Asian Journal of Chemistry; Vol. 25, No. 3 (2013), 1613-1617

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2013.13470

Fractionation of Extract from *Ligusticum chuanxiong* (*Apiaceae*) by High Speed Counter Current Chromatography and Their Efficacy in Rice Against S-metolachlor Injury

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(*Received*: 18 January 2012; *Accepted*: 24 September 2012) AJC-12173

Herbicide S-metolachlor with the high efficacy toward the weed from *Cyperaceae* and *Poaceae* is never solely applied in rice field due to its grave inhibition to rice growth. This study was conducted to separate the active compound from *Ligusticum chuanxiong* Hort, which could protect rice from the injury of S-metolachlor. High speed counter current chromatography was applied to the separation of the crude extract of *L. chuanxiong* Hort. The crude extract obtained by supercritical fluid extraction was fractionated into four fractions after eluted in the head-to-tail pattern with a *n*-hexane/ethyl acetate/ethanol/water solvent system (5:4:3:2, v/v/v/v). Two components were successfully separated by high speed counter current chromatography and identified as Z-ligustilide and senkyunolide A by liquid chromatography-mass spectrometry, gas chromatography-mass spectrometry, proton nuclear magnetic resonance (^{1}H NMR) and carbon nuclear magnetic resonance (^{13}C NMR). The corresponding yields with the purity of over 96 % were 40 mg and 15 mg from 500 mg of crude extract in a single run, respectively. In bioassay experiments, results showed that the level of the growth recoveries conferred by Z-ligustilide and senkyunolide A were 100 % and 91 % of untreated control in plant height as well as 94 % and 83 % of untreated control in roots length, respectively. Z-ligustilide has the potential to be a safener with the capability of reversing the effects on rice caused by S-metolachlor.

Key Words: Rice, *Ligusticum chuanxiong* Hort, High speed counter current chromatography, Z-ligustilide, Safener.

INTRODUCTION

The lesion of herbicides to crops is a major problem world-wide. Many selective herbicides used in crops field not only eliminate weeds, but also produce the damage to crops and directly lead to the loss of production. To reverse the effects on crops caused by herbicides, mostly, there are chemically synthesized safeners to be used for the protection of crops from herbicides injury, which include fenchlorazole ethyl and mefenpyr diethyl for aryloxyphenoxy propionate against black-grass[1], benoxacor and fluxofenim protecting maize and sorghum from chloroacetamides damage[2,3] and fenclorim enhancing herbicides detoxification in rice[4-6], *etc*. Meanwhile, a few natural safeners were reported, including the *Rhodococcus sp*. strain N186/21 isolated from the rhizosphere of maize with the capability of degrading thiocarbamate herbicides[7]. To our best knowledge, S-metolachlor from chloroacetamides is a selective pre-emergence herbicide having a high activity against the weed from *Cyperaceae* and *Poaceae* and some dicotyledonous weed. It is commonly used in the field of some dry crops. Due to its grave inhibition to rice growth, it is barely used in rice field against weed. It also has not been reported that S-metolachlor combined with safener is applied in rice field against weed to date. In contrast to the synthesized, natural safeners could be more compatible with environment, barely causing the environmental pollutions. Therefore, our study was concerned to obtain the natural safeners from *L. chuanxiong* Hort, which could reverse the effects on rice caused by S-metolachlor.

L. chuanxiong Hort, well-known in traditional Chinese medicine, could firmly facilitates blood flow and disperses blood stasis[8-10] and is used extensively for the treatment of cardiovascular or cerebrovascular diseases, headache and menstrual disorders, *etc*.[11-13]. It is mainly composed of essential oil, alkaloids, phenolic acid and so on[14,15]. In essential oil, the primary bioactive components are phthalides such as Z-ligustilide, senkyunolide A and butylidenephthalide, *etc*.[16,17]. Among them, Z-ligustilide and senkyunolide A have been attracting considerable attentions for their properties such as insecticidal[9,18], antifungal[19,20] and antioxidant[21] activity, which give them potential for the important applications. However, there is no report about their activity as safeners for herbicides.

In previous reports, silica gel column was used for the separation of compounds from *L. chuanxiong* Hort[18,19,22,23].

Affinity chromatography with affinity materials immobilized in silica packed column is also used for the separation[24]. Compared to the silica packed column, high speed counter current chromatography is a fast and effective way in separation and purification of many natural and synthetic products in past[25]. It is a liquid-liquid separation method with the advantage of less solvent consumption, small amounts of expensive solvents and no loss of the irreversible absorption of solid matrix. It was reported that Z-ligustilide and senkyunolide A (Fig. 1) have been separated from the extract of *L. chuanxiong* Hort[16] as well as chuanxiongzine[26] by high speed counter current chromatography. In this paper, we had successfully separated extract from *L. chuanxiong* Hort by high speed counter current chromatography with the solvent system which was different from the former research.

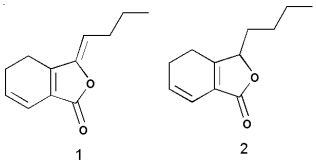

Fig. 1. Structures of phthalides Z-ligustilide (1) and senkyunolide A (2) from *L. chuanxiong* Hort

Hence, the purpose of this study was to establish a method for fractionation of extract from *L. chuanxiong* Hort by high speed counter current chromatography and determined the effects of fractions through bioassays to discover active components in protecting rice against the damage of herbicide S-metolachlor as safeners.

EXPERIMENTAL

Acetonitrile for high performance liquid chromatography (HPLC) was obtained from Tedia. Analytical grade S-metolachlor (97 % pure) was purchased from Nutrichem Laboratory Co. Ltd. (Beijing, China). Other solvents were of analytical grade from Sinopharm Chemical Reagent Co. Ltd. (Beijing, China).

A TBE-300A high speed counter current chromatography instrument (Tauto Biotech, Shanghai, China) was applied in this research with three multilayer coil separation columns (1.6 mm diameter and 280 mL volume) connected in series and a 20 mL sample loop, whose revolution radius and β values is consistent with the description mentioned by literature[27]. The system is equipped with a model TBD-2000 UV detector (Tauto Biotech, Shanghai, China) interfaced with Jinda Biochem chromatography workstation (Biochem Instruments Co. Ltd., Shanghai, China), a TBP-50A pump (Tauto Biotech, Shanghai, China) and LX-300 constant-temperature controller (Scientific Instruments Company, Beijing, China).

The HPLC equipment is Shimadzu system (Shimadzu, Kyoto, Japan), including a SPD-20A Prominence UV/VIS detector, two LC-20AT pump, a Shimadzu LC solution workstation and a C_{18} column of CNW (250 mm length by 4.6 mm diam, 5 μm particle diam).

^{1}H NMR (300 MHz) and ^{13}C NMR (300 MHz) spectra were recorded on a Varian INOVA-300 (Varian, Inc., USA) spectrometer. Mass spectra were obtained by GC-MS on a Hewlett-Packard 5973 Series mass spectrometer interfaced with a Hewlett-Packard 6980 gas chromatograph fitted with a column (HP-5, 30 m length by 0.25 mm diam) or by LC-MS on Agilent 1100 series LC-MSD fitted with a column (ZORBAX Extend-C_{18}, 250 mm length by 4.6 mm diam, 5 μm particle diam).

Supercritical fluid extraction instrument and extract preparation: The applied supercritical fluid extraction Instrument is HA231-50-06 including CO_2-supplying steal bottle, pressure-controlling system ranging from 0 to 50 Mp, temperature controller ranging from 0 to 95 ºC, separator and extraction tank (Huaan Super Critical Extraction Co. Ltd., Nantong, China).

After sieved through sifter (2 mm diam) and dehydrated in freeze-dryer at -40 ºC, sun-dried *L. chuanxiong* Hort purchased from LBX Pharmacy (Changsha, China) was extracted by supercritical fluid extraction under conditions as follows: extraction in the extraction tank (5 L) with CO_2 at 50 ºC and 35 Mp, subsequent separation in the separator from CO_2 at 55 ºC and 8 Mp. The crude extract was stored in -20 ºC, directly used for high speed counter current chromatography separation without going through the time-consuming silica column.

Selection and preparation of two-phase solvent system: The solvent system composed of *n*-hexane/ethyl acetate/ethanol/water (5:5:3:7, v/v/v/v) was chosen to test partition coefficients (K values: upper phase area/lower phase area under the corresponding peaks) for target peaks in crude sample according to the reference[26]. The mixed solvents in separatory funnel were thoroughly equilibrated. Each of the two phases (2 mL) was delivered into a 5 mL capped graduated cylinder, followed by measurement of the settling times *t* of the two phases in terms of the method mentioned by Ito[25]. Then an appropriate amount of crude sample was added into the two phases in the graduated cylinder and gently inverted 5 times. After equilibration, each of the two phases (100 μL) dissolved in 2 mL ethanol was applied to HPLC detector for the determination of K values. Because the K values of the target peaks 1 to 4 were 0.63, 23.6, 12.98 and 24.6 (Table-1), respectively, mostly distributed in the upper phase, several other ratios toward more hydrophobic were tested for the suitable K values[25].

For the determination of the % retention of the stationary phase, the solvent remaining inside the column was totally pumped out by the nitrogen and then collected into a graduated cylinder. The % retention of the stationary phase was estimated by the recovered stationary phase dividing by the column capacity[25].

The selected solvent system used for high speed counter current chromatography was prepared by adding the solvents into the separatory funnel proportionally and equilibrated thoroughly. The two phases were collected separately and degassed for 30 min with sonication prior to use.

Sample solution and separation procedure: The crude product (500 mg) was dissolved in 10 mL upper stationary phase and then a little lower mobile phase was added until the cloudy appearance. The dissolved crude was degassed several minutes and injected into the sample loop after the upper phase was entirely pumped into the spiral column at 10 mL min^{-1}

TABLE-1
K VALUES OF THE TARGETED PEAKS 1–4 IN SEVERAL DIPHASE SOLVENT SYSTEMS

n-Hexane/ethyl- acetate/ethanol/water (v/v)	K_1	K_2	K_3	K_4	a_1	a_2	a_3	Settling time *t* (s)
5:5:3:7	0.63	23.62	12.98	24.6	20.6	1.82	1.04	24
5:4:3:4	0.26	3.19	8.05	15.72	12.27	2.52	1.95	45
5:4:3:3	0.2	1.04	3.8	6.99	5.2	3.65	1.84	42
5:4:3:2	0.18	0.5	2.01	3.4	2.78	4.02	1.7	34

without hydrodynamic equilibrium. When the apparatus was rotated at 900 rpm and 26 ºC and the mobile phase was eluted through the column at 1 mL min^{-1}, the effluent was monitored by the absorption of 254 nm and the peak fractions were manually collected.

Purity detection by HPLC and identification of fractions: The peak fractions from high speed counter current chromatography separation determined by HPLC were performed on a C_{18} column at 35 ºC under the monitoring of 254 nm wavelength. The mobile phase consisted of water and acetonitrile was used in the gradient elution mode as follows: water from 60 % to 52 % for 40 min, from 52 % to 60 % for more 15 min, 60 % for last 5 min. The flow rate was 1 mL min^{-1}. The fractions with high purity of HPLC were further identified by LC/MS, GC/MS, H-NMR and C-NMR.

Rice culture conditions: Rice seeds (*Oryza sativa* L. Zhuliangyou-90: indica type) were purchased from Nongfeng Seed Industry Co. Ltd. (Changsha, China) and germinated in conformity with the literature[5]. The uniformly-selected germinated seeds were sown in the 250 mL beakers containing 150 mL 0.3 % agar medium and grew in the incubator under the 16 h photoperiod of the light intensity of 7500 lux at from 28 to 26.5 ºC.

Bioassay for fractions: For the detoxification experiment in agar medium, the four separated fractions at a series of concentrations ranging from 0.15 to 2.4 mg were tested to reverse the effects on rice caused by 0.03 mg S-metolachlor. S-metolachlor and the combinations of the fractions and S-metolachlor were each dissolved in 1 mL acetone and distributed into 150 mL 0.3 % agar medium and then the germinated seeds were placed in it, replicated three times. The germinated seeds grew for 8 days after the treatment and then the height and roots length of the rice seedlings were measured. Data were the means of three replications ± standard deviation.

RESULTS AND DISCUSSION

High speed counter current chromatography solvent system: The essential oil extracted by supercritical fluid extraction was performed on HPLC with the aforesaid selected condition (Figure 3A) and then several solvent systems were tried to determine the K values of the targeted peaks 1, 2, 3 and 4 by HPLC according to the earlier stated method. The results are showed in Table-1. The solvent system composed of *n*-hexane/ethyl acetate/ethanol/water at the ratio of 5:4:3:2 (v/v/v/v) was selected for the high speed counter current chromatography operation. The acceptable K values found for this solvent system were 0.18, 0.50, 2.01 and 3.40 for the peaks 1, 2, 3 and 4 respectively. This solvent system with the advantage of less water that made it easier to dry fractions was different from that of *n*-hexane/ethyl acetate/methanol/water/acetonitrile at the ratio of 8:2:5:5:3 (v/v/v/v) used to separate the essential oil of *L. chuanxiong* Hort in the previous study[16]. Albeit the settling time of 34 s more than 20 s recommended in literature[25] could lead to the heavy loss of stationary phase, a low eluted flow rate and a high revolution speed could increase the retention of stationary phase. Consequently, the separation of the above four peaks was successfully achieved with the 60 % retention of stationary phase in a preparative run when the mobile phase was eluted through the column at 1 mL min^{-1} and the apparatus rotated at 900 rpm immediately after 500 mg of crude sample in 10 mL stationary phase was injected into the column without the achievement of hydrodynamic equilibrium (Fig. 2). Another important parameter considered was the separation factor that should be more than 1.5 as recommended in literature[25]. The separation factors among four peaks were 2.78, 4.02 and 1.70 (Table-1). Hence, it is easy to separate them. The high purities for the targeted peaks 3 and 4 on HPLC were fulfilled through manually collecting the fractions from the peak slope except for the peaks 1 and 2. The collected fractions were directed for the further bioassay and identification.

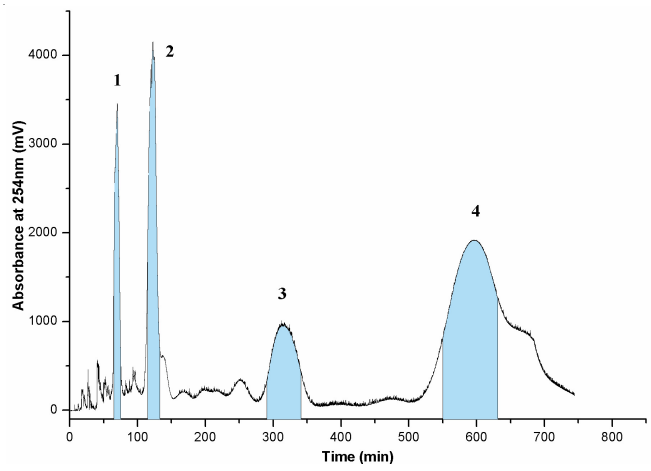

Fig. 2. Fractionation of crude extract from *L. chuanxiong* Hort by HSCCC. The solvent system of *n*-hexane/ethyl acetate/ethanol/water [5:4:3:2 (v/v)] is used to eluted from the head-to-tail at 1 mL min^{-1}, apparatus is rotated at 900 rpm and 26 ºC with the injection of 500 mg of crude extract in 10 mL stationary phase. The % retention of stationary phase is 60 %. Fractions assignment: unknown (1 and 2), senkyunolide A (3) and Z-ligustilide (4)

Identification of the fractions: After a single run of high speed counter current chromatography, the peak fractions 1, 2, 3 and 4 were led to HPLC analysis. The purities of the peak fractions 2, 3 and 4 on HPLC were all over 96 %, while the purity of the fraction 1 were 80 % (Fig. 3). The solvent and water of the fractions 1, 2, 3 and 4 was removed under reduced pressure to give the corresponding yields of 8 mg, 10 mg, 15 mg and 40 mg. The peak fraction 2 which can not be gasified for the analysis of GC-MS was determined by LC-MS. Spectra

showed it was a mixture of two compounds. Therefore, the peak fractions 1 and 2 need the further separation. The peaks 3 and 4 were then identified as senkyunolide A (2) and Z-ligustilide (1) by GC-MS, LC-MS, ^{1}H and ^{13}C NMR, respectively.

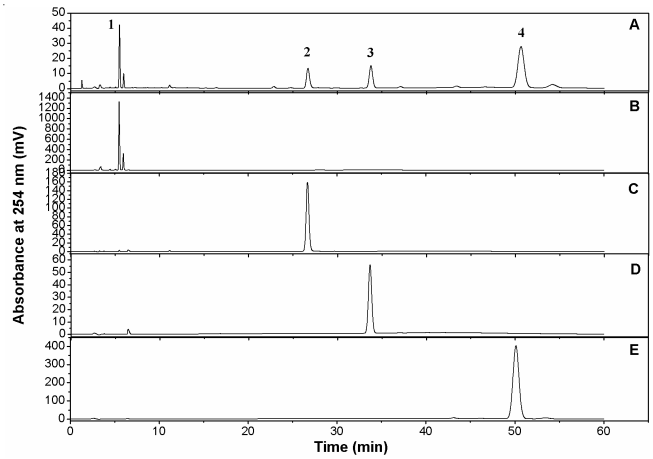

Fig. 3. HPLC chromatograms of crude extract from *L. chuanxiong* Hort (A), unidentified fractions B and C, Z-ligustilide (D) and senkyunolide A (E). Respectively, panels B-E refer to fractions 1-4 shown in Fig. 2

Compound **3** yellowish oil was (S)-3-butyl-4, 5-dihydro-isobenzofuran-1 (3*H*)-one (buty-4, 5-dihydro-phthalide) ($C_{12}H_{16}O_2$). LC-MS (APCI, Pos.): *m/z* 193.1 $[M+H]^+$. GS-MS: *m/z* (relative intensity, %) 192 (27, M^+), 163 (3), 135 (5), 108 (9), 107 (100), 79 (20), 78 (8), 77 (21), 57 (5), 41 (3). ^{1}H NMR (acetone, δ): 6.08 (1H, dt, *J* = 9.6, 2.1 Hz, H-7), 5.91-5.97 (1H, m, H-6), 5.04 (1H, br dd, *J* = 7.2, 3.3 Hz, H-3), 2.55 (2H, d, *J* = 9.0 Hz, H-4), 2.40-2.48 (2H, m, H-5), 1.88-1.99 (1H, m, HH-8), 1.49-1.59 (1H, m, HH-8), 1.31-1.43 (4H, m, HH-9, HH-10), 0.90 (3H, t, *J* = 9.0 Hz, H-11). ^{13}C NMR (D_2O, δ): 171.04 (C-1), 163.15 (C-3a), 129.45 (C-6), 124.46 (C-7a), 117.16 (C-7), 82.87 (C-3), 32.39 (C-8), 29.03 (C-9), 23.03 (C-10), 22.81 (C-4), 21.04 (C-5), 14.13 (C-11). Data is similar to published data[16,18,23].

Compound **4** yellowish oil was (*Z*)-3-butylidene-4,5-dihydroisobenzofuran-1 (3*H*)-one (*Z*-ligustilide) ($C_{12}H_{14}O_2$). LC-MS (APCI, Pos.): *m/z* 191.1 $[M+H]^+$. GS-MS: *m/z* (relative intensity, %) 190 (70, M^+), 161 (100), 148 (80), 134 (15), 133 (14), 106 (31), 105 (40), 91 (10), 78 (19), 77 (20), 55 (33). ^{1}H NMR ($CDCl_3$, δ): 6.28 (1H, dt, *J* = 9.6, 2.1 Hz, H-7), 5.98-6.04 (1H, m, H-6), 5.23 (1H, t, *J* = 8.1 Hz, H-8), 2.62-2.67 (2H, m, HH-4), 2.43-2.51 (2H, m, HH-5), 2.33 (2H, p, *J* = 7.5 Hz, HH-9), 1.50 (2H, p, *J* =7.8 Hz, HH-10), 0.96 (3H, t, *J* = 7.1 Hz, H-11). ^{13}C NMR ($CDCl_3$, δ): 167.62 (C-1), 148.55 (C-3), 147.05 (C-3a), 129.87 (C-6), 123.98 (C-7a), 117.04 (C-7), 12.90 (C-8), 28.10 (C-9), 22.40 (C-5,C-10), 18.51 (C-4), 13.75 (C-11).

Protective effects on rice against S-metolachlor injury: Chloroacetamides including pretilachlor, acetochlor, metolachlor and S-metolachlor are the prominent pre-emergence herbicides which can not be solely applied in rice field to eliminate weed. To extend the use of these herbicides to the rice field against weed, it is of necessity to develop safeners for them. It was reported that safener fenclorim at 150 g ha^{-1} (active ingredient) or higher doses totally reversed the effects of the rates of pretilachlor ranging from 150 to 900 g ha^{-1} (a. i.)[28] and their combination was commercialized in application to kill weed in rice field. But there is no report about developing safeners to protect rice from the damage of others.

In present study, S-metolachlor was proved to inhibit rice growth 8 days after its treatment. The height of S-metolachlor-treated plants was round 70 % of untreated control (Figs. 5A and 4). Moreover, the roots length was also significantly influenced (Fig. 5B and 4), which was similar to the previous report that pretilachlor diminished the roots length of rice seedlings[5]. Aiming to protect rice from the lesion of S-metolachlor, the effects of extracts from different botanical materials were studied previously in our lab, showing that the extract from *L. chuanxiong* Hort distinguishingly protected rice from the damage of S-metolachlor (unpublished). However, little is known about the active compounds of extract and their role in protecting rice from S-metolachlor injury. With expectation to obtain active components, this study examined the protective effects of fractions obtained by high speed counter current chromatography on rice against S-metolachlor. The results demonstrated that *Z*-ligustilide was more active in rice protection against S-metolachlor injury than senkyunolide A (Fig. 5), while fractions 1 and 2 didn't have such activity. When *Z*-ligustilide was applied at the concentrations ranging from 0.15 mg to 0.6 mg, the level of the reversion of inhibitive effects on rice was from 86 % to 100 % of untreated control in plant height as well as from 79 % to 94 % of untreated control in roots length, while its application from 1.2 mg to 2.4 mg led to the decline of protective ability. Senkyunolide A also presented the similarity to *Z*-ligustilide in reversing the effects of S-metolachlor on rice. The results indicated those two compounds at the higher concentration caused toxicity to the rice seedlings. This study has already conformed that phthalides *Z*-ligustilide and senkyunolide A take on role in protecting rice from S-metolachlor injury. Especially, *Z*-ligustilide totally reversed the effects on rice by S-metolachlor at an appropriate rate (0.3 to 0.6 mg), possessing the potential to be developed as a new natural safener.

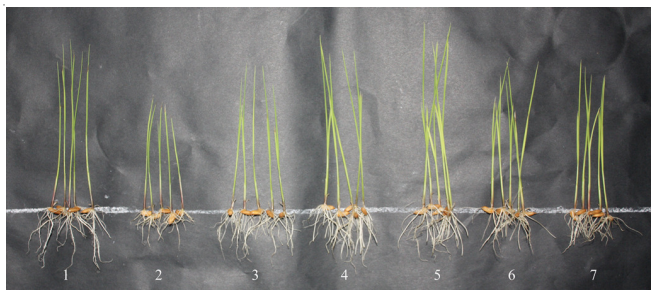

Fig. 4. Activities of *Z*-ligustilide in protecting rice from S-metolachlor injury. Picture showed untreated control (1), S-metolachlor-treated seedlings (2) and seedlings treated with the combinations of S-metolachlor and *Z*-ligustilide (3-7)

According to the relationship between their structures and efficiencies, a clue was found that the sole difference in the bond between C8 and C9 of these two compounds produces disparate efficiencies. That suggests comparison with other similar natural products could guide us to find other might-be active compounds.

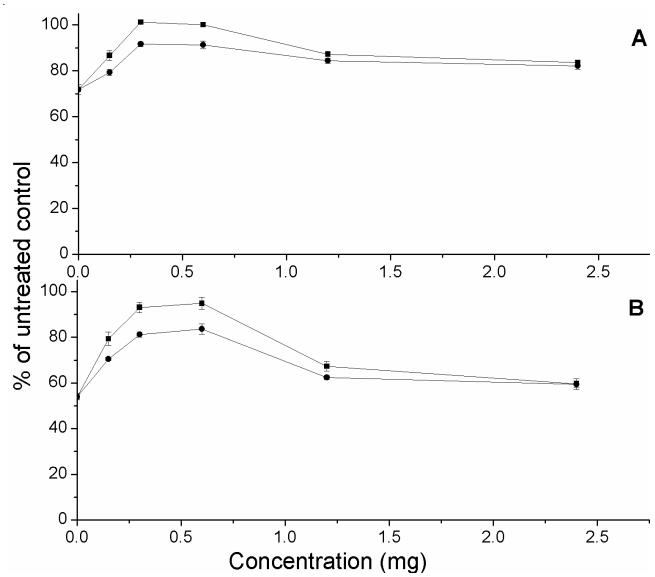

Fig. 5. Activities of *Z*-ligustilide and senkyunolide A in protecting rice from injury of herbicide S-metolachlor. *Z*-ligustilide (■) or senkyunolide A (●) reversed the effects on rice in plant height (A) and roots length (B) that were caused by S-metolachlor. Data shown were the means of three replications ± standard deviation

Conclusion

High speed counter current chromatography is a powerful technique for the separation and purification of compounds *Z*-ligustilide and senkyunolide A, which are newfound to reverse the effects on rice caused by S-metolachlor. Our study on the natural safeners for herbicides from Chinese traditional medicine provides another new path for the research of saferners. The further studies will focus on the development of formulations to improve efficiency and stability and on the mode of action.

ACKNOWLEDGEMENTS

This research was funded by National Natural Science Foundation of China (No. 30971951 and 31171891) and Hunan Leading Academic Discipline Project (No. 090403).

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