ml + sample C 2 ml + water 2 ml	+++
	F	Protoanemonin 6 mg + water 6 ml (1 mg/ml)	---

<sup>a</sup>The water extract of *P. koreana* leaves with the lipophilic or middle polar materials discarded.

<sup>b</sup>The water extract of *P. koreana* leaves heated in the microwave for 3 min to destroy the plant enzymes.

<sup>c</sup>RGR (%): > 90; +++, 90-70; ++, 70-50; +, 50-30; -, 30-10; --, < 10; ---.

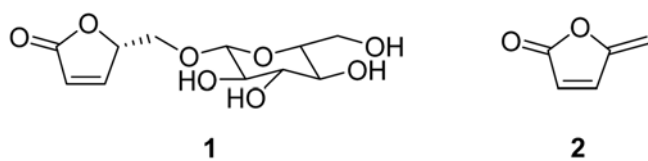


Fig. 1. Structures of ranunculin (1) and protoanemonin (2).

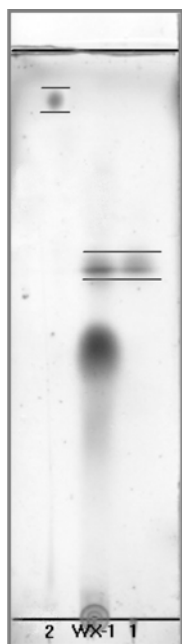


Fig. 2. Comparison of  $R_f$  values of protoanemonin (2,  $R_f=0.98$ ), the water extract (WX-1) of *P. koreana* leaves, and ranunculin (1,  $R_f=0.61$ ) on thin layer chromatography (Kieselgel, eluting solvent;  $\text{CHCl}_3\text{-MeOH-H}_2\text{O}=3:3:1$ , sprayed with 10%  $\text{H}_2\text{SO}_4$  and heated).

in Table 2, sample B did not have any germination inhibitory activity (RGR: >95%), whereas sample A showed a very strong inhibition activity against the germination of *L. sativa* seeds. This result suggested that the substance responsible for the germination inhibitory effect could be directly related to the enzymatic reactions. In other words, the inhibitory substance was generated by the enzymatic reaction. Extensive studies have been conducted with regard to the material known as ranunculin, 1 (Fig. 1), which showed a variety of physiological activities and DNA-polymerase inhibition activities, which affect the cellular function<sup>9-10</sup>. The  $R_f$  value of water extract of *P. koreana* leaves (WX-1) and ranunculin were compared on TLC (Fig. 2). Figure 2 shows the extract was composed of a significant amount of ranunculin. Therefore, to estimate the inhibitory activity of ranunculin,

sample C was prepared by dissolving 6 mg of ranunculin synthesized in our laboratory with 6 ml of water. Samples D and E were prepared with sample C, WX-1, and Heat WX-1 (Table 2), and their inhibitory activity against germination were measured. Samples C and E, which were composed of ranunculin, had 22-24 units out of total 25 stocks displaying signs of germination (RGR: 88-96%, Table 2). On the other hand, sample D composed of an active enzyme containing WX-1 demonstrated stronger germination inhibitory activity compared to sample C, even though sample D had 1/3 of ranunculin compared to sample C (RGR: 16-21%). Considering these results, the enzymatic reactions play an essential role in exhibiting the germination inhibitory activity of ranunculin within the plant tissues. Yili *et al.* proposed that ranunculin is autolytically released as a toxin or during the formation of protoanemonin by  $\beta$ -glucosidase (EC 3.2.1.21) within the plant<sup>11</sup>. The lactone protoanemonin, 5-methylene-2-oxodihydrofuran, 2 (Fig. 1) is the irritant oil responsible for the vesicant properties attributed to the members of the buttercup family including species of *Helleborus*, *Anemone*, *Clematis*, *Ceratocephalus*, and most commonly *Ranunculus*. The oil is derived by autolysis during maceration of fresh plant tissue and produces an intense burning sensation when chewed or erythema, dermatitis, and blistering when applied to the skin<sup>12</sup>. It also has antifungal and antibiotic properties<sup>13-14</sup>. Protoanemonin (6 mg) was added to prepare sample F. Sample F displayed a very strong inhibition activity with RGR values ranging from 4 to 8% unlike sample C, which was based on ranunculin.

In conclusion, ranunculin in the water extract of *P. koreana* leaves automatically generated protoanemonin and this secondary substance demonstrated more than 90% inhibition of germination against *L. sativa* at concentration of 1 mg/ml, suggesting that protoanemonin is one of the active principle of the allelopathy substances, which were generated by the *P. koreana* to prevent the invasion of other plants. This discovery of protoanemonin which was an independent substance embedded with the allelopathy characteristic and could be a very meaning outcome of this research. A more concrete and systematical research on protoanemonin and the development of the natural herbicides applying these *P. koreana* leaves are highly recommended.

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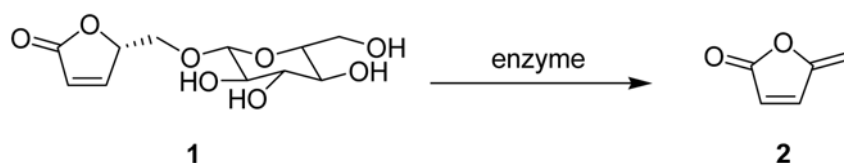


Fig. 3. Biosynthetic process of protoanemonin (2) from ranunculin (1) by ranunculase.

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