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Table: -, Figs : 8

Bioactivity of allelochemicals isolated from the roots of *Echinacea purpurea* L. Moench on *Amaranthus viridis* L., *Portulaca oleracea* L. and *Microcystis aeruginosa*

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ABSTRACT

Based on the bioassay-guided strategy, we isolated 6-six allelochemicals [cichoric acid **(I)**, 1,3-dicaffeoylquinic acid **(II)**, 4,5-dicaffeoylquinic acid **(III)**, chlorogenic acid **(IV)**, 1-hydroxy-2-phthoic acid **(V)**, echinacoside **(VI)]** from the roots of *Echinacea purpurea* (L.) Moench. Their structures were identified by nuclear magnetic resonance (NMR) and electrospray ionization mass spectrometry (ESI-MS) spectroscopic data. The bioassays studies included allelopathic and algicidal activities to test the effects of extracts and isolated fractions against the test weeds (*Amaranthus viridis* L., *Portulaca oleracea* L. and *Microcystis aeruginosa* Kutzing). At 100 µg/mL, compound **(II)** inhibited the shoot length and germination of *A. viridis* and *P. oleracea* weeds with the germination RI of -0.95±0.04 and -0.95±0.02, respectively. Furthermore, compound **(III)** showed the strongest inhibition of root length of *P. oleracea* L. We also found that compounds **I-VI** have algicidal activity. The compound **(I)** at low inoculum (5.0×10^2 cells mL^{-1}) and high inoculum (1.0×10^4 cells mL^{-1}), showed the highest algicidal activity of 78 % and 87.67 % 6 h after the treatment at 5 µg mL^{-1} respectively.

Keywords: Algicidal bioassay, allelochemicals, *Amaranthus viridis*, bioactivity, *Echinacea purpurea*, herbicides, *Microcystis aeruginosa*, NMR, *Portulaca oleracea*

INTRODUCTION

Weed infestation in crops causes severe losses in yield and quality of corn (3). The magnitude of weed-related crop yield reduction depends on weed species, time of weed emergence relative to crop emergence, weeds distribution and weed density etc (28). *Amaranthus viridis* L (Family Amaranthaceae) and *Portulaca oleracea* L. (Family Portulacaceae) (Figure 1 and 2) are two major weeds of corn crop (29). *A. viridis* L. is also weed in vegetable fields, corn, soybeans, cotton, mint, sweet potatoes (39). *P. oleracea* L. is drought tolerant with, strong vitality, and drastically reduces the crops growth. Its adult plants are resistant to stress, large leaf volume, many branches made them dominant in competitive advantage and thus reduce crop yields (16,34,38-40). The weeds can be controlled by mechanical, biological, chemical and cultural methods. Among them,

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synthetic herbicides are most effective (5), but there is global concern about their negative effects [environmental pollution, development of weed resistance and effects on non-target organisms etc]. (10). Hence, it is necessary to find the natural products that possess better and eco-friendly efficacy. Allelopathy is generally considered to be direct or indirect effects of one living plant, including microorganisms, on another through the production of special chemicals released into the environment (27). The effect of allelopathy is intricate, including inhibitory and stimulatory reciprocal biochemical interactions (31). Such particular chemical components are called allelochemicals. Under some conditions, these may be released in quantities suppressive to developing weed seedlings (2). For example, Wang *et al* suggested that essential oils of *Geranium wilfordii Maxim* L. (Geraniaceae) can be used as plant growth regulator (33).

Figure 1. The pictures of *Amaranthus viridis* L.

Figure 2. The pictures of *Portulaca oleracea* L.

Harmful algal blooms (HABs) are major public problems due to their negative impacts on water ecosystems and human health (8,9,19,30). *Microcystis aeruginosa* is most dominant and ecologically destructive species in freshwater lakes (7). However, traditional control strategies usually induce the secondary pollution due to chemical pesticides (1,18,37). Now biological products can control harmful algae (22,25). Therefore, to find new bioactive compounds in plants have become important. The inhibition of algal growth by plant litter has been mainly due to allelochemicals such as phenolic compounds (17,24).

In our screening for herbicidal and algicidal metabolites from plants, we found that *A. viridis* and *P. oleracea* weeds rarely grow near *Echinacea purpurea* (Family Asteraceae) a perennial herb (Figure 3). This plant is cultivated in North America and Europe and widely used as an herbal medicine and dietary supplement worldwide (20). It is popular herbal medicines to treat common colds and respiratory disorders (15,35). We found that ethanol extract of *E. purpurea* root, were highly phytotoxic against *A. viridis* L., *P. oleracea* L. and *M. aeruginosa*. Further Investigation, lead to the isolation of six compounds **(I-VI)**. Their structures were determined using 1D NMR and ESI-MS spectroscopic data analysis.

Figure 3. The picture of *Echinacea purpurea* (L.) Moench

This study aimed to determine the chemical composition, allelopathic and algicidal activities of the extracs from the roots of *E. purpurea* and identify the potent allelochemicals by bioassay-guided isolation and identification of the bioactive constituents. Establishing a theoretical foundation for the development of *E. purpurea* allelochemicals into herbicides and algicide.

MATERIALS AND METHODS

Plant Samples

The roots of *Echinacea purpurea* (L.) Moench during the flowering period were collected in August 2016 from Changchun Park, Changchun, (N 43°05’～45°15’, E 124°18’～127°05’). Changchun has temperate continental humid climate. The height above sea level: 200-400 m, annual rainfall : 600-700 mm and maximum and minimum temp: 40°C and -36.5°C]. The *E. purpurea* Plants were identified by Professor Ying Wu, College of Plant Science, Jilin University (Specimen No. S20160515) and stored at 4 ℃.

Algae Material

One axenic strain of *Microcystis aeruginosa* (FACHB-315), obtained from Culture Collection of Freshwater Algae, Institute of Hydrobiology, Chinese Academy of

Sciences, Wuhan, China was selected for bioassay. The cultures were grown in sterilized BG11 liquid medium under a 12:12 h light: dark cycle light intensity of 2500 lux at 25 ± 1 ºC and manually shaken twice daily during the incubation. The cells in exponential phase were used for growth inhibition tests.

Extraction and isolation

The 4.5 kg air-dried ground roots of *E. purpurea.* were extracted thrice in 30 L ethanol at 25 ℃ supported with ultrasonic cleaner. After removing ethanol under reduced pressure, the crude extract (52 g) was obtained. This crude residue was partitioned successively with water and petroleum ether, ethyl acetate (EtOAc), and then n-butanol to yield three extracts. The crude combined EtOAc extract (38 g) was subjected to silica gel column and eluted in order of increasing polarity, with a gradient of CH_2Cl_2/MeOH (100% CH_2Cl_2, 50:1, 30:1, 20:1, 10:1, 5:1, 100 % MeOH) and approximately 2 L for each ratio was eluted to obtain 7 fractions as per TLC profiles (developed with a volume ratio of CH_2Cl_2/MeOH = 30/1). It was found in preliminary test that the fractions 4 and 6 were inhibitory to the tested weeds *Amaranthus viridis* L. and *Portulaca oleracea* L. and *M. aeruginosa* from the preliminary test. At 2 mg/mL concentration, the inhibition of *A. viridis* germination was 71.26 % and 64.82 %, while the inhibition of *P. Oleracea* germination was 52.47 % and 58.37 %, respectively. Subsequently, fraction 4 and 6 were further separated by column chromatography and eluted with dichloromethane and methanol to obtain compounds **VI** (6.1 mg), **I** (5.4 mg), and a mixture. The mixture (46 mg) was further purified by Sephadex LH-20 column (MeOH) to yield **III** (7.3 mg) and **IV** (6.7 mg). Fr. 6 (8.4 g) was passed through a Sephadex LH-20 column (MeOH) to obtain three subfractions, Frs. 3.1-3.3. Frs. 3.2 (1.9 g) was further subjected by CC on ODS (gradient MeOH/ H_2O 20-10 0%) to afford **II** (8.4 mg), **V** (7.5 mg). The structures of 6-compounds were identified based on ^{1}H nuclear magnetic resonance (^{1}H-NMR), EI-MS and by comparison of their data literature.

Chemical analysis

NMR spectra were recorded on Bruker AVANCE III (500 MHz) instrument. ESI-MS with a Thermo LCQ Fleet instrument. Column chromatography (CC) was performed on silica gel (90-150 μm; Qingdao Marine Chemical Inc., Qingdao, China), MCI gel (75-150 μm; Mitsubishi Chemical Corp., Tokyo, Japan), Sephadex LH-20 (40-70 μm; Amersham Pharmacia Biotech AB, Uppsala, Sweden), and Lichroprep RP-18 gel (40-63 μm; Merck, Darmstadt, Germany). GF254 plates (Qingdao Marine Chemical Inc.) were used for thin-layer chromatography (TLC).

Seed germination and seedling growth bioassay

The allelopathic effects were assayed in Petri dishes as per Lvancheva and Palaciosa (14,21) with some modifications. The weed seeds were imbibed in deionized water for 2 h, soaked in 0.5 % $KMnO_4$ solution for 15 min, and washed with sterile water until they were colourless. The Petri dishes (9 cm dia) were lined with two layers of filter paper, impregnated with 5 ml distilled water (control) or extract at test doses as per treatments. In each petri dish, 30 seeds of *A. viridis* or *P. oleracea* were placed on top of

filer paper. Different doses of compounds (100 µg/mL) added in water-DMSO mixture (99.5:0.5) were loaded on the filter paper attached to the inner side of the Petri dish cover and then sealed with Parafilm to test the inhibitory effects of six compounds (Figure 4). Controls of water-DMSO mixture alone showed no differences than water control. Petri dishes were placed in growth chamber (25 °C, 12 h/12 h dark/light). The germination and root lengths of seedlings were measured after 3 days treatment. Data of three replications were transformed to percent of control. The allelopathic response index (RI), which measured each treatment response (T) to control (C), was calculated as under:

$$\text{If } T \geq C, \text{ then } RI = 1 - (C/T); \text{ if } T < C, \text{ then } RI = (T/C) - 1 \quad (29)$$

Where, RI: Response index, T: Treatment response, C: Control response, RI > 0: Stimulation and RI < 0: Inhibition

Figure 4. The schematic diagram of germination bioassay

Algicidal Activity

The algicidal activity of test compounds against *Microcystis aeruginosa* were determined by the modified method of Oh *et al.* (18). The various doses (0, 1, 3 and 5 µg /mL) of test compounds were completely dissolved in DMSO then added to a 96-well plate. Then 100 cells (low inoculum) and 2,000 cells (high inoculum) of *M. aeruginosa* were inoculated in 200 µL of BG 11 medium per well, respectively. The incubation durations were 1 and 6 h at 25 ± 1 ºC. To further determine the susceptibility of *M. aeruginosa* to test compounds, different concentrations (0, 0.2, 0.4, 0.8, 1.6 and 3.2 µg/mL) of test compounds were completely dissolved in DMSO then added at 0.64 µg per well per well. One hundred cells of *M. aeruginosa* were inoculated in 200 µL of BG 11 per well. The experiments were repeated thrice in Complete Randomised Design. After incubating under previous culture conditions for 1 h, the surviving cells were counted under a microscope with a Hausser hemocytometer. The number of cells was counted and transformed to algicidal rate (%). The formula used was as under:

$$\text{Algicidal Rate } (\%) = (1 - T / C) \times 100$$

Where, RI: Response index, T: Treatment response, C: Control response, RI > 0: Stimulation and RI < 0: Inhibition.

Statistical analyses

All treatments were replicated thrice Design with twice Parallel test and the results were reported as mean ± SD. The data were analyzed by one-way ANOVA; statistically significant effects were further analyzed and means were compared using Duncan's multiple-range test. Statistical significance was determined at $P < 0.05$. The algicidal activity was analyzed using one-way analysis of variance and compared using Duncan's test at a significance level of $P<0.05$ using SPSS v10.1.

RESULTS AND DISCUSSION

Allelopathic activity

E. purpurea is an indigenous medicine of Europeans and the native American Indians with multiple biological activities, such as anti-oxidation, anti-inflammation, anti-proliferative, anti-bacteria, antihypertensive and immunomodulation effects for hundreds of years (32). The medical plants were also food supplements that used to be infectious diseases in children, old people and animals in Europe and North America (4). A previous report showed that the total phenols and caffeic acid derivative contents of the different parts of *E. purpurea* were in the ascending order as follows: root > stem >leaf <flower (13). Another literature exhibited that *E. purpurea* are rich in caffeic acid, choric acid, and other polyphenols and glycoproteins. Among them, glycoproteins, polysaccharides and alkylamides in roots of *E. purpurea* are considered to be main components for their immunomodulatory properties (26).

As serious agricultural weeds, *A. viridis* is most prevalent in plantation agriculture (14), which produces a lot of seeds a year and can be dispersed by wind, water as well birds, with manure, with movement of farm machines and as a contaminant in crop seeds (10). While, *P. oleracea* is common weed in turfgrass areas and in field crops (17). The results of statistical analysis show that the allelopathic activities of the six compounds have significant differences ($P<0.05$). Six phenolic acid compounds isolated from the roots of *E. purpurea* inhibited the seed germination of *A. viridis* and *P. oleracea* significantly (Figure 5). Among them, compound **(II)** inhibited germination of *A. viridis* (RI =-0.95±0.04) and *P. oleracea* (RI =-0.95±0.02) most effectively. Compound **(III)** showed better effect to *A. viridis* (RI =-0.89±0.02) and *P. oleracea* (RI =-0.94±0.02). However, the phytotoxicity of compound **(VI)** exhibited lower inhibition to *A. viridis* (RI =-0.09±0.06) and *P. oleracea* (RI =-0.09±0.07).

Seedling growth, shoot and root length of two weeds (*Amaranthus viridis* L. and *Portulaca oleracea* L.) were all inhibited by the six phenolic acid compounds. Compound **(II)** inhibited the root length of *A. viridis* (Figure 6, RI =-0.95±0.02). The shoot length of *P. oleracea* was more sensitive to compound **(III)** (RI=-0.97±0.02). Compound **(VI)** inhibited the shoot length of *A. viridis* weakly. (Figure 6, RI =-0.24±0.02). The inhibitory effect to the root length of *P. oleracea* by compound **(VI)** was the weakest (RI =-0.07±0.05).

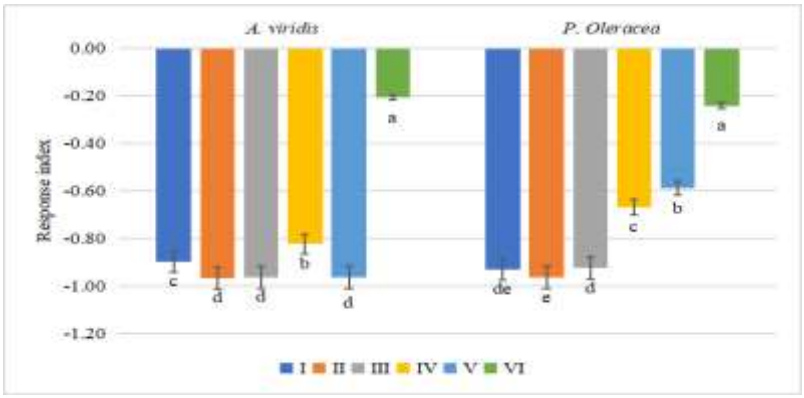

Figure 5. Inhibitory effects of six isolated active compounds on seed germination of *A. viridis* and *P. Oleracea*.

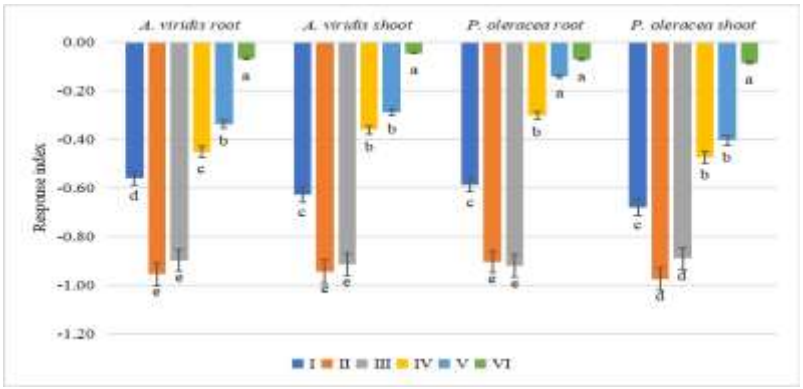

Figure 6. Effects of six isolated active compounds on root and shoot growth of *A. viridis L.* and *P. oleracea L.*

Algicidal Activity

Freshwater cyanobacteria *Microcystis* proliferate in many aquatic ecosystems, due to climate and anthropogenic changes and their blooms are predicted to increase at fast speed. *Microcystis* blooms strongly impacts the functioning of aquatic ecosystems and pose risk to human and animal health due to ability to release destructive/harmful toxins (6).

To further explore the potential of six phenolic acid compounds **(I-VI)** in agricultural applications, an algicidal activity test was done. The statistical analysis of results showed that the algicidal activity of 6-compounds significant by differed ($P < 0.05$). The results of algicidal activity of compounds **I-VI** at various concentrations (1, 3, and 5 µg mL^{-1}) and different treatment times (1 and 6 h) against different cells number of *M. aeruginosa* were shown in Figure 7. The higher concentration or the longer treatment time of compounds were algicidal to *M. aeruginosa*. All compounds were more effective at *M. aeruginosa*. Furthermore, no matter low inoculum (5.0×10^2 cells mL^{-1}) or high

Figure 7. Algicidal activity of six compounds on *M. aeruginosa* from roots of *E. purpurea* (o1l: 1μg/mL treatment for 1 h and low inoculum, o6l: 1μg/mL treatment for 6 h and low inoculum, o1h: 1μg/mL treatment for 1 h and high inoculum, o6h: 1μg/mL treatment for 6 h and high inoculum, t1l: 3μg/mL treatment for 1 h and low inoculum, t6l: 3μg/mL treatment for 6 h and low inoculum, t1h: 3μg/mL treatment for 1 h and high inoculum, t6h: 3μg/mL treatment for 6 h and high inoculum, f1l: 5μg/mL treatment for 1 h and low inoculum, f6l: 5μg/mL treatment for 6 h and low inoculum, f1h: 5μg/mL treatment for 1 h and high inoculum, f6h: 5μg/mL treatment for 6 h and high inoculum).

inoculum (1.0×10^4 cells mL^{-1}), the compound **I** showed the strongest algaicide activity. At 5 µg mL^{-1} and for 6 h treatment time, the reduction rates were 78 %±3.79 for low inoculum and 87.7 %±1.53 for high inoculum, respectively. However, compound **(VI)** showed lower algicidal activity at all concentrations tested. At 5 µg mL^{-1} and for 6 h treatment time, the reduction rates were 39.7 % and 51.3 %, respectively. The algicidal activity of 6-compounds from followed the order: >**V >II >III >IV >VI**. This suggested that phenolic acids have better algicidal activity, especially compound **(I)**. We conclude that the biological activity depends on the presence of various functional groups in the phenolic acids structure. Based on previous references, we concluded that carboxyl group changes the insecticidal activity of six compounds. This is the first report on algicidal activity of compounds **I-VI,** isolated from the roots of *E. purpurea*. The effects of phenolic acids on other algae and the mechanism of algicidal activity need further research in the future.

Identification of allelochemicals

Chemical investigations of crude extracts from the roots of *E. purpurea* by multiple chromatographic procedures, isolated 6-compounds: Cichoric acid **(I)**, 1,3-Dicaffeoylquinic acid **(II)**, 4,5-Dicaffeoylquinic acid **(III)**, Chlorogenic acid **(IV)**, 1-Hydroxy-2-phthoic acid **(V)**, Echinacoside **(VI)** (Figure 8). Their structures were identified based on the basis of comparison of their NMR and MS data with those reported compounds in literature. (10,11,12,23,36).

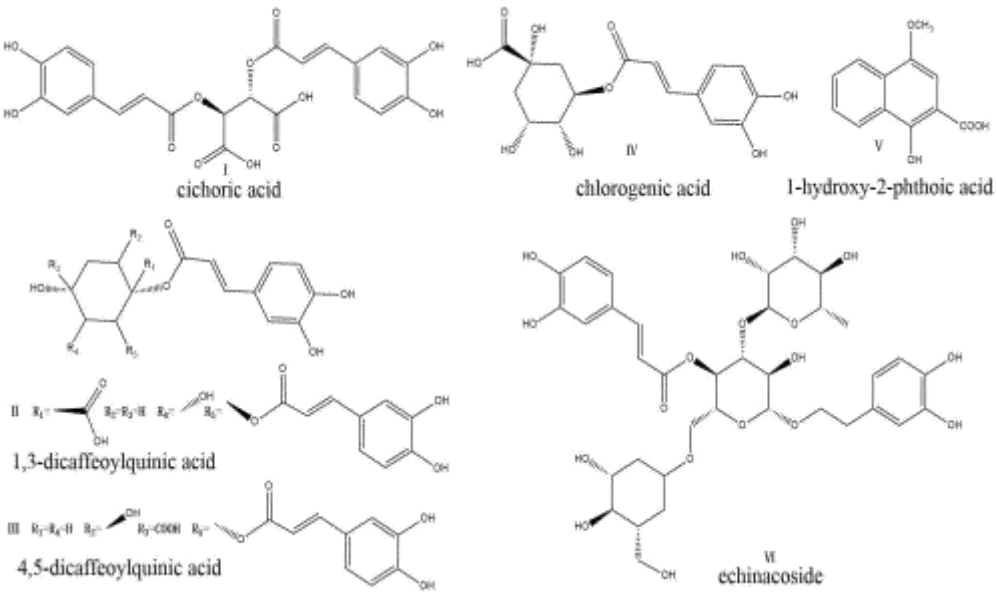

Figure 8. The structure of six isolated active compounds from the roots of *Echinacea purpurea* (L) Moench.

CONCLUSIONS

We isolated, 6-structurally different phenolic compounds from the roots of *E. purpurea*. We first time studied the bioactivity of 6-allelopathic phenolic acids from the roots of *E. purpurea* against *A viridis*, *P. oleracea* and *M. aeruginosa*. These findings indicated that allelopathic compounds from the roots of *E. purpurea* have potential to be developed as the natural herbicides to control weeds and algicidal agents to control *M. aeruginosa.*

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