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Herbicidal activity of allelochemicals from the root bark of *Periploca sepium* Bunge

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ABSTRACT

This study aimed to examine the role of the allelochemicals in the root bark of *Periploca sepium* Bunge in weed management. The allelochemical was isolated using a bioactivity-guided method and characterised as 2-hydroxy-4-methoxy-benzaldehyde by ^{1}H and ^{13}C 1D NMR spectroscopic data. The herbicidal activity of 2-hydroxy-4-methoxy-benzaldehyde against 6 common weeds (*Amaranthus retroflexus, Alternanthera philoxeroides, Humulus Scandens, Conyza canadensis, Mikania micrantha, Portulaca oleracea*, *Setaria viridis*) was evaluated in growth bioassays, contact bioassays and rooting bioassays. The Application of 2-hydroxy-4-methoxy-benzaldehyde at 1mg/ml completely inhibited the seeds germination and also significantly inhibited the growth of taproot and caulis in test weeds. The results of contact bioassays showed dose-dependent toxicity of 2-hydroxy-4-methoxy-benzaldehyde to the leaves of all test weeds. The test weeds leaves wilted, after the treatment and the injured leaves did not recover. With the decreasing concentration of 2-hydroxy-4-methoxy-benzaldehyde, the toxicity symptoms of the tested on leaves were gradually reduced. Besides, the rooting bioassays results showed that 2-hydroxy-4-methoxy-benzaldehyde at 0.0625 mg/ml concentration significantly inhibited the rooting in *Alternanthera philoxeroides* Griseb. To conclude, 2-hydroxy-4-methoxybenzaldehyde might be a potential broad-spectrum herbicide.

Key Words: Allelochemical, *Alternanthera philoxeroides, Amaranthus retroflexus,* bioassay, *Conyza canadensis,* herbicidal activity, 2-hydroxy-4-methoxybenzaldehyde, *Mikania micrantha*, *Periploca sepium, Humulus Scandens, Portulaca oleracea*, seed germination, seedlings growth, *Setaria viridis,* toxicity, weed

INTRODUCTION

Weeds are amongst the major causes of losses in crop yields together with pests and diseases (3, 22). Weeds compete with crops for resources, lowered the crop yields and contaminate crops seeds with weed seeds, thereby increasing the weeds problem in

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subsequent growing seasons. Current approaches for alternative weed control are: sanitation of plant material and seeds, use of mulch, solarisation, hand weeding, heating etc. (1). Regardless of the alternatives, used for weed control, the intensive use of synthetic herbicides poses causes environment damage and the development of resistant weeds to these herbicides. The exposure to synthetic herbicides increases the risk of cancer and Parkinson's diseases (8,15). In last 20 years, the problem of development of resistance in weed populations have increase manifolds (23). These concerns shifted the researchers attention to alternative weed control technologies based on natural products (23). The allelochemicals that suppress or eliminate the competing plant species near the source plant have received special attention due to their agricultural potential as selective natural herbicides (5). Herbicides and agrochemicals based on natural products are attractive for variety of reasons. There are 34 species of resistant weeds in China, ranking 5th in the world. The chemical control of crops weeds in China faces the era of "resistance management", and weed resistance must adopt an integrated management strategy. Natural products are environment friendly (13). Bioactive secondary metabolites are natural products that require further research for the development of new products. Development of natural products as novel herbicides is useful strategy for management of herbicide resistance in weeds.

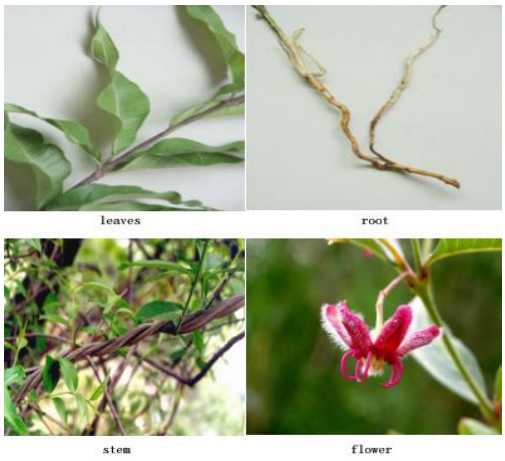

Figure 1. Shape of *P. sepium* Bunge

The root bark of *Periploca sepium* Bunge (Asclepiadaceae) (Figure 1), "xiangjiapi", has been used as traditional Chinese herbal medicine to treat rheumatoid arthritis and wounds for > 2000 years in China. The plant is widely distributed in northwestern areas of China. Previous phytochemical studies on *P. sepium* identified several major compounds [pregnane glycosides, cardiac glycosides, oligosaccharides, flavonoids, coumarins and triterpenoids (6,9-14, 18-25,27-29,31,33)]. The *P. sepium* extract possesses insecticidal activities against *Mythimna separate*, *Plutella xylostella*, *Schizaphis graminum*, *Pieris rapae*, *Musca domestica* and *Solenopsis invicta* Buren (16,18,19). Several insecticidal compounds

have been isolated e.g. periplocoside A, E, N, X, and NW and the insecticidal mechanisms of periplocoside X and NW have been reported (7,17). However, the allelopathy and chemical structure of allelochemicals of *P. sepium* have not been reported. To determine the bioactive principles and deeply explore this plant, the methanol extract of *P. Sepium* was evaluated by bioactivity-guided method. This study aimed to determine the bioactive principles and evaluate the methanol extract of *P. Sepium* was by bioactivity-guided method.

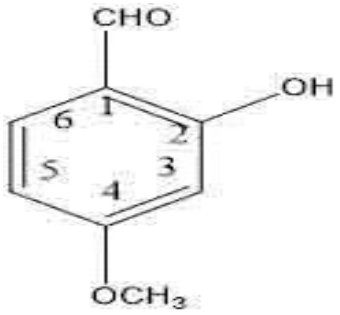

Figure 2. Structure of 2-hydroxy-4-methoxybenzaldehyde

MATERIALS AND METHODS

Ⅰ. Plant Material

The root barks of *P. sepium* were collected on 15 August, 2015 in Yanan, Shaanxi Province, China (Northern latitude, 35.21°; East longitude, 107.41°; Mean altitude 1070 m; Annual rainfall, 550 mm; Minimum and Maximum temperature, -25.4 °C and 39.7 °C, respectively), and labelled as B-1. The sample was identified by Dr. Gao-ying Liu and voucher specimen (sample number: 198410132) has been deposited in the Shaanxi Institute of Pharmaceutical Industry. The tested weed plants were identified by Dr. Ze-huan Wang. For bioassays uniform and healthy seeds of *Mikania micrantha* H.B.K. were collected from the Experimental Farm, South China Agricultural University. The seeds of *Setaria viridis* (L.) Beauv., *Amaranthus retroflexus, Portulaca oleracea* L., and *Conyza canadensis* (L.) Cronq. were collected from the roadside of Science City, Guizhou province. The seeds were stored at 4 °C for till future use. *Alternanthera philoxeroides* Griseb was obtained by vegetative cuttings from Anhui Academy of Agricultural Sciences. Experiments were done from August 2015 to May 2017 in Guiyang. Guizhou province (Northern latitude, 26.35°; East longitude, 106.42°; Mean altitude, 1100 m; Annual rainfall, 1072.8 mm; Minimum and Maximum temperature, -7.3 °C and 35.1 °C, respectively).

II. Extraction and Isolation

For the supersonic extraction, the model KH-500 DE supersonic cleaning bath with heater and timer (500 W power and 40 KHz frequency, Hechuang Ultrasonic Instrument Co., Ltd, Kunshan, Jiangsu, China) was used. The air-dried root bark powder of *P. sepium* (2.5 kg) was supersonically- extracted with MeOH (6 Extraction time: 30 min; Extracted: 3 time;

Interval of extraction: 30 min) at 25 °C. The residue was extracted twice using supersonic extraction, all extraction solutions were mixed. The extract was concentrated *in vacuo* to yield crude residue (512 g). The residue was suspended in water and extracted with $CHCl_3$ (2 liters × 3) to obtain a chloroform fraction (106 g). A part of this fraction (100 g) was subjected to chromatography on silica gel (100-200 mesh) and eluted successively with gradient petroleum ether-EtOAc (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, and 0:10, v/v) to obtain fractions A-1 to A-17. The A-3 fraction was obtained from the acetone to yield 2-hydroxy-4-methoxy-benzaldehyde (13.6524 g, 0.546 % yield). The bioassay for allelopathy showed that 2-hydroxy-4-methoxy-benzaldehyde is an allelochemical of *P. sepium*.

III. Bioactivity-guided Assay

In bioactivity-guided assay, the A-1 to A-17 fractions bioactivity was determined on the germination of *M. micrantha*. The empty seeds floating in tap water were discarded for germination studies. Only the fully developed seeds settled at bottom were selected for this investigation. The A-1 to A-17 fractions were dissolved in acetone at 5 mg/ml concentration, while the concentration of crude extract was 10 mg/ml. Petri dishes (7 cm dia) were lined with one Whatman No. 1 filter paper and 200 µl solution was added per Pteri dish as per treatment. After solvent evaporation, 10-seeds were sown equidistant in each Petri dish. To keep the filter papers moist, little distilled water was added in the Petri dishes. In control, 200 µl acetone was added per Petri dish. Treatments were replicated thrice. All Petri dishes were placed in growth chamber [25 ± 2 °C, 60% RH and 16 h photoperiod]. The Petri dishes were monitored daily. After 3-days, seeds with radicle emergence >1 mm were germinated. Seeds germination Inhibition rate was calculated with Formula 1 given below (45).

$$\text{Inhibition rate of seed germination (\%)} = \frac{\text{Number of germinated seeds in control} - \text{Number of germinated seeds in treatment}}{\text{Number of germinated seeds in control}} \times 100 \quad \text{Formula 1}$$

IV. Germination and Growth Bioassay

All bioassays were done under Laboratory conditions. The seeds of weeds (*M. micrantha*, *S. viridis*, *A. retroflexus*, *P. oleracea L.* and *C. canadensis*) were selected for the germination and growth bioassay. Before the bioassay seeds of test weeds were soaked in tap water for determine their germination (1-3 days) and floating seeds were discarded. Only the swollen seeds settled at bottom were used in bioassay. 2-hydroxy-4-methoxy-benzaldehyde concentrations from 1000 mg/ml to 0.00125 mg/l (1000, 500, 250, 125, 62.5, 12.5, 1.25, 0.125, 0.0125, and 0.00125 mg/l) were evaluated for dose-dependent herbicide activity against the selected weed species. 2-hydroxy-4-methoxy- benzaldehyde was dissolved in acetone for the above series of concentrations. Petri dishes (7 cm dia) were lined with one Whatman No. 1 filter paper and 200 µl solution was added per Pteri dish as per treatment. After solvent evaporation, 10-seeds were sown in each Petri dish. To keep the filter papers moist, little distilled water was added in the Petri dishes. In control, 200 µl acetone was added per Petri dish. Treatments were replicated thrice. All Petri dishes were placed in growth chamber [25 ± 2 °C, 60% RH and 16 h photoperiod]. The Petri dishes were monitored daily. After 3 days, the seeds showing radicle emergence >1 mm were considered germinated. After 7 days, taproot and caulis growth speeds were calculated as per Formula

2 and 3 for each trial (34).

$$\text{Inhibition rate of taproot growth (\%)} = \frac{\text{Mean length of taproot in control} - \text{Mean length of taproot in treatment}}{\text{Mean length of taproot in control}} \times 100 \quad \text{Formula 2}$$

$$\text{Inhibition rate of caulis growth (\%)} = \frac{\text{Mean length caulis in control} - \text{Mean length caulis in treatment}}{\text{Mean length caulis in control}} \times 100 \quad \text{Formula 3}$$

V. Contact Activity Assay

The 2-hydroxy-4-methoxy-benzaldehyde dose-dependent contact activity was determined against the selected test weed species at concentrations of 10 mg/ml to 0.625 mg/ml (10, 5, 2.5, 1.25, and 0.625 mg/ml). This range was selected based on our Pre-experiments. *M. micrantha* were grown in plastic pots (15 cm dia) and placed in greenhouse. Nine-week old plants were used for examination. For this bioassay long-duration plants of *Humulus Scandens* (Lour.) Merr., *S. viridis*, *C. canadensis, Lactuca serriola* and *Artemisia lavandulaefolia* DC. were selected from the roadside of Science City, Guizhou province. 2-hydroxy-4-methoxy-benzaldehyde was dissolved in acetone at the determined series of concentrations. Then, with soft brush the solutions were uniformly painted on leaves of test weeds. The control was painted only with acetone. Treatments were replicated thrice. *M. micrantha* was maintained in growth chamber [25 ± 1 °C, 60 % RH and 12 h photoperiod] to prevent its spread in Guizhou Province. While other plants collected from the roadside of Science City, Guizhou province, were maintained in natural conditions (Temperature: 25 °C-30 °C, Relative humidity: 50 % RH). After 24 h, the results were observed, recorded and photographed.

VI. Rooting Bioassay

2-hydroxy-4-methoxy-benzaldehyde dose-dependent rooting activity was evaluated against *A. philoxeroides* at concentrations of 1 mg/ml to 0.0625 mg/ml (1, 0.5, 0.25, 0.125, and 0.0625 mg/ml). A certain amount of 2-hydroxy-4-methoxy-benzaldehyde was dissolved in 1 ml acetone and 1 ml methyl oleate was added as surfactant. The final volume was made with distilled water for the desired concentration. Twenty ml of the solutions were added in the 50 ml centrifuge because the ordinary test tubes are small and fragile. The top 3-internodes of selected branches of *A. philoxeroides* were removed. Three internodes (No. 4, 5, and 6) were kept, and the sixth internode was immersed in the solutions kept in the centrifuge tubes. Distilled water with same amount of methyl oleate and acetone served as control, while, the same amount of distilled water served as blank control. The treatments were replicated thrice. The test branches were maintained in growth chamber [25 ± 1 °C, 60% RH and 16 h photoperiod]. After 4 days, the results were observed, recorded and photographed.

RESULTS AND DISCUSSION

I. Chemical Structure

2-hydroxy-4-methoxy-benzaldehyde (Figure 2), an allelochemical of *P. sepium* was obtained as colourless needle like crystals and possessed the unique smell of *P. sepium* and characterized as 2-hydroxy-4-methoxy-benzaldehyde based on comparison of its 1D NMR spectroscopic data with those reported in literature from the ^{1}H, ^{13}C NMR spectral data (20-21,30). For the first time, we isolated a phenolic compound with allelopathic activity from the root barks of *P. sepium* using a bioactivity-guided method. The compound was also separated from *Decalepis hamiltonii* and *P. sepium*, and several activities were reported (30,20,2,32). However, the allelopathy of the compound has never been reported. Compared with other natural allelopathic compounds, the simple structure of 2-hydroxy-4-methoxy-benzaldehyde suggested it is easier and economical to synthesize and perform the structure modification. Therefore, we will attempt to modify the allelochemical structure of this compound in our future study to obtain allelochemical with more herbicidal potential.

II. Bioactivity-guided Assay

The A-1 to A-17 fractions were bioassayed for further separation experiments (Table 1). The inhibition rates of seed germination of A-3 fraction and A-4 fraction reached 100% and 70%, respectively, however, the inhibition rates of other fractions were < 50%. Notably, the active compound was collected in A-3 fraction. Thus, A-3 fraction was further separated.

Table 1. Inhibitory effects of fractions (A-1 to A-17) applied at 5mg/ml on the seed Germination of *M. micrantha*

Fractions	Inhibition rate of seeds germination (%)	Fractions	Inhibition rate of seeds germination (%)
Crude extract	93.33 ± 1.1547	A-9	0
A-1	3.33 ± 0.5773	A-10	0
A-2	43.33 ± 0.5773	A-11	0
A-3	100	A-12	0
A-4	70.00 ± 1.0000	A-13	0
A-5	30.00 ± 1.0000	A-14	0
A-6	10.00 ± 1.0000	A-15	33.33 ± 0.5773
A-7	10.00 ± 1.0000	A-16	30.00 ± 1.0000
A-8	0	A-17	10.00 ± 1.0000

The concentration of crude extract was 10 mg/ml. Data are means ± SE from 3 Replications.

III. Germination and Growth bioassay

2-hydroxy-4-methoxy-benzaldehyde significantly inhibited the seed germination, taproot and caulis growth of the test weeds (Table 2). A dose-dependent variation was observed in the germination and growth indices. At 1000 mg/l, seeds germination taproot and caulis growth of *S. viridis, A. retroflexus* and *M. micrantha* were completely inhibited i.e. 100 % inhibition. At < 12.5 mg/l, there was no inhibition in seed germination of all tested weeds. Obviously, inhibition rates of seed germination and taproot growth of *P. oleracea* and *C. Canadensis* were lower than above tested weeds. At < 1.25 mg/l there was no

inhibition in taproot and caulis growth bioassays of all tested weeds.

Table 2. Inhibitory effects of 2-hydroxy-4-methoxy-benzaldehyde from *P. sepium* on seed germination, caulis and taproot growth of test weeds

Test weeds	Concentration (mg/l)					
	12.5	62.5	125	250	500	1000
	Seeds germination inhibition (%)					
S. viridis	0	0	10.00 ± 1.00	80.00 ± 1.00	100	100
A. retroflexus	0	30.00 ±1.00	56.67 ±0.57	73.33 ±2.08	86.67 ±1.52	100
P. oleracea	0	0	0	6.66 ±1.15	23.33 ±0.57	63.33 ±0.57
M. micrantha	0	0	13.33 ± 0.78	53.33 ±1.15	80.00 ±1.00	100
C. canadensis	0	0	0	40.00 ± 1.00	76.67 ±2.08	83.33 ± 1.15
	Tap root growth inhibition (%)					
S. viridis	8.32 ± 0.34	23.70 ± 0.88	32.92 ± 1.85	77.37 ± 2.12	100	100
A. retroflexus	0	22.65 ± 0.35	34.66 ± 0.14	81.53 ± 0.14	96.70 ± 0.69	100
Q. oleracea	0	0	4.74 ± 0.26	40.28 ± 0.45	55.99 ± 0.08	65.64 ± 0.72
M. micrantha	0	13.71 ±0.20	41.11 ± 0.81	60.47 ± 0.17	80.95 ±0.19	100
C. canadensis	0	0	1.36 ± 0.58	25.38 ± 0.91	56.00 ± 0.88	83.33 ± 0.28
	Caulis growth inhibition (%)					
S. viridis	36.01 ± 0.66	42.37 ± 0.16	66.94 ± 0.81	95.76 ±1.56	100	100
A. retroflexus	11.43 ± 0.35	35.55 ±0.25	62.21 ± 2.18	96.26 ± 0.20	100	100
R. oleracea	0	24.31 ± 0.62	30.86 ± 0.43	34.86 ± 0.16	60.84 ± 0.20	100
M. micrantha	5.44 ±0.66	15.13 ±0.31	48.97 ±0.50	83.56 ±0.04	100	100
C. canadensis	0	0	18.68 ± 0.89	44.02 ± 0.33	100	100

IV. Contact activity Bioassay

The application of different concentrations of 2-hydroxy-4-methoxy-benzaldehyde on leaves of test weeds showed the toxicity symptoms after 24 h (Fig. 3). The results showed that the degree of injury was dose-dependent. There were no obvious alterations in the leaves of *H. scandens* in control group (Fig. 3). However, 2-hydroxy-4-methoxy-benzaldehyde induced the dose-dependent toxicity symptoms (chlorosis and wilting) in leaves of *H. scandens*.

After treatment with 2-hydroxy-4-methoxy-benzaldehyde > 2.5 mg/ml, the tested leaves of *S. viridis* exhibited toxicity symptoms (yellowing and partial wilting) than control (Fig. 3). However, the tested leaves treated with 1.25 mg/ml and 0.625 mg/ml of 2-hydroxy-4-methoxy-benzaldehyde showed only rolling without yellowing or wilting. Notably, the contact activity of 2-hydroxy-4-methoxy- benzaldehyde against *S. viridis* was weaker than against *H. scandens.*

The figure 3 showed that the toxicity symptoms of tested leaves of *A. lavandulaefolia* differed in comparison to above tested weeds. Part of the tested leaves changed from green to brown and the brown area gradually reduced with decreasing concentration of 2-hydroxy-4-methoxy-benzaldehyde.

As shown in Figure 3, the tested leaves of *M. micrantha* exhibited toxicity symptoms (wilting and shriveling). The degree of injury of tested leaves was gradually reduced with the decrease in dose of 2-hydroxy-4-methoxy-benzaldehyde.

Figure 3 showed that the tested leaves of *C. canadensis* exhibited toxicity symptoms

(chlorosis, curling and withered). The injured area of tested leaves was reduced with decrease in dose of 2-hydroxy-4-methoxy-benzaldehyde.

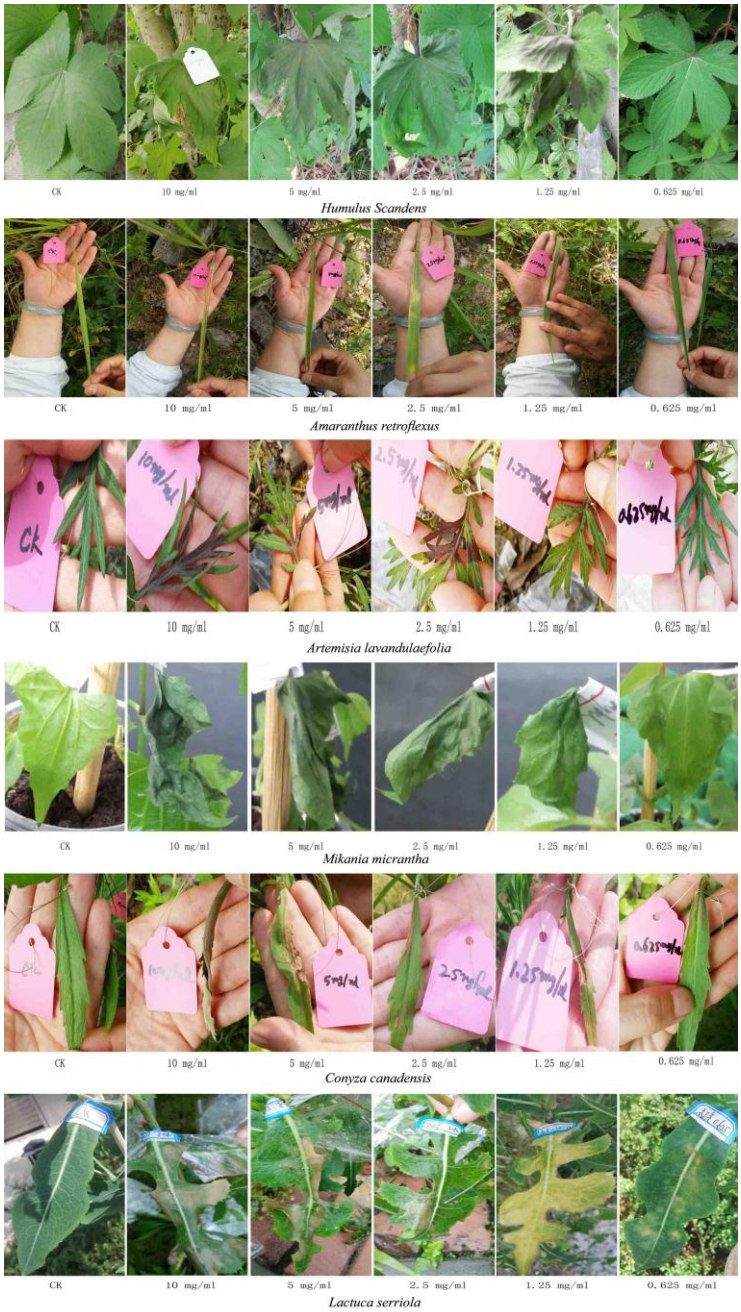

Figure 3. Toxicity symptoms of test leaves of test weeds treated with different concentration of 2-hydroxy-4-methoxy-benzaldehyde from *P. sepium*at 24 h after treatment.

As shown in Figure 3, 10 mg/ml of 2-hydroxy-4-methoxy-benzaldehyde made the tested leaves of *L. serriola* widely turned brown. With the decreasing concentration of 2-hydroxy-4-methoxy- benzaldehyde, the toxicity symptoms of tested leaves were gradually reduced. At 0.625 mg/ml, the tested leaves showed several yellow spots.

Subsequent observations showed that the injury areas did not recover in all test leaves of test weeds. After several days, all test leaves dried up but did not fell off. At the same time, the untreated leaves of the selected weeds appeared normal. The contact activity of phenolic allelochemicals has been rarely reported and our study provided additional information about the contact activity of phenolic compounds.

In this experiment, none of the untested leaves showed toxicity symptoms in contact bioassay, but the entire tested branches presented toxicity symptoms at 1.0 mg/ml and 0.5 mg/ml in rooting bioassay. These results imply that 2-hydroxy-4-methoxybenzaldehyde is conducted upward in weeds. Symptom observation of contact bioassays showed the different toxicity symptoms of the tested weeds. These findings suggest that the allelochemical act on multiple targets, although how the impact of allelochemical will be implemented remains unclear. Thus, the mechanism of action will be achieved in future studies for development of new products. The isolation and bioactivity results of this experiment will provide scientific foundation for rational development and utilization of this compound. Further studies will be required to determine the suitability and effectiveness of the compound for controlling weed populations under field conditions.

Figure 4. Rooting inhibition activity of 2-hydroxy-4-methoxy-benzaldehyde against *A. philoxeroides*

V. Rooting Bioassay

Figure 4 showed the results of rooting bioassays against *A. philoxeroides* after treatment with different concentration of 2-hydroxy-4-methoxy-benzaldehyde for 4 days. The results indicated that rooting inhibition activity of 2-hydroxy-4-methoxy-benzaldehyde presented a dose-dependent positive relationship. No obvious difference was observed in rooting between the blank and parallel control groups (Fig. 4). The branches treated with different concentrations of 2-hydroxy-4-methoxy- benzaldehyde showed significant inhibition in rooting. At 1.0 mg/ml and 0.5 mg/ml, the test branches didn't produce roots and exhibited toxicity symptoms (wilt, shriveling, yellowing). At 0.25 mg/ml, no root was observed, but the tested branch appeared normal. At 0.125 mg/ml, several root tips were observed. The average root length was 0.1 cm at 0.0625 mg/ml concentration. Evidently, 2-hydroxy-4- methoxy-benzaldehyde can be translocated to the leaves through the stems.

CONCLUSIONS

Our study supported the idea that phenolic compounds could play important roles as allelochemicals in weed management. Our research considers the possibility that other types of allelochemicals also are present in *P. sepium* plant, which needs to be identified in future. This article had shown the allelopathic potential of phenolic compounds to solve various ecological problems, especially for the sustainable development of agriculture, forestry, natural resources and environment conservation.

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