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Article

Allelopathic Potential and Mechanism of Rosebay Willowherb [*Chamaenerion angustifolium* (L.) Scop.] Demonstrated on Model Plant Lettuce

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Abstract: Allelopathic plants are important resources for the discovery of bioherbicides. Rosebay willowherb [*Chamaenerion angustifolium* (L.) Scop. syn. *Epilobium angustifolium* L.] widely distributes in Western Asia, Europe, and North America, and behaves as a dominant species within the community due to the production of substances that restrict growth of other plants. This study aims at investigating the allelopathic potential of rosebay willowherb by evaluation of the effects of aqueous extracts from different parts on seed germination and seedling growth in lettuce (*Lactuca sativa* L.), as well as measuring the accumulation of reactive oxygen species and structural analysis of root tips via scanning electron and transmission electron microscopy. It was observed that the aqueous extracts from the leaves of rosebay willowherb had the strongest inhibitory effect on the germination index, germination energy and total germination of lettuce seeds, followed by capsular fruits and flowers, and the inhibition effect of stems was the weakest. All aqueous extracts (100 mg/mL) showed a significant inhibitory effect on radicle elongation of lettuce seedlings. Additionally, after treatment with the aqueous extract of rosebay willowherb leaves, accumulation of reactive oxygen species increased in columella cells, which correlated with disruption of root tip structure.

Keywords: *Chamaenerion angustifolium* (L.) Scop.; phytotoxicity; seed germination; seedling growth; morphology; reactive oxygen species

1 Introduction

Weeds are plants that are not valued where they are growing, at least from human being point of view, and are usually of vigorous growth, competing with crops for sunlight, space, water, and nutrients. In order to maximize the production and reduce the economic losses, chemosynthesis herbicides have been used extensively in modern day agricultural systems to prevent and control damages caused by weeds [1,2]. However, the massive and continuous application of herbicides results in environmental pollution, ecological disturbance, human health problems, and resistance to the bioactive products [3–5]. Scientific evidences have revealed that pesticide residues are closely related to human diseases, such as formation of tumors, reproductive and neurodegenerative disorders, and other chronic diseases, which have become a major international community issue [6–9]. Among the approaches of weed control developed and used in recent years, the development of botanical herbicides is promising, owing its diverse active ingredients, specific mechanisms of action, overcoming the acquired resistance of chemical herbicides in weed control, and the safety to non-target organisms such as humans and animals. [10]. The fact that plants, microorganisms, viruses and fungi can harmfully or beneficially affect the growth and development of natural and agricultural ecosystems by releasing secondary metabolites into the environment was defined as allelopathy by the International Allelopathy Society [11]. With the deep-going understanding of the properties of allelopathic plants, increasing attention has been paid to utilize their antagonistic potential for the control of weeds.

Rosebay willowherb [*Chamaenerion angustifolium* (L.) Scop. syn. *Epilobium angustifolium* L.], a perennial herbaceous plant belonging to Onagraceae family, is widely distributed in Western Asia, Europe, and North America [12]. As a traditional folk medicine, it was used to treat gastrointestinal tract, prostate, kidney and urinary tract, and skin and mucosa diseases [13]. Many preparations containing rosebay willowherb extracts are sold in the European market and are considered to be effective drugs for the treatment of prostate diseases [14]. Pharmacological studies have demonstrated its anti-inflammatory, antimicrobial, antioxidant, and immunomodulatory properties [13]. According to a previous study, polyphenols are main chemical constituents of rosebay willowherb among which oenothein B (tannin), quercetin 3-O-arabinoside (flavonoid), and gallic acid and ellagic acid (phenolic acids) are potential bioactive constituents [13,15].

In the general survey of the plant resources of Huitengxile grassland in Xilingol district, Inner Mongolia of China, researchers found that other plants are difficult to grow in the community with rosebay willowherb, which behaves as the dominant species, and whether this natural phenomenon is related to allelopathy remains to be demonstrated. Based on this point, the effects of aqueous extracts from different parts of rosebay willowherb on seed germination and seedling growth in lettuce (*Lactuca sativa* L.) were tested, which supports its use as a botanical herbicide. Then, by measuring the accumulation of reactive oxygen species (ROS) and assessing structural changes in root tips via scanning electron and transmission electron microscopy, the phytotoxic effects of the aqueous extract of rosebay willowherb leaves could be demonstrated in the present study.

2 Materials and Methods

2.1 Plant Materials

The aerial parts of rosebay willowherb were collected from Xilingol district (N 43° 46′, E 116° 11′), Inner Mongolia of China, in August 2016, and authenticated by Prof. Zhiduo Niu, Jilin Academy of Chinese Medicine Sciences, China. A voucher specimen (CA-LL-201608001) was deposited in the Department of Natural Medicine and Pharmacognosy, School of Pharmacy, Qingdao University, China. The samples were separated into four different parts (stems, leaves, flowers, and capsular fruits) and air dried, chopped into fine powder, respectively. Seeds of *Lactuca sativa* L. var. *romana* Hort. (lettuce) were purchased from Sichuan Zhongdu Seed Company (Sichuan, China).

2.2 Preparation of the Aqueous Extracts

The aqueous extracts of willowherb were prepared as previously described with some modifications [16]. The air-dried powders (10 g) of plant materials were respectively extracted by soaking in 100 mL distilled water on a rotary shaker for 24 h, with the conditions of 20°C and 150 rpm. Afterward, the extraction solutions were filtered through Whatman No. 1 filter paper to remove the plant debris and yield four aqueous solutions with concentration of 10 g dry tissue / 100 mL water (100 mg/mL, 10% *w/v*). Several solutions at 33, 10, 3, and 1 mg/mL were also prepared by subsequent dilutions with sterilized distilled water. The distilled water was used as a control in the bioassay procedure. All solutions were stored in the refrigerator at 4°C.

2.3 Evaluation of Phytotoxic Activity on Seed Germination

The seed germination test was performed by the plate-culture method according to the literature [16]. In brief, the test lettuce seeds were surfaced-sterilized by immersion in 10% sodium hypochlorite (Shuangshuang Chemical Company, Yantai, China) solution (*w*/*v*) for 10 min, rinsed three times with distilled water and then sown on filter paper in sterilized Petri dishes (90 mm in diameter), each containing 30 seeds and receiving 5 ml of the above aqueous extracts. All dishes were cultured in a dark growth chamber kept at 20 ± 1°C, 75 ± 2% relative humidity. Distilled water was used as a control. Three replicates were performed for each treatment. Seeds were deemed germination until the radicle length was at least 1 mm. The germination count was recorded at 48 h and used to calculate the final germination rate, also known as total germination. The germination potential was recorded on 24 h. Germination index was calculated according to the following relationship [17]: germination index = $\sum(G_i/N_i)$, where G_i is the number of germinated seeds in time interval, and N_i is the number of test times after sowing. Number of seeds germination was monitored at 8 h intervals over a 2-day period to obtain germination index.

2.4 Evaluation of Phytotoxic Activity on Seedling Growth

The seedling growth test was conducted using the plate-culture method as previously described [16]. 20 germinated seedlings were placed into the Petri dishes on filter paper and 5 mL of different concentrations of aqueous extracts were added to each well. After being incubated in a constant-temperature humidity chamber kept 20 ± 1°C, 75 ± 2% relative humidity and a 12 h / 12 h dark/light photoperiod for 5 days, the radicle length of lettuce seedlings were measured. Three replicates were performed for each treatment.

2.5 Morphological Examination

The samples for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were processed according to the literature [18]. In brief, root tips (0.5 cm) were excised from lettuce seedlings after treatment with 10 and 100 mg/mL aqueous extracts, and fixed for 4 h at 4°C in 2.5% glutaraldehyde solution. The solution was discarded by washing with 0.1 M phosphate buffer three times. Then 1.5% potassium permanganate solution was added to fix for 3 h. The fixed seedlings were washed in 0.1 M phosphate buffer and dehydrated through a graded series of ethanol aqueous. The selected dehydrated seedlings were rinsed with isoamyl acetate solution followed by critical point dried for examination by SEM (JSM-840, JEOL, Tokyo, Japan). Additionally, the rest of dehydrated seedlings were rinsed with acetone and embedded in Embed 812. Ultra-thin sections (70 nm) were cut with an ultramicrotome (Ultracut, Reichert-Jung, Austria), and then stained with 3% uranyl acetate in ethanol for examination by TEM (JEM1200, JEOL, Tokyo, Japan).

2.6 ROS Production

ROS production was measured according to the literature with slight modifications [19]. 2′,7′-Dichlorofluorescein diacetate (DCF-DA, Aladdin Chemical Reagent Company, Shanghai, China) was applied to label the ROS. Treated seedlings were washed 5 times with distilled water and dyed for 15 min in the dark using DCF-DA (20 μM, 1% dimethyl sulfoxide, Aibi Chemical Reagent Company, Shanghai, China). Then the dye residues were washed off in water, and the root tips were carefully excised to be viewed using the fluorescence microscope (Nikon A1R MP, Tokyo, Japan) with excitation wavelength 488 nm and emission wavelength 520 nm.

2.7 Statistical Analysis

Fisher's least significant different test and one-way analysis of variance (ANOVA) were used to mark the significant differences between the tested group and the control group via SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). The allelopathic reaction index (RI) of each indicator was adopted to analyze the allelopathy of aqueous extracts from different parts of rosebay willowherb on seedling growth using the following relationships [20]: RI = 1−C/T (when T ≥ C) and RI = T/C−1 (when T < C). RI refers to the extent or intensity of allelopathy, T stands for the treatment value, and C refers to the control value for a particular investigated parameter. A positive RI value refers to stimulatory effect and vice versa.

3 Results and Discussion

3.1 Phytotoxic Effects of Crude Extracts from Rosebay Willowherb on Lettuce Seed Germination

Lettuce (*Lactuca sativa* L.), has been widely used to investigate the allelopathic potential of plants by biological monitoring in laboratory, owing to its fast germination and high uniformity for growth. In the present study, the effects of crude extracts from stems, leaves, flowers, and capsular fruits of rosebay willowherb were evaluated on the germination of lettuce. Results revealed that all the aqueous extracts from different parts of rosebay willowherb have significant effects on germination rate, germination potential, and germination index of lettuce at higher concentrations. As shown in Fig. 1, the germination rate of all the aqueous extracts from stems, flowers, and capsular fruits of rosebay willowherb were significantly decreased compared to control samples ($p < 0.01$) in the concentration of 100 mg/mL (100 mg dry weight of plant material equivalent aqueous extract/mL, same below), while there is no statistical significance at concentrations of 1, 3, 10, and 33 mg/mL. However, aqueous extract of leaves with 33 and 100 mg/mL showed significant phytotoxic effect on lettuce seed germination. It was surprising that the inhibition ratio of seed germination was up to 100% when treated with 100 mg/mL aqueous extracts of flowers and leaves. The germination potential and germination index, indicating the speed of germination and seed uniformity, are important parameters of seed vigor. In the present study, results showed that both

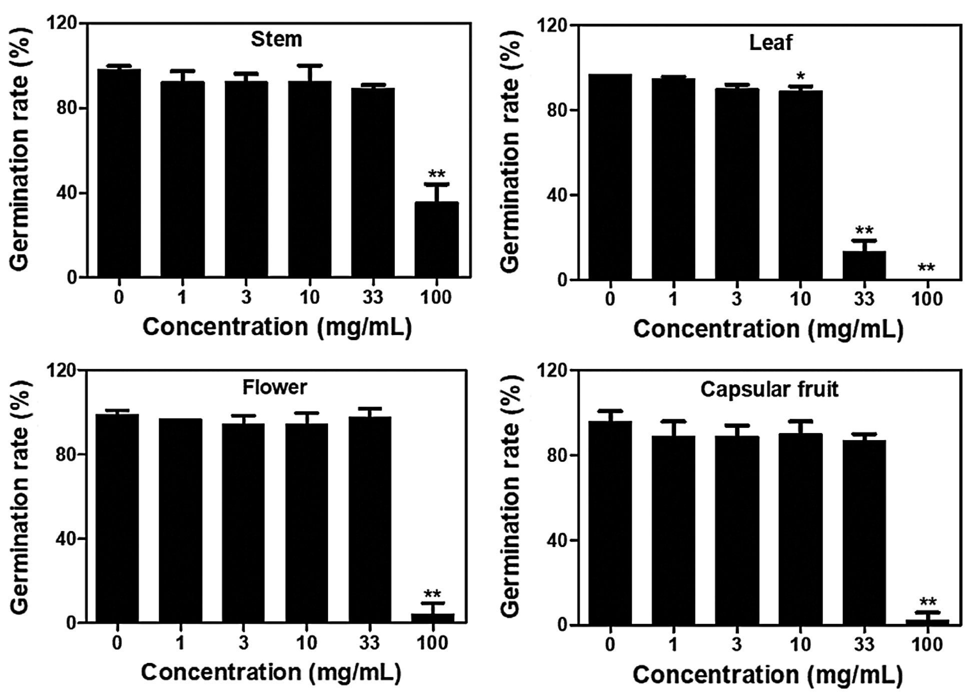

Figure 1: Phytotoxic effect of aqueous extracts from different parts of rosebay willowherb on germination rate of lettuce seeds. Values are expressed as a percentage of the mean compared to 30 seeds. Error bars are one standard deviation of the mean ($N = 3$). Means that significantly differ from the controls are indicated with an asterisk * (one way ANOVA; $p < 0.05$) or two asterisks ** ($p < 0.01$)

the germination potential and germination index were significantly decreased compared to controls ($p < 0.01$) at concentrations of 33 and 100 mg/mL of all the aqueous extracts of rosebay willowherb (Figs. 2 and 3). All the variables declined with increased treatment concentrations indicating the dose-suppression effects are evident in both germination potential and germination index. Indeed, of the four parts of rosebay willowherb treatments tested in this study, the strongest inhibition of germination rate, germination potential, and germination index were caused by aqueous extracts of leaves at higher concentrations (33 and 100 mg/mL) compared to the controls. Comparative results indicated that the germination of lettuce seeds was delayed at higher concentrations of all crude extracts compared to lower concentrations, especially exposed to aqueous extract from leaves of rosebay willowherb.

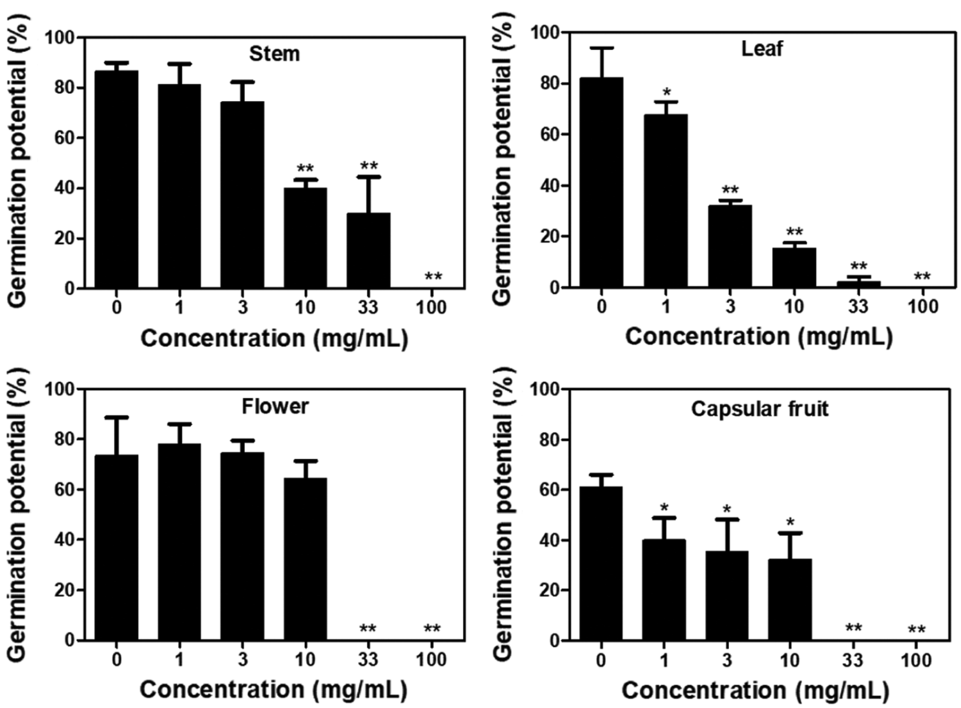

Figure 2: Phytotoxic effect of aqueous extracts from different parts of rosebay willowherb on germination potential of lettuce seeds. Values are expressed as a percentage of the mean compared to 30 seeds. Error bars are one standard deviation of the mean ($N = 3$). Means that significantly differ from the controls are indicated with an asterisk * (one way ANOVA; $p < 0.05$) or two asterisks ** ($p < 0.01$)

Seed germination is a key step in the propagation and cultivation of most crop species. Since seeds are most sensitive to allelochemicals, bioassay of seed germination has been widely used in allelopathy research. For example, the allelopathic effects of *Luetzelburgia auriculata* on seed germination of lettuce were investigated, and the results indicated that aqueous extracts of leaves and roots caused phytotoxic effects on germination index and total germination [21]. Carvalho et al. reported that the leaf ethanol extracts from five *Amaranthus* species (*A. spinosum*, *A. viridis*, *A. deflexus*, *A. hybridus*, and *A. retroflexus*) inhibit the germination rate and germination speed index of lettuce seeds in a dose-dependent manner [22]. Bravetti et al. [23] reported that *Cortaderia speciosa* extract has phytotoxic effects against mono- and dicotyledonous species, and enabled the isolation of six phenolic acids contributing to the inhibition of germination. These studies provide a viable strategy for weed management.

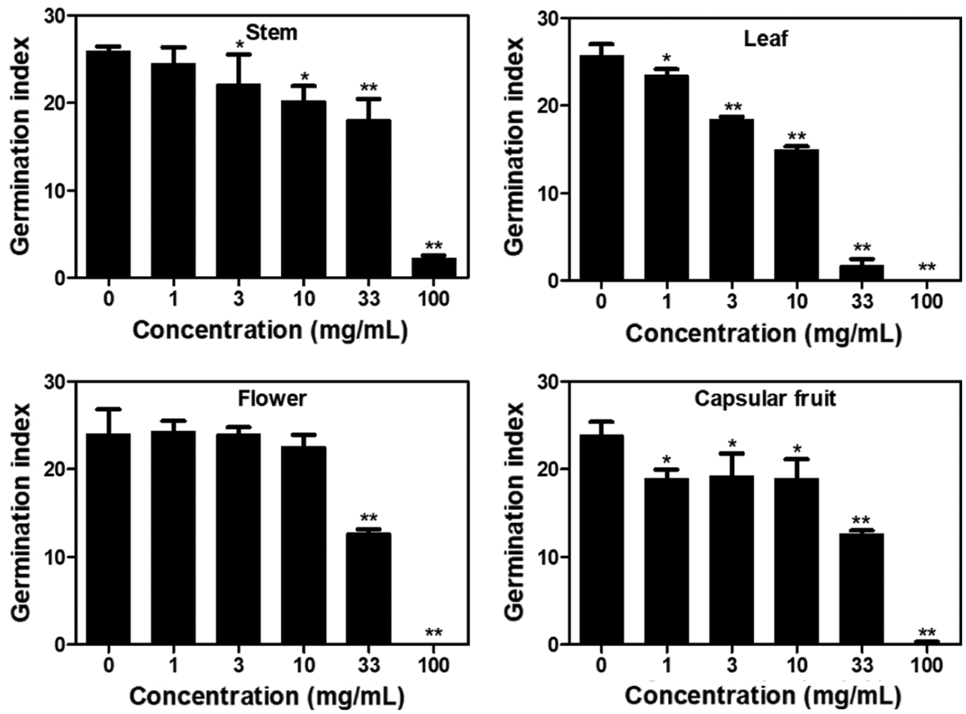

Figure 3: Phytotoxic effect of aqueous extracts from different parts of rosebay willowherb on germination index of lettuce seeds. Values are expressed as a percentage of the mean compared to 30 seeds. Error bars are one standard deviation of the mean ($N = 3$). Means that significantly differ from the controls are indicated with an asterisk * (one way ANOVA; $p < 0.05$) or two asterisks ** ($p < 0.01$)

3.2 Phytotoxic Effects of Crude Extracts from Rosebay Willowherb on Lettuce Seedling Growth

Our results evidenced a high correlation between the presence of crude extracts from rosebay willowherb (stems, leaves, flowers, and capsular fruits) and the seedling growth inhibition of lettuce. Different parts of rosebay willowherb showed phytotoxic effects on radicle elongation of lettuce (Fig. 4). The flower extracts showed dose-dependent influence of growth, with low-promoting or high-inhibiting effects. The radicle length of lettuce was increased by 19.8% and 15.1% following flower extract treatments of 1 and 3 mg/mL ($p < 0.01$), while significantly decreased by 39.9%, 93.6%, and 100% compared to the control ($p < 0.01$) when subjected to 10, 33, and 100 mg/mL, respectively. In contrast, the stems, leaves, and capsular fruits extracts decreased the radicle length of lettuce at different degrees, and thus showed only inhibitory effects (Fig. 4). Higher concentrations exerted much stronger suppression. The most significant reductions were observed for radicle growth in lettuce in the presence of leaves extracts (100%) compared to stems (91.7%) and capsular fruits (97.2%) at concentration of 100 mg/mL.

Recently, considering allelopathy as an ecological approach to manage weeds, crude extracts and natural compounds have been evaluated for phytotoxic effects on different bioindicator species by examining seedling growth. For example, two phenolic derivatives (syringic acid and methyl syringate) isolated from *Schumannianthus dichotomus* were reported to inhibit shoot and root growth of cress and Italian ryegrass in a concentration-dependent manner [24]. Besides, extracts from medicinal plants [25,26], ornamental plants [27], invasive plants [16], fireweed [22,28], and seaweed [29], rich in biological active compounds, have potential as bioherbicides.

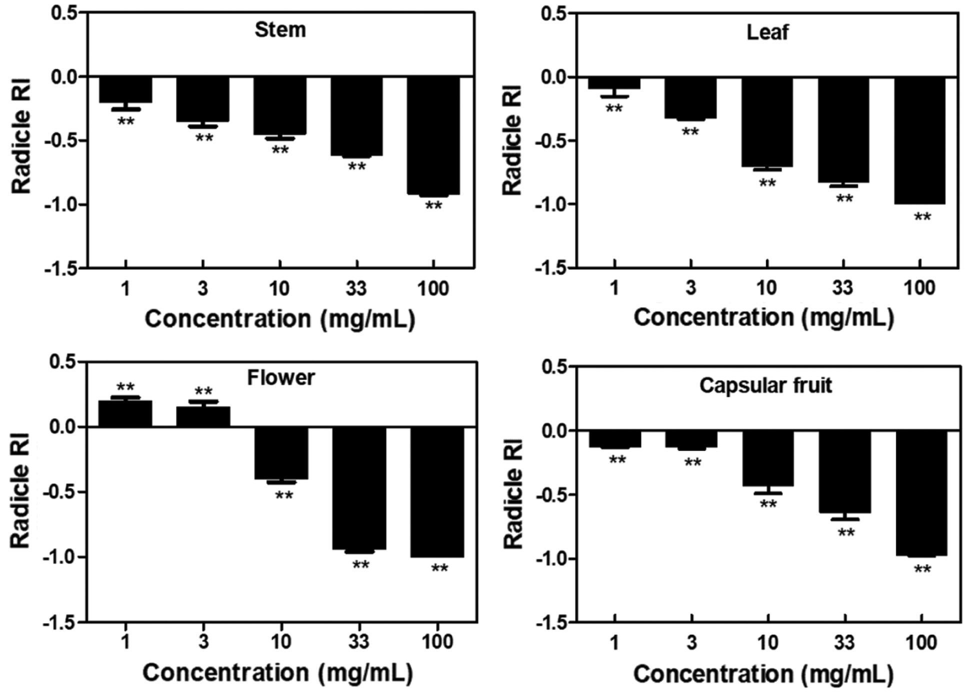

Figure 4: Allelopathic reaction index of aqueous extracts from different parts of rosebay willowherb on radicle growth of lettuce seeds. Values are expressed as a percentage of the mean compared to the control. Error bars are one standard deviation of the mean ($N = 3$). Two asterisks ** represent statistically significant differences between the treated lettuce seedlings and the control at $p < 0.01$

In the last few decades, various polyphenols including gallic acid, ellagic acid, caffeic acid, chlorogenic acid, rutin, quercetin, and oenothein B were reported to be main phytochemicals in rosebay willowherb [13,15]. Many studies have proved that these compounds were among the allelochemicals commonly cited as being responsible for their allelopathic activity. For example, gallic acid, verified in tea, fruits and several plants, was shown allelopathic effect on lettuce seeds when concentration was greater than 512 μg/mL [30]. Besides, gallic acid was toxic to cowpea, sorghum, and microalgaes [31–33]. Quercetin, isolated from aqueous extract of *Pulicaria odora*, show a strong allelopathic effect on the seed germination and seedling growth of *Medicago sativa* and *M. sativa subspfalcata* [34]. Chlorogenic acid displays significant inhibitory effects on root length and fresh weight of lettuce at concentrations of 10 μmol/L and more, and the phytotoxic mechanism was related to ROS accumulation [35]. Thus, in the present study the allelopathic activity of aqueous extracts from rosebay willowherb may be due to these polyphenols.

3.3 Morphological Analysis

The morphology of lettuce root tips was analyzed by electron microscopy using SEM and TEM. Representative photographs of the effects of different extracts from rosebay willowherb on growth of lettuce seedlings are shown in Fig. 5. As described above, higher concentrations of rosebay willowherb extracts resulted in serious disorders in lettuce seedling growth, and the leaf extract exhibited the most remarkable effect. At concentration of 100 mg/mL a large number of lettuce seedlings died and decayed after 5 days of germination. The SEM images (Fig. 5A) showed that intact cells were regularly arranged in root tips in control groups; while with 100 mg/mL of leaf extract treatment, the root tips were strongly damaged, ruptured, and large spaces appeared between cells. Ultra-structure analysis through TEM

(A)

(B)

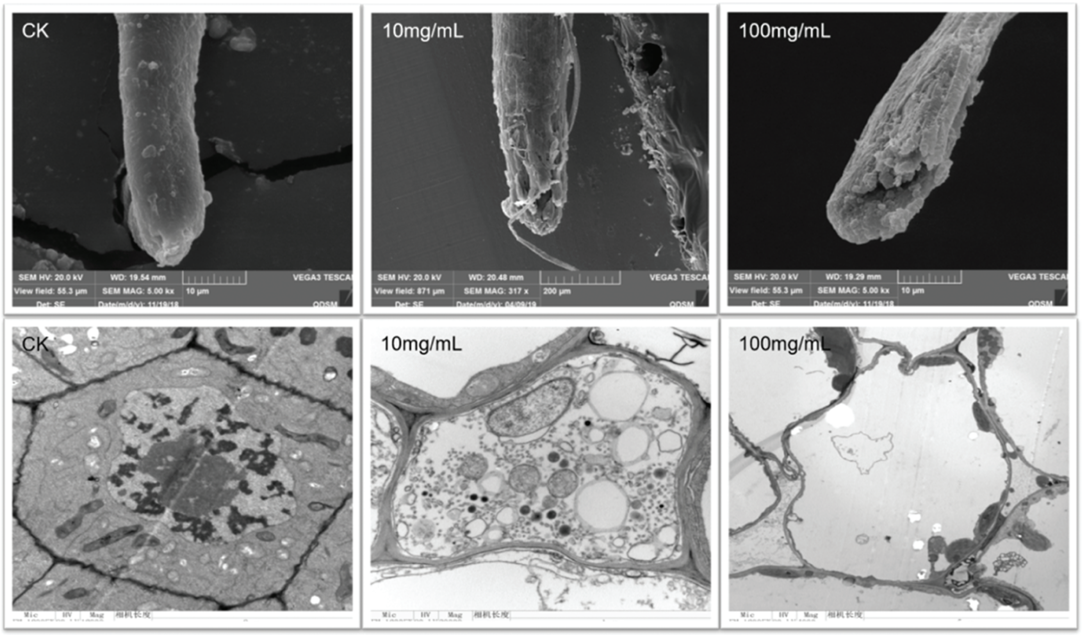

Figure 5: Representative scanning electron microscope images of lettuce radicle surface (A) and transmission electron microscope images of the ultrastructure of lettuce radicle cells (B) under control and treatment with 10 and 100 mg dry weight equivalent aqueous extracts of rosebay willowherb leaves/mL

(Fig. 5B) indicated a normal cell structure, with clearly visible cell membrane, nuclei and organelles in the control; while treatment with 100 mg/mL of leaf extract, cell damage was observed, including cell membrane shrinkage, appearance of vacuoles, and disruption of organelles.

Electron microscopy is an important tool in revealing the ultrastructural variations induced by allelochemicals. In previous research, of the effects of phytotoxic extracts from peach root bark, TEM showed that most of organelles were destroyed, which was accompanied by cell deformation, and nucleolar dysfunction in the root tips when treated with ethanol-based peach root extracts [36]. Another study showed the ultrastructural variations that included the rupturing and shrinking of cells along epidermis in cassia leaf through SEM [37]. Thus, imaging of the root tips clearly evidenced the phytotoxic nature of rosebay willowherb extracts.

3.4 Effect of Crude Extracts from Rosebay Willowherb on ROS Accumulation

The ROS levels in the root tips of lettuce seedlings treated or not with leaf extracts of rosebay willowherb were analyzed by DCF-DA staining. The effect of leaf extracts on ROS production is shown in Fig. 6. At low concentration (1 mg/mL), fluorescence images showed slight fluorescence and the intensity of fluorescence increased remarkably in roots treated with the highest concentrations (10 and 33 mg/mL), compared to the control. At 100 mg/mL of the processing group, the structure of radicle tips were ruptured, resulting in death without detection of fluorescence.

ROS comprise a kind of oxygen-containing compounds with high biological activity produced by intracellular aerobic metabolism or exogenous oxidants, including superoxide anion, hydrogen peroxide, hydroxyl radical, and nitric oxide. Plants can produce ROS under the oxidative stress, led to cell membrane peroxidation damage, protein and lipid destruction, and restrained antioxidant system of receptor plants, which affect the cell vitality, and may cause cell apoptosis [38]. Oxidative damage contributed strongly to the morphological changes mentioned above and the growth deficit observed

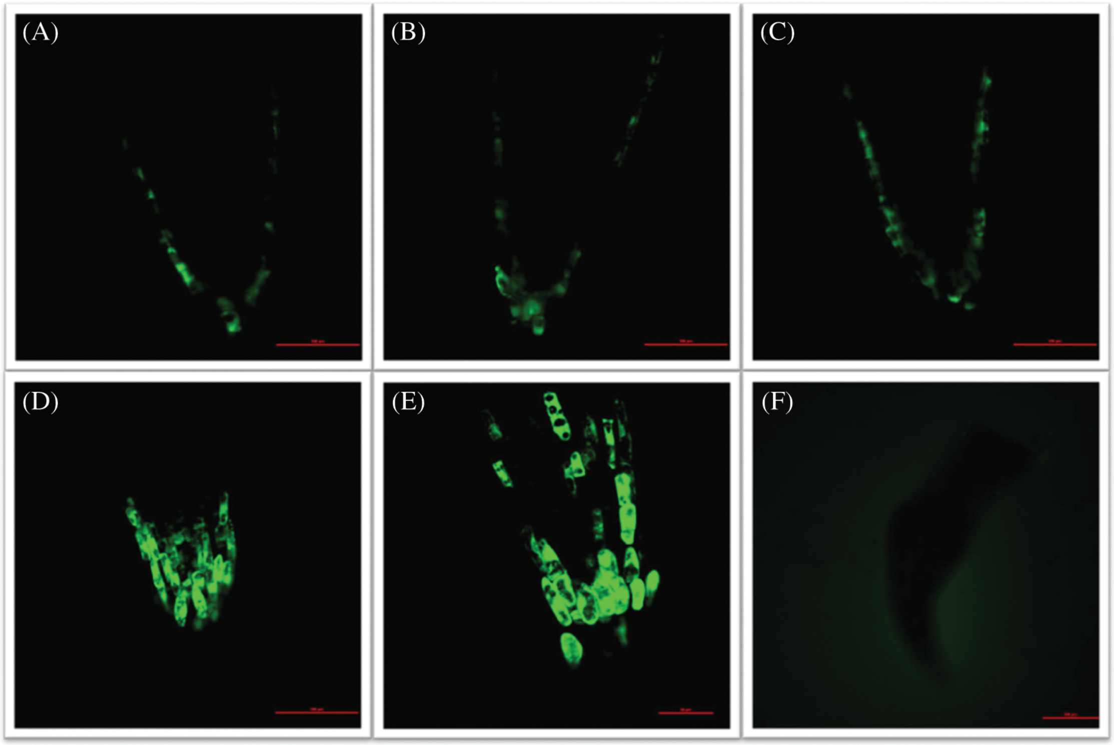

Figure 6: Representative photographs of root tips showing the accumulation of reactive oxygen species in response to extracts of rosebay willowherb leaves (A: control, B: 1 mg/mL, C: 3 mg/mL, D: 10 mg/mL, E: 33 mg/mL, F: 100 mg/mL)

mainly in lettuce. Recently, other studies demonstrated the importance of ROS-mediated damages induced by allelochemicals. Xin et al. (2019) [39] pointed out that imperatorin, vanillin and ferulic acid inhibited the growth of lettuce and *A. sinensis* seedlings via ROS overproduction. In our study, leaf extract of rosebay willowherb with 10 mg/mL also induced the accumulation of ROS in columella cells of root tips, suggesting that ROS may be a key factor underlying root growth inhibition.

4 Conclusions

In this study, the aqueous extracts from stems, leaves, flowers, and capsular fruits of rosebay willowherb were reported to possess phytotoxic potential on seed germination and seedling growth of lettuce. Morphological analysis revealed that the cell structures of lettuce radicle tips turn deformed and atrophied after treatment with 100 mg/mL of the crude extracts, and the accumulation of ROS may be a primary factor explaining the phytotoxic mechanism. While the chemical nature of the bioactive extracts remains to be elucidated, the aerial part of rosebay willowherb would be a promising candidate as a source of natural herbicides.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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