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Discovery and Weed Inhibition Effects of Coumarin as the Predominant Allelochemical of Yellow Sweetclover (*Melilotus officinalis*)

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Abstract

This research aimed to isolate, identify and quantify the predominant allelochemical of yellow sweetclover through organic-solvent extraction, chromatography, thin-layer chromatography (TLC), Gas Chromatography-Mass (GC-MS), and Nuclear Magnetic Resonance (NMR), as well as to evaluate its inhibitory effects on weeds through bioassays. The most active allelochemicals of yellow sweetclover were extracted using petroleum ether. A simple coumarin, identified as 2H-1-benzopyran-2-one, was isolated and recognized as the most active allelochemical, and the chemical structure of this coumarin was determined. The coumarin content of original yellow sweetclover extract was 46.78 μg mL^{-1}, which accounted for 1.152% of dry matter of the extract. At 40 μg mL^{-1}, and this coumarin significantly inhibited the seed germination and seedling growth of Italian ryegrass (*Lolium multiflorum* Lam.), common knotgrass (*Polygonum aviculare*), red clover (*Trifolium pratense*), veronica (*Veronica persica*), annual bluegrass (*Poa pratensis* L.), common lambsquarters (*Chenopodium album*), and plantain (*Plantago asiatica*) ($P < 0.05$). At 80 μg mL^{-1}, except for a slight promotion effect on the seed germination of grainamaranth, coumarin exerted significant inhibition effects on both the seed germination and seedling growth of all tested plants ($P < 0.05$). Coumarin also completely inhibited the seed germination and seedling growth of Italian ryegrass, common knotgrass, and red clover. The coumarin 2H-1-benzopyran-2-one was further found to be the predominant allelochemical of yellow sweetclover. This coumarin had strong inhibition effects on seed germination and seedling growth in many weeds. Therefore, coumarin could be used as a natural herbicide. © 2016 Friends Science Publishers

Keywords: Allelochemical; Yellow-sweetclover water extract; Coumarin (2H-1-benzopyran-2-one); Weeds inhibition; GC-MS

Introduction

Synthetic herbicides play important roles in weed suppression in agricultural fields, gardens, and roadsides. However, they can have detrimental effects on crops, groundwater, and soil and human health and cannot effectively control herbicide-resistant weeds (Macías *et al.*, 2001). The allelopathic suppression of weeds is receiving increased attention in recent years as an alternative weed-management tool in sustainable, intensive crop production (Farooq *et al.*, 2011). Numerous crops reportedly possess allelopathic suppression potential for associated weeds. Natural compounds could partially replace synthetic herbicides or to serve as starting materials for the chemical synthesis of biodegradable herbicides (Jabran *et al.*, 2010; Dilipkumar and Chuah, 2013). Allelopathic plants or its allelochemicals are believed to be less harmful to the environment than synthetic herbicides because the former can readily undergo degradation in the environment (Petroski and Stanley, 2009). To date, a number of allelochemicals have been isolated and investigated to develop new natural herbicides (Kelton *et al.*, 2012).

Yellow sweet clover (*Melilotus officinalis)* has been shown to have strong allelopathy potential, as evidenced by the inhibition of seed germination and seedling growth of Italian ryegrass (*Lolium multiflorum* Lam.), veronica (*Veronica persica*), and annual bluegrass (*Poa pratensis* L.) by the yellow sweetclover extract; even stronger than alfalfa and hairy vetch (Wu *et al.*, 2010). Moreover, yellow sweetclover has been used as an alternative weed-suppression agent for a long time. Cultivated as green manure, residues of yellow sweetclover effectively suppress weeds during fallow season and control the perennial weeds dandelion (*Taraxacum officinale*) and perennial sowthistle (*Sonchus arvensis* L.), as well as the annuals kochia (*Kochia scoparia* L.), flixweed (*Descurainia sophia* L.), Russian thistle (*Salsola iberica*), and downy brome (*Bromus tectorum*) (Blackshaw *et al.*, 2001). Killing sweetclover with a wide blade cultivator and leaving residues on the surface could suppress weeds, and in some cases, virtually eliminate these weeds for the rest of the season (Moyer *et al.*, 2007). In addition, yellow sweetclover powdered

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biomass drastically inhibits weed growth, and is used for weed control in crops through intercropping, rotation, or soil mulching (Wu *et al.*, 2010).

The chemical composition of melilotus, particularly its pharmacological characteristics, has been extensively studied. Numerous biological active substances such as coumarin, flavonoids, phenolic acids, triterpenes, and saponins have also been isolated and quantified (Stoker and Bellis, 1962; Kang *et al.*, 1988). However, reports on allelopathic active substances of yellow sweetclover are few, and only some phenolics and polar compounds have been isolated and identified as potential allelochemicals of yellow sweetclover (Macías *et al.*, 1998; Macías *et al.*, 1999). To date, the predominant or most active allelochemical in yellow sweetclover is still unknown.

In the present work, organic-solvent extraction, column chromatography, and thin-layer chromatography (TCL) were used to isolate allelochemicals from yellow sweetclover with strong phytotoxic effects. Then, Gas Chromatography-Mass (GC-MS) and nuclear magnetic resonance (NMR) were used for the qualitative analysis of these allelochemicals. Eight weed seeds were collected and used as recipient plant in bioassays. We aimed to determine the predominant or most active allelochemical of yellow sweetclover and evaluate its capacity to inhibit weed growth.

Materials and Methods

Plant Material

Yellow sweetclover was grown on the research farm of Yangzhou University in Yangzhou, China from October 2011 to June 2012. The farm located in east longitude 119°22′ to 119°25′, latitude 32°20′ to 33°23′ (Fig. 1). Stems and leaves of the plants in the flowering stage were collected to produce an extract. Italian ryegrass was used for the bioassay to determine the biological activity of the isolated fractions of yellow sweetclover extract. Seeds of Italian ryegrass, barnyard grass (*Echinochloa crusgalli* L.), veronica, red clover (*Trifolium pratense*), common lambsquarters (*Chenopodium album*), grain amaranth (*Amaranthus hypochondriacus*), common knotgrass (*Polygonum aviculare*), plantain (*Plantago asiatica*), and annual bluegrass (*Poa annua* L.) were also used as test plants for the bioassay of the allelopathic activity. Seeds of Italian ryegrass and yellow sweetclover were provided by Clover Co., Ltd. Beijing, China. Seeds of grain amaranth, barnyard grass, veronica, red clover, common lambsquarters, common knotgrass, plantain and annual bluegrass were collected in the wild from June to October 2011.

Water Extraction of Yellow Sweetclover

Leaves and stems (100 g, fresh weight) of yellow sweetclover were cut into small pieces (2 cm length) and soaked in 1000 mL of pure water for 72 h at 4°C. The water extract was filtered by filter paper (pore size: 30 μm) and membrane (pore size: 0.45 μm). The filtrate was stored at 4°C for extraction by organic solvent. The filtrate was used as the water extract of yellow sweetclover.

Isolation of the Most Active Allelochemical in Yellow-sweetclover Water Extract

Allelochemicals were isolated from yellow-sweetclover water extract according to the method of Bertin *et al.* (2003) with modifications. Yellow-sweetclover water extract (10 L) was rotary evaporated (45°C, −0.1 MPa) into a 1 L concentrate. The concentrate was then successively extracted using petroleum ether, ethyl acetate, and *n*-butanol. These three organic solvents had increasing polarities. Each organic solvent was extracted thrice, and the same organic fractions were combined. After collecting the residues, water in these organic extracts was removed using anhydrous sodium sulfate. The residue, the organic-solvent extracts, and some of the initial yellow sweetclover extract were then evaporated to dryness. Part of each dried fraction was used to make a 100 μg mL^{-1} solution to determine the biological activity by bioassay, whereas the other parts were prepared for further isolation and purification.

According to the bioassay results (Fig. 2), the fractions showing strong biological activity, including petroleum ether and ethyl-acetate extracts, were further isolated by chromatography on a silica gel column and then eluted. The petroleum-ether extract was chromatographed on a silica-gel column (30 × 450/24; 300–400 mesh; wet-packed column) and eluted with an eluent composed of an ethyl acetate and petroleum ether mixture at a 1:5 ratio. The petroleum-ether extract was chromatographed on a silica-gel column (30 × 450/24; 300–400 mesh; wet packed column) and eluted with the eluent of a mixture of ethyl acetate and petroleum ether at a ratio of 2:3.

The components of the eluents were classified by TCL color results on silica-gel plates and detected using an UV analyzer (Ghafar *et al*., 2001). The same class of fractions were collected and combined. The biological activity of each fraction was evaluated by bioassay, and the fractions showing higher biological activity was repeatedly chromatographed until the components in the fractions cannot be further isolated. Subsequently, the most active fraction was prepared for qualitative analysis by GC-MS detection.

Bioassay for Biological Activity of Each Extraction Separated from Yellow-sweetclover Water Extract

Italian ryegrass seeds were sterilized by soaking in 1% NaClO solution for 30 min. Sterilized seeds were then washed six times with distilled water. Fifty sterilized Italian ryegrass seeds were sown in a Petri dish fitted with quartz sand, and 10 mL solution was added to each dish.

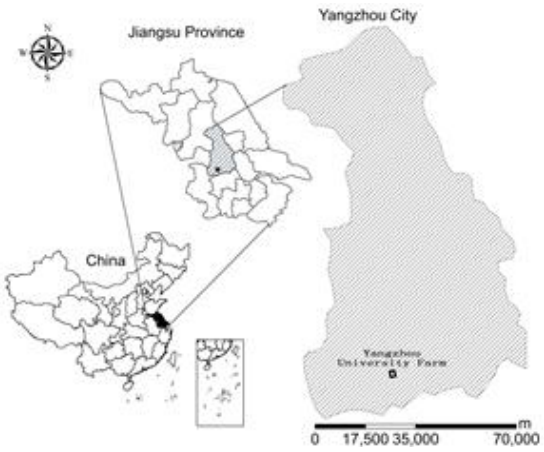

Fig. 1: Location of experimental site, Yangzhou University Farm

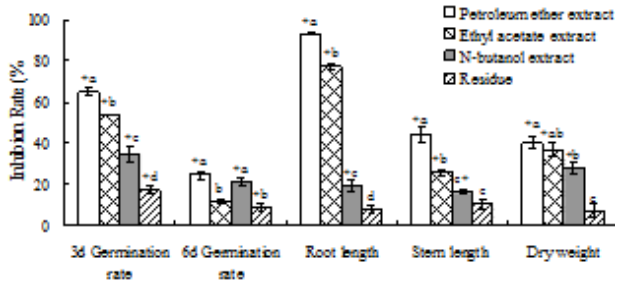

Fig. 2: Inhibition rate of seed germination and seedling growth of Italian ryegrass treated with 100 μg·mL^{-1} solvents of different organic extract of yellow-sweetclover water extract

Values with * indicate statistical differences between the treatment and control (P < 0.05). Values with different letters indicate statistical differences among the treatments at (P < 0.05)

The dishes were in randomly arranged in an illumination incubator (25°C; light: 4000 lx; 12 h/d). Germination rate was calculated after sowing for 3 and 6 d. On d 6, 10 seedlings were randomly selected from each dish. The root and stem lengths of each seedling were determined, and then all 10 seedlings were wrapped in filter paper, dry weighed, and dried for 48 h at 70°C. After cooling in a desiccator, the paper bags with seedlings were weighed and the average dry weight of each seedling was calculated. Inhibition rate of seed germination, stem and root length and dry weight were calculated. Each treatment had three replicates.

$$\text{Inhibition rate} = \frac{(\text{Control value} - \text{Treatment value})}{\text{Control value}} \times 100$$

Qualitative Analysis

A 30 mm × 0.25 mm × 0.32 μm silica capillary column (Stabilwas-DA) was used. The initial temperature was 50°C, which was maintained for 3 min, and then increased to 300°C at a speed of 10°C/min, and then heated to 330°C at a speed of 20°C/min. The injection volume was 1 μL. Each sample was measured thrice by GC-MS to check the repeatability of the process. ^{1}H- and ^{13}C-NMR spectra were recorded with a NMR spectroscopy system (AVANCE 600 Superconducting Ultra Shield TM Fourier-Transform).

Coumarin Quantification

Concentrations of coumarin in yellow-sweetclover water extract and petroleum-ether extract was determined by LC-MS (Agilent 6460) with a C_{18} column (4.6 mm × 250 mm×5 μm). The mobile phase was a mixture of methanol and water (65:35). Detection wavelength was 276 nm, and column temperature was 35°C. The flow rate was 1 mL/min.

Bioassay of the Allelopathic Biological Activity of Coumarin

After sterilization in 1% NaClO solution for 30 min, 50 seeds of recipient weeds were sown in each glass Petri dish containing quartz sand. A coumarin solution or yellow-sweetclover water extract (10 mL) was added to each Petri dish. In control experiments, 10 mL of distilled water was used. Inhibition rates of seed germination was determined after incubation for 3 and 6 d at 25°C with light of 4000 lx at 12 h/d. Inhibition rates of seedling root elongation, stem elongation, and dry weight were determined 6 d after sowing. Each treatment had three replicates. Petri dishes were in a complete random arrangement.

Statistics

Bioassay data were analyzed by one-way ANOVA using SPSS software (Ver. 16.0) for Windows.

Results

Biological Activity of Different Organic Solvent Extracts of Yellow-sweetclover Water Extracts

Petroleum-ether, ethyl-acetate and n-butanol extracts significantly inhibited seed germination and seedling growth of Italian ryegrass (P < 0.05; Fig. 2). Petroleum-ether extract showed the strongest inhibition effect, followed by ethyl-acetate extract. Inhibition rates of germination rate of 3 d and seedling root length of 6 d of Italian ryegrass treated by petroleum ether extract were 65.14% and 93.75%, respectively; significantly higher than the treatments of ethyl-acetate extract, *n*-butanol extract and residue (P<0.05). The *n*-butanol extract and the treatments exhibited weaker inhibitory effects, especially residue treatment, which had the lowest inhibition rate on seed germination and seedling growth of Italian ryegrass and

were significantly lower than the other treatments. Results indicated that after extraction using the three organic solvents with increasing polarity, the most active allelochemical was retained in the petroleum ether. Therefore, the petroleum-ether extract was further isolated and analyzed.

Isolation and Identification of the Predominant Allelochemical in the Petroleum-ether Extract

After three times of chromatography and elution through a silica gel column, the most active fraction that cannot be further isolated was obtained. This fraction was collected, evaporated to dryness, and then analyzed with GC-MS. Results showed that this fraction has only one detectable major peak, which was preliminarily identified as coumarin.

This coumarin was further characterized by NMR. ^{1}H-NMR (600 MHz, CDCl3): δ 7.69 (d, J = 9.5 Hz, 1H), 7.52 (t, J = 7.8 Hz, 1H), 7.47 (d, J = 7.7 Hz, 1H), 7.32 (d, J = 8.3 Hz, 1H), 7.26 (t, J = 7.5 Hz, 1H), 6.41 (d, J = 9.5 Hz, 1H). ^{13}C-NMR ($CDCl_3$, 151 MHz): δ 161.56, 154.32, 143.61, 132.05, 128.07, 124.64, 119.08, 117.16, 116.97 ppm.

By comparing with literature data, the substance was identified as 2H-1-benzopyran-2-one, which is a simple coumarin. The chemical structure of this coumarin is shown in Fig. 3.

Coumarin-Content Determination

LC-MS results showed that coumarin concentration in the initial yellow-sweetclover water extract was 46.78 μg mL^{-1}. Moreover, 2.03 g of dry matter was obtained after rotary evaporation of 500 mL of the water extract, which meant that coumarin accounted for 1.152% of the dry matter of yellow-sweetclover water extract. Coumarin content in the dry matter of petroleum-ether extract was 820.13 μg·mg^{-1}, which accounted for 82.013% of the dry matter of petroleum-ether extract.

Allelopathic Activity of the Coumarin

Coumarin concentration in yellow-sweetclover water extract was 46.78 μg mL^{-1} (Table 1). Accordingly, 20, 40, 60, 80 and 100 μg mL^{-1} coumarin solutions were assayed for their allelopathic activity. Results showed that At 20 μg mL^{-1}, coumarin had no significant effect on the seed germination of Italian ryegrass, but significantly inhibited both seedling root and stem lengths, with inhibition rates of 22.54% and 81.29%, respectively (Fig. 4). At 40 μg mL^{-1}, coumarin significantly inhibited not only the seed germination but also the root and shoot lengths of Italian ryegrass ($P < 0.05$). All inhibition rates exceeded 50%. At ≥ 80 μg mL^{-1}, both seed germination and seedling growth of Italian ryegrass were almost completely inhibited by coumarin.

With a concentration of 46.78 μg mL^{-1} of coumarin, the inhibition effect of yellow-sweetclover water extract on the seed germination of Italian ryegrass was equivalent to the 60 μg mL^{-1} coumarin (Fig. 4), whereas the inhibition effect of yellow-sweetclover water extract on the root length of Italian ryegrass was equivalent to 40 μg mL^{-1} coumarin. Varieties of allelochemicals existed in the yellow-sweetclover water extracts, and coumarin accounted for only 11.52%. Antagonistic and synergistic effects possibly existed simultaneously in the yellow-sweetclover water extracts, but the combined effects of all these allelochemicals were almost equivalent to the same coumarin concentration.

Table 1: Coumarin concentration

yellow-sweetclover water extract (μg mL^{-1})	Petroleum-ether extract of yellow-sweetclover water extract (g mg^{-1})
46.78±0.0760	820.13±34.06

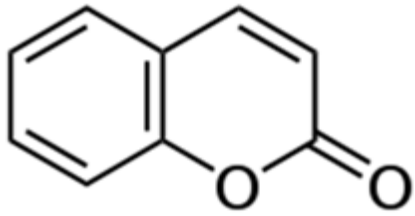

Fig. 3: Chemical structure of coumarin

Effect of Coumarin on the Seed Germination and Seedling Growth of Eight Weeds

Based on the effect of coumarin on Italian ryegrass, 40 and 80 μg mL^{-1} coumarin were used for weed-inhibition tests on eight weeds (Fig. 5 and 6). Results showed that coumarin had the strongest effect on the seed germination and seedling growth of common knotgrass and red clover. At 40 μg mL^{-1}, the inhibition rates of 3 d germination of common knotgrass and red clover were 100% and 95.8%, respectively and the inhibition rates of 6 d germination were 79% and 96.63%, respectively followed by veronica, common lambsquarters, plantain, and annual bluegrass (Fig. 5). For barnyard grass, no significant inhibition in seed germination was found at 40 μg mL^{-1}, but significant inhibition in root length and seedling dry weight was observed. However, at 40 μg mL^{-1}, coumarin showed significantly higher seed germination of grain amaranth. At 80 μg mL^{-1}, coumarin showed significant inhibition effects on both seed germination and seedling growth of all the tested plants, except for a slight stimulation of seed germination of grain amaranth ($P < 0.05$; Fig. 6). These results indicated that coumarin had strong inhibition effects on a considerable number of weeds. However, different plants showed different degrees of sensitivity to the phytotoxic activity of coumarin.

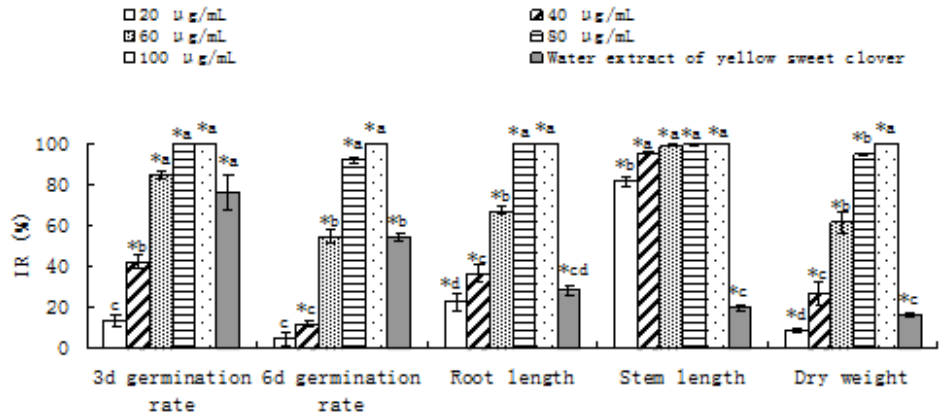

Fig. 4: Inhibition rates of different concentrations of coumarin on seed germination and seedling growth of Italian ryegrass
Values with * indicate statistical differences between the treatment and control ($P < 0.05$). Values with different capital letters indicate statistical differences among the treatments ($P < 0.05$)

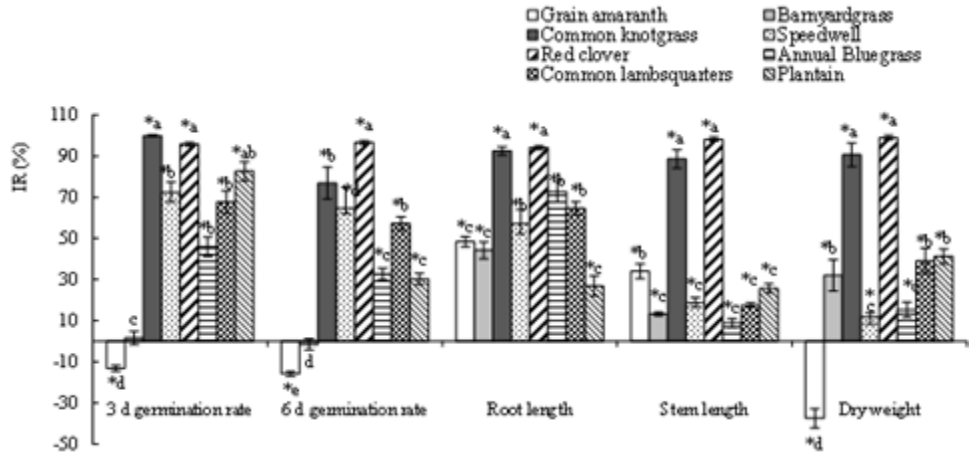

Fig. 5: Effect of 40 μg·mL^{-1} coumarin on the seed germination and seedling growth of eight weeds
Note: Values with * indicate statistical differences between the treatment and control ($P < 0.05$). Values with different capital letters indicate statistical differences among the plant species ($P < 0.05$)

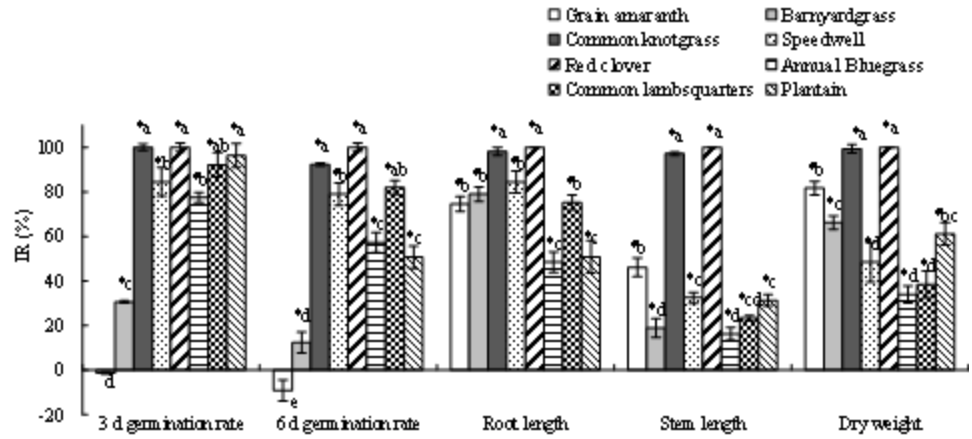

Fig. 6: Effect of 80 μg·mL^{-1} coumarin on the seed germination and seedling growth of eight weeds
Note: Values with * indicate statistical differences between the treatment and control ($P < 0.05$). Values with different capital letters indicate statistical differences among the plant species ($P < 0.05$)

Discussion

Allelochemicals are an integral part of allelopathy research, which is incomplete until the allelochemicals present under specific experimental conditions are isolated, identified and characterized. In the present study, we isolated and identified the predominant allelochemical of yellow sweetclover. In numerous studies on the extraction, isolation, and identification of allelochemicals, organic solvents such as methanol and ethanol have been used to soak the plant to extract more allelochemicals. Although such methods could extract plants chemical substances including allelochemicals, some of the extracted substances are not released into the environment under natural conditions to show allelopathic activity and thus cannot be called allelochemicals. Under natural conditions, plants release allelochemicals into the environment by root exudation, leaf and stem leaching, volatilization, stump decomposition and other natural ways. Therefore, the extraction of plant allelochemicals using water is better than using organic solvents (Salam and Kato-Noguchi, 2011). Accordingly, in the present work, allelochemicals of yellow sweetclover extracted by water was set as the basic research condition for isolation and identification.

Allelochemicals are the products of secondary metabolism and are non-nutritional primary metabolites (Weir *et al.*, 2004; Iqbal and Fry, 2012). These compounds belong to numerous chemical groups including trike-tones, terpenes, benzoquinones, coumarins, flavonoids, terpenoids, strigolactones, phenolic acids, tannins lignin, fatty acids and non-protein amino acids (Soltys *et al.*, 2013). Among these compounds, coumarins are known as a large group of plant secondary metabolites mainly from the shikimic-acid pathway. These compounds are widely distributed in the Fabaceae, Apiaceae, Rutaceae, and Asteraceae families of plants. Coumarins exist in plant roots, stems, leaves, flowers, fruits, and seed coats. Most of their functions are related to plant self-protection, such as anti-microbial functions, grazing prevention, ultraviolet shielding, germination inhibition of surrounding plants, and so on. The compounds are divided into two subgroups, namely, simple and furano coumarins. In the present work, a simple coumarin (Fig. 3) was isolated and identified as the most active allelochemical in the yellow-sweetclover water extract.

Coumarin is often reported for its allelopathic activities (Razavi, 2011; El-Shahawy and Abdelhamid, 2013; Mahmood *et al.*, 2013), including antibacterial, nematocidal, and insecticidal activities, and phytotoxic activity on other plants. Some coumarins exert strong antibacterial effects on animal pathogen strains (Razavi *et al.*, 2009a, b). Imperatorin, a prenylated fouranocoumarin, exhibits antifungal activity and entirely inhibits mycelial growth of a fungus at a concentration of 1000 μg mL^{-1} (Razavi *et al.*, 2010a). Some psoralen derivatives such as 8-methoxy, 5-methoxy, 5,8-dimethoxy, and 5-geranyloxy psoralen have been shown to have insect-antifeedant ability (Stevewnson *et al.*, 2003). Some studies have demonstrated the phytotoxic activity of some Rutaceae plants, such as *Esebeckia yaxhoob* and *Stauranthus perforatus* (Mata *et al.*, 1998; Anya *et al.*, 2005), as well as Apiaceae species, such as *Prangos uloptera* and *Zosima absinthifolia* (Razavi *et al.*, 2009a; Razavi *et al.*, 2010a). This activity had been confirmed due to the presence of coumarin in their plants. Coumarin has also been discovered as the predominant allelochemical in *Gliricidia sepium*, which was reported to possess high total activities for inhibition of radicle growth in various plants (Takemura *et al.*, 2013).

Inhibition of seed germination of other plants is one of the most common allelopathic strategy employed by the yellow sweetclover (Wu *et al.*, 2010; Wu *et al.*, 2015). In the same way, coumarin can inhibit the seed germination of several plants. Two simple coumarins, namely, 7-prenyloxy coumarin and auraptene, entirely stunt the seed germination, root, and shoot growth of lettuce at >100 μg mL^{-1} (Razavi *et al.*, 2010b). Aviprin, a oxyprenylated furanocoumarin, has indicated phytotoxic activity against lettuce and entirely suppresses seed germination at 500 μg mL^{-1} (Razavi *et al.*, 2010c). Xanthyletin, a pyranocoumarin, shows very high phytotoxic activity on the seed germination of *Amaranthus hypochondriacus* with IC_{50} values of 59.9 and 69.5 μg mL^{-1} (Anya *et al.*, 2005). At 10 μM to 100 μM, coumarin inhibits the seed germination of Bidens (*Bidens pilosa* L.) to different levels; at lower concentrations, coumarin as a cytostatic delays the seed germination of Bidens (Pergo *et al.*, 2008). In the present study, we found that coumarin apparently delayed the seed germination of all tested plants (Fig. 5). In addition, different plants showed different degrees of sensitivity to the phytotoxic activity of coumarin, which was consistent with the reports of Pergo *et al.* (2008).

Apart from inhibiting seed germination, coumarin also evidently inhibited plant seedling growth, particularly the root growth of other plants. Imperatorin significantly reduced the root and shoot growth of lettuce at >100 μg mL^{-1}, and the effect on roots was more pronounced (Razavi, 2011). It was found that the root length of corn exponentially decreased with increasing coumarin concentration (Hegde and Miller, 1992). Furthermore, 10^{-3} M of coumarin could significantly inhibited the root growth of alfalfa and barnyard grass with inhibition rates reaching more than 95% (Chon *et al.*, 2002). Although, Inhibition of seedling growth, especially the root growth of other plants, is another common allelopathic strategy employed by the yellow sweetclover (Wu *et al.*, 2010; Wu *et al.*, 2015). In our previous study, yellow clover water extract also had significant effect on root length of red clover, annual bluegrass, grain amaranth, speedwell, barnyardgrass, common knotgrass, common lambsquarters and Chinese ixeris (*Ixeris chinensis*) ($P < 0.05$). In the present work, low-concentration (40 μg mL^{-1}) coumarin significantly inhibited the root growth of all eight tested weeds. Inhibition rates of root length of common knotgrass and red clover reached

92.56% and 94.21%, respectively and inhibition rates of root length of grain amaranth, veronica, annual bluegrass, and common lambsquarters also exceeded 50%. At a high concentration (80 μg mL^{-1}), inhibition rates of root length of all eight tested weeds exceeded 50%.

Effects of coumarin inhibiting plants seed germination and seedling growth exactly consistent with the effect of yellow-sweetclover water extract on other plants. (Wu *et al*., 2015). In our another work, it was found that coumarin was the highest allelochemical contents in the ethyl-acetate extract of yellow-sweetclover water extract, which also showed strong inhibitoy activity that second only to the petroleum-ether extract. And coumarin contributed greatly to the inhibitory effect of the ethyl-acetate extract (Wu *et al*., 2014). These results demonstrate that coumarin plays a predominant role in the yellow sweetclover inhibitory activity.

Nowadays, successful weed control using synthetic herbicides is accompanied by negative effects on environments and humans; moreover, weed species ultimately and rapidly develop resistance to specific herbicides, which has led to cross-resistance within entire chemical classes, underlining the constant need for natural chemicals (Reigosa *et al.*, 2006). Therefore, some coumarins are promising potential bioherbicides with their new target sites (Razavi, 2011). Most allelopathins are also totally or partially water soluble, which makes them easier to apply without additional surfactants (Vyvyan, 2002; Dayan *et al.*, 2009). The chemical structure of coumarins is also more environmentally friendly than synthetic herbicides, because the former possess higher oxygen- and nitrogen-rich molecules with relatively few "heavy atoms" as halogen substitutes and without "unnatural" rings. These properties decrease a chemical's environmental half-life, thus preventing the accumulation of the compound in soil and eventually inhibiting effects on non-target organisms (Razavi *et al.*, 2010c). Based on these characteristics, coumarin could be used as a natural herbicide in various ways (Pergo *et al.*, 2008). For example, using plants with high coumarin content for intercropping or crop-rotation systems to reduce weeds, which are already in practice in yellow sweetclover. Thus, extraction methods should be improved to obtain higher coumarin content of plant extracts that could be directly applied to fields for weed control. Coumarin could also be applied after abstracting from plants. In future studies, we will test for more weeds to clarify the scope of weed control of coumarin and determine its effective doses.

Conclusion

Extract of the petroleum-ether of yellow-sweetclover water extract inhibited seed germination and seedling growth of Italian ryegrass most among all the organic solvents extraction. A most inhibitory compound was isolated from the petroleum ether extract and identified as coumarin by GC-MS and NMR. Authentic coumarin had a concentration of 46.78 μg mL^{-1} in the crude water extract. Authentic coumarin showed significant inhibition effect on several weeds seed germination or seedling growth. Coumarin is the most active allelochemical of yellow sweetclover and plays a predominant role in the yellow sweetclover inhibitory activity. Use of coumarin as a natural herbicide is very promising.

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