Bioherbicidal activity of a germacranolide sesquiterpene dilactone from *Ambrosia artemisiifolia* L.

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Abstract

Ambrosia artemisiifolia L. (common ragweed) is an invasive plant which allelopathic properties have been suggested by its field behaviour and demonstrated through phytotoxicity bioassays. However, the nature of the molecules responsible for the allelopathic activity of commom ragwed has not been explored. The main objective of this study was to identify the phytotoxic molecules produced by A. *artemisiifolia.* A preliminary investigation has indicated that a methanol extract of A. *artemisiifolia* completely inhibited the germination of cress and radish. Semi-preparative fractionation of the methanol extract allowed separating the phytotoxic fraction which contained a single compound. The structure of this compound was elucidated by LC-MS/MS, HRMS, NMR and FTIR as the sesquiterpene lactone isabelin ($C_{15}H_{16}O_4$). The effect of pure isabelin was tested on four different weed species, confirming the inhibitory activity of the molecule. The results indicate directions for future studies about herbicidal specific activity of isabelin, as pure molecule or in the crude extract, as potential candidate for biological weed control.

Keywords: Allelopathy, allelochemical, herbicide, *Ambrosia artemisiifolia L.*, ragweed, isabelin.

Introduction

Allelopathy is a phenomenon in which a plant, by the release of a chemical product (allelochemical), usually deriving from the secondary metabolism, directly or indirectly interacts with the organisms in the surrounding environment.[1] Allelochemicals, in particular those with herbicidal action, could lead to new molecules useful for the chemical industry, due to the emergence of resistant weeds to older synthetic molecules and to the necessity for less harmful compounds for the environment.[2-4]

Common ragweed (*Ambrosia artemisiifolia* L.) is an annual weed indigenous of North America, imported to Europe since the 18th century. Besides being an agricultural weed, it is a pioneer colonizer of urban exposed or abandoned lands.[5] Today *A. artemisiifolia* is a stable weed in North America, [6] while it is an important invader in several European countries such as France, [7,8] Hungary, [9,10] Austria, [11] as well as in China. [12,13] In addition to the invasive ability, it is well known due to its highly allergenic pollen [14,15] which causes severe problems to the population with allergy sensitivity. Allelopathic activity of *A. artemisiifolia* could be one of the reasons for its invasive success, as supported by the "novel weapon hypothesis", [16] the production of phytotoxic molecules can advantage the invader against the native species, which did not evolve specific defences. Different studies assessed the allelopathic activity of *A. artemisiifolia* in field and greenhouse experiments. [10,17,18] In a previous study conducted in our department, [19] the relationship between allelopathic behaviour of *A. artemisiifolia* and the invasiveness potential against crops and weeds was reported: ragweed plants residues influenced the yields of crops such as tomato and lettuce, and the germination of the weed *Digitaria sanguinalis*. Although the allelopathic behaviour of *A. artemisiifolia* has been largely demonstrated, little is known concerning the nature of the allelochemicals responsible for its potential phytotoxicity. Shetty et al.[20] attributed the inhibition of the growth of Brazilian pepper to the thiarubrine-A found in the ragweed roots.

The aim of this work was to check the action of ragweed extracts on the germination of sensible seeds, isolate the toxic fraction from common ragweed extract, with the purpose of identifying the compounds responsible for the inhibitory effect, and test the phytotoxic activity of the pure molecules against different weed families.

Materials and methods

Chemicals

All reagents were analytical or LC-MS grade and were obtained from Sigma-Aldrich, Milan, Italy.

Plant material

Ambrosia artemisiifolia L. (common ragweed) plants (shoots and roots) were collected in the campus of Turin University located in Grugliasco (45°04'02.8"N; 7°35'37.9"E), prior to the flowering, from streets and field edges, where the plant was widespread and forming big colonies. Samples were stored in plastic bags in freezers at -20°c. Cress seeds (*Lepidum sativum* L.) were purchased from Green Paradise s.r.l. (Milan, Italy). Radish seeds (*Raphanus sativus* L. var. Saxa 2) were purchased from OBI (Milan, Italy). Rapeseed (*Brassica napus* var. Helga), red clover (*Trifolium pratense* var. Altaswede) and darnel (*Lolium italicum* var. Barmultra) seeds were purchased from Biasion (Bolzano, Italy).

Extraction of plant material

Crude ragweed extract was obtained from whole plants, cleaned from soil residues and chopped with an electric mixer. Subsamples of plant (50 g) were extracted with 100 mL solvent, on an orbital shaker (120 rpm), for 24 hours at room temperature. The tested solvents were methanol, ethyl ether and n-hexane.

Germination test

The phytotoxicity of the crude extracts as whole or after fractionation was evaluated through germination tests on cress and radish and, in some cases, on darnel, red clover and rapeseed. Round filters placed in 35 mm i.d. Petri dishes were moistened with 300 µL of plant extract or purified

solution. After evaporation of the solvent, 150 µL water was added in each dish and 25 seeds were placed on the filter paper in five separated batches per condition. The Petri dishes were closed and germination was conducted in germination chamber at 25 °C for 48 h, with a light/darkness cycle of 16/8 hours. Petri dishes moistened with demineralized water alone or pre-treated with extraction solvent were used as control. The germination index (I_g) was calculated by the number of germinated seeds (n) and the mean length of the radicle (m) in sample (s) and control (c), according to equation 1.

$$Ig = \frac{n_s * m_s}{n_c * m_c} * 100 \qquad (1)$$

Purification of the crude extract

The crude methanol extract was fractionated by elution on C_{18} SPE column (Sigma Aldrich, Milan) with different polarity solutions. Each fraction was then tested for its phytotoxicity on cress and the active fractions were analysed by liquid chromatography, UV detection, using a Spectraphysic P2000 liquid chromatograph equipped with a C_{18} SupelcolSil (25 cm, 4.6 mm, 5 µm) column, a 10 µL Rheodyne injection valve and a UV-100 detector set at 220 nm. The mobile phase was water acidified to pH 3 with H_3PO_4 (A) and acetonitrile (B), in a 75% A, 25% B ratio with a flow of 1 $mL.min^{-1}$.

In order to obtain higher amounts of the active fractions the SPE fractionation was substituted by a semi-preparative chromatography using the LC system described above but with a semi-preparative column ($^{®}C_{18}$, 10x150 mm, GL Sciences column, Milan, Italy), a 2mL Rheodyne injection valve and a flow of 4 $mL.min^{-1}$.

A liquid-liquid purification of the crude extract was also conducted prior to semi-preparative chromatography in order to eliminate the large amounts of polar compounds interfering with the chromatographic separation: 30 mL of methanol crude extract were brought to dryness, re-dissolved in the same volume of H_2O acidified with 0.2% H_3PO_4 and LL extracted with 30 mL of dichloromethane. The dichloromethane fraction was dried by rotavapor and re-dissolved in 3 mL acetonitrile, obtaining a ten-fold concentration of the purified extract.

LC-MS/MS analysis

LC-MS system was a Varian MS-310 triple quadrupole mass spectrometer equipped with an ESI source, and 212 LC pump (Agilent, Milan, Italy). Separation was performed on a Luna C_{18} column, 5 μm particle size, 50 × 2.0 mm (Phenomenex, Torrance, CA, USA). The mobile phase solvents were water (A) and acetonitrile (B), both containing 0.1% (v/v) acetic acid. The mobile phase gradient was from 90 to 10% A in 10 min with a flow rate of 0.2 mL/min. ESI conditions used in negative polarization were: needle potential -3500V, shield -500V, capillary -30V. Gas conditions were set with 20.0 psi of air as nebulizing gas and 25.0 psi at 300°C N_2 as drying gas. The respective ion transitions were as follows: m/z 259 → 147.1 (collision energy 16.5 V), m/z 259 → 170.8 (collision energy 12.0 V), m/z 259 → 214.6 (collision energy 9.5 V). The m/z 214.6 ion was used for quantification..

LC-HRMS analysis

High-resolution mass spectral data were obtained using a LC Ultimate 2000 system (DIONEX, Thermo Fisher Scientific Inc., Massachusetts, USA) equipped with a Luna C18 column, 5 μm particle size, 50 × 2.0 mm (Phenomenex, California, USA) connected to a LTQ Orbitrab (Thermo-

Fisher Scientific Inc., Massachusetts, USA) with a ESI source in negative ionisation mode. Separation was carried out using same condition as for LC-MS/MS Analysis. ESI source conditions were: negative polarization mode, capillary temperature 270°C, source voltage 3500V; with sheath and auxiliary gas flows respectively set on 35 and 15 psi.

NMR analysis

A Bruker Avance 300 (300 MHz and 75 MHz for ^{1}H and ^{13}C, respectively) was used. Spectra were registered in $CDCl_3$, at 25°C, and chemical shifts were calibrated to the residual $CHCl_3$ proton and carbon resonances.

FTIR analysis

Infrared spectra (FTIR) were recorded as a thin film, using CH_2Cl_2 as a solvent, and the Spectrum BX FT-IR System (Perkin Elmer, Milan, Italy).

Statistical analysis

Statistical analysis of the germination tests conducted on crude extracts, different fractions obtained from preparative and inhibition curve was performed through ANOVA, using R-E-G-W-Q as post-hoc test. Differences between crude extracts and pure allelochemical were evaluated by Mann-Whitney test. Statistics were performed using GraphPad Prism for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

Results and discussion

171

172 ***Phytotoxicity of crude extracts***

173

174 The results of the germination test performed on the crude extracts compared with those of the pure
175 solvents are reported in Table 1. The crude methanol and ethyl ether extracts completely inhibited
176 the germination of both cress and radish while the n-hexane extract did not have any significant
177 effect. These results suggest that the phytotoxicity was due to medium-high polarity compounds
178 insoluble in n-hexane, therefore this solvent has been excluded from further investigation.
179 Considering that both methanol and ethyl ether extracts gave the same results, further tests have
180 been conducted using methanol extract, due to its lower toxicity in comparison to ethyl ether.

181

182 ***Purification and identification of the phytotoxic compound***

183

184 The crude methanol extract (1 mL) was fractionated on a C_{18} SPE eluted with methanol. The
185 fractions were collected according to their colour and tested for their phytotoxicity through
186 germination test. A single toxic fraction, eluted between 2.4 and 2.8 mL elution volume, was found.
187 A LC-UV investigation pointed out that this contained too many compounds to allow a chemical
188 characterisation without further purification.
189 The toxic fraction obtained by SPE was brought to dryness in rotavapor and diluted in 2 mL
190 acetonitrile in which it was completely soluble. The solution was then loaded on the SPE cartridge
191 and eluted with a acetonitrile-water solution (1:4, V/V), collecting ten fractions of 1 ml eluate. Each
192 fraction was tested for its phytotoxicity through germination test and analysed by LC-UV. It was
193 observed that the phytotoxicity was correlated with the presence in the fraction of a peak with
194 retention time 10 min.
195 In order to simplify the purification procedure including two successive SPE fractionations we
196 tentatively purified the crude methanol extract by LL separation with dichloromethane. The

dichloromethane phase was brought to dryness, and the residue was re-diluted in a water and acetonitrile (1:1, v/v) solution in which it was soluble. The LC-UV analysis of the purified extract indicated that LL separation allowed to eliminate most of the polar compounds (with retention times lower than 6 min) while the compound with retention time 10 min was recovered. Consequently, the LL purified extract was used for semi-preparative chromatography allowing separating 4 fractions which were assayed for their phytotoxicity versus cress through germination tests. The germination indexes were as follows: 1st fraction: 69.5 ± 3.1 %; 2nd fraction 10.1 ± 3.5 %; 3rd fraction: 52.1 ± 1.6 %; 4th fraction: 68.5 ± 9.4 %. The statistical analysis indicated that the germination index of the 2nd fraction was significantly different than that of the others suggesting that it should be the richest in toxic compounds. The fractions were then submitted to LC-MS/MS analysis in order to investigate the main constituent compounds.

LC-MS/MS and HRMS analysis of the phytotoxic fraction

Full scan analysis of the fractions allowed correlating the phytotoxicity with the presence of a peak at 5.4 min, in negative polarization mode, relative to the signal of m/z 259 which was the only peak in the 2nd fraction. This fraction was therefore used for further investigation. MS/MS breakdown from parent ion gave two main fragments at m/z 215 and 171, with collision energy of 9.5 and 12.0 V respectively. The two consequent losses of 44 Da could be related to two losses of CO_2. The HRMS spectrum (Fig. 1.) indicated an exact mass of 259.0902 Da (M-H ion), suggesting a molecular formula of $C_{15}H_{16}O_4$ (with an error of 6.834 mDa). HRMS analysis confirmed two consequent losses of 43.9884 Da, leading to the ions m/z 215.1018 and m/z 171.1135 and allowed to calculate that those losses can be related to CO_2 (with an error of 1.429 mDa) confirming the MS/MS data. NMR and FTIR analysis was then performed in order to obtain the chemical structure of the molecule.

NMR analysis

Chemical shifts and coupling constants obtained by ^{13}C and ^{1}H NMR are shown in Table 2. The ^{13}C NMR spectrum, showing more signals than the 15 expected from HRMS analysis, was not consistent with the number of C atoms. However, ^{13}C NMR multiple signals suggest the presence of two different conformers in the purified solution. Values obtained in ^{13}C NMR spectra matched with those assigned by Jimeno et al.[21] for the two conformations of the sesquiterpene lactone isabelin. In particular isabelin shows specific ^{13}C signals at δ 171.39 and 172.16 which, in some studies, were seen as a single shift at δ 171.71 ppm, related to the second lactone ring present in the molecule in two conformational conditions. Chemical shift values for ^{1}H NMR were also identical to those reported by Yoshioka and Mabry [22], in their study about isabelin in which variation of conformation was assessed for the first time by ^{1}H NMR experiments at different temperatures.

FTIR analysis

In order to confirm the identification, FTIR was conducted on the purified compound (Fig. 2). The assignment of the main FTIR bands was based on Bellamy [23] and Lin-Vien et al. [24] .The two bands at 2927 and 2852 cm^{-1} are typical of the in-phase and out-phase vibrations of the hydrogen atoms of the CH_2 group. The 2927 cm^{-1} band exhibits a shoulder at higher frequency, which should be the asymmetrical stretching mode of the CH_3 group while the symmetrical stretching band, expected at 2872 cm^{-1}, is likely overlapped by the CH_2 stretching. The weak band at 3095 cm^{-1} should correspond to the =CH stretching vibration. The C=O stretching band at 1756 cm^{-1} is in the range of the five-membered ring unsaturated lactones (1795-1740 cm^{-1}). This assignment is confirmed by the IR spectra of some germacranolides exhibiting an absorption close to 1560 cm^{-1}.[25] The FTIR spectrum is in accordance with the supposed structure except for the absorption at

3490 cm^{-1} which is typical of O-H stretching and could be due, besides the 1658 cm^{-1} band, to the absorption of traces of water.

Identification of the phytotoxic compound

The analytical characterization led to the identification of the compound as the sesquiterpenoid isabelin (Fig.3). This is a known germacranolide found in *Ambrosia psilostachia*, [22] as in *Zexmenia valerii*, [26] and *Mikania cynanchifolia*. [27] Recent studies have focused on sesquiterpenoids produced by *A. artemisiifolia* for pharmacological applications or for their antimicrobial activity. [28-30] Isabelin has been reported in *A. artemisiifolia* by Porter and Mabry [31] and, recently, a study by Taglialatela-Scafati et al. [14], about allergenic compounds of pollen, reported high concentration of isabelin in *A. artemisiifolia* samples collected in the Piedmont region. Although isabelin is not a newly discovered molecule, its phytotoxic effect had never been reported. This effect could be related to the microtubules development in cell mitosis, as was observed for other sesquiterpene lactones from *Artemisia annua* by Dayan et al., [32] but further physiologic tests are needed to better understand isabelin mechanisms of action.

Quantification and anatomical distribution of isabelin in ragweed

Isabelin solutions at known concentration, prepared by quantitatively diluting pure isabelin obtained by LC separation, were used for the quantification of the compound. The mean concentration of isabelin in ragweed plant samples was on average 1 ± 0.3 $mg.g^{-1}$ plant on a dry weight basis. Approximately 83% of the compound was found in leaves while 11% and 6% in stems and roots respectively. The presence on leaves could be related to the concentration of the compound in leaf trichomes, specific organs in which the high biosynthesis of sesquiterpenoids in similar species has been reported [33].

Assessment of the phytotoxicity of pure isabelin

The concentration of isabelin in methanol extract, which provided total inhibition of cress germination, was on average 0.6 mM. In order to confirm the phytotoxicity of isabelin, germination tests were performed using a methanolic solution of pure isabelin at the same concentration. Germination tests at decreasing pure isabelin concentrations were also conducted on cress in order to obtain a dose/effect relationship (Fig. 4). The 0.6 mM isabelin solution, although significantly compromising the germination of cress (Ig =16.1%), did not inhibit it completely as the crude extract at the same concentration. The curve confirmed the relationship between the concentration of the molecule and the inhibition of the seeds germination, showing a significant effect of inhibition at concentration higher than 0.12 mM pure isabelin in the solution and allowing calculating a 50% reduction of the I_g at 0.27 mM concentration. In order to assess the phytotoxicity of isabelin on other species, methanol extract and 0.6 mM isabelin solution were tested on cress, red clover, rapeseed, darnel and radish. The corresponding germination indexes are reported in table 4. Methanol extract shown a significant inhibition on all the tested seeds, with I_g values ranging from 0.0 for radish to 7.8 ± 2.1 for darnel. In all cases the effect of the crude extract was major than that of pure isabelin. The minor effect exerted by pure isabelin as compared with the crude extract could probably be due to a synergistic effect related to other compounds present in the whole extract, as it often happens for the bioactive molecules. Furthermore, germination tests carried out with pure isabelin showed a wider range of effect on different species. Cress showed the most intense response, an intermediate effect was obtained on red clover and rapeseed, while radish and darnel seemed to be the most resistant species. Diverse responses in the inhibitory effect on different species were observed by Vidotto et al., [19] on plants grown on soils containing ragweed residues. In germination tests this could be due to mechanisms of action or to different seed size as for specific characters of the species.

CONCLUSION

This study has assessed that the sesquiterpene lactone isabelin, largely present in *Ambrosia artemisiiflolia*, inhibited the germination of different seeds. This suggests that the molecule, when released by the plant, could contribute to prevent the germination of surrounding species, therefore to promote its invasive ability. This hypothesis should be confirmed by further studies aimed to understand the ways of release of the molecule and its persistence in the environment. On the other hand the bioactivity and results obtained with sesquiterpene lactones so far makes it possible to propose them as leads for future natural product based herbicide development, [34] therefore isabelin, as pure molecule or in the crude extract, could be a potential candidate for such application.

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FIGURE CAPTIONS

Figure 1. HRMS spectrum of the phytotoxic compound..

Figure 2. FTIR spectrum of the phytotoxic compound.

Figure 3. Molecular structure of isabelin.

Figure 4. Effect of isabelin solutions at different concentrations on the germination indexes of cress.

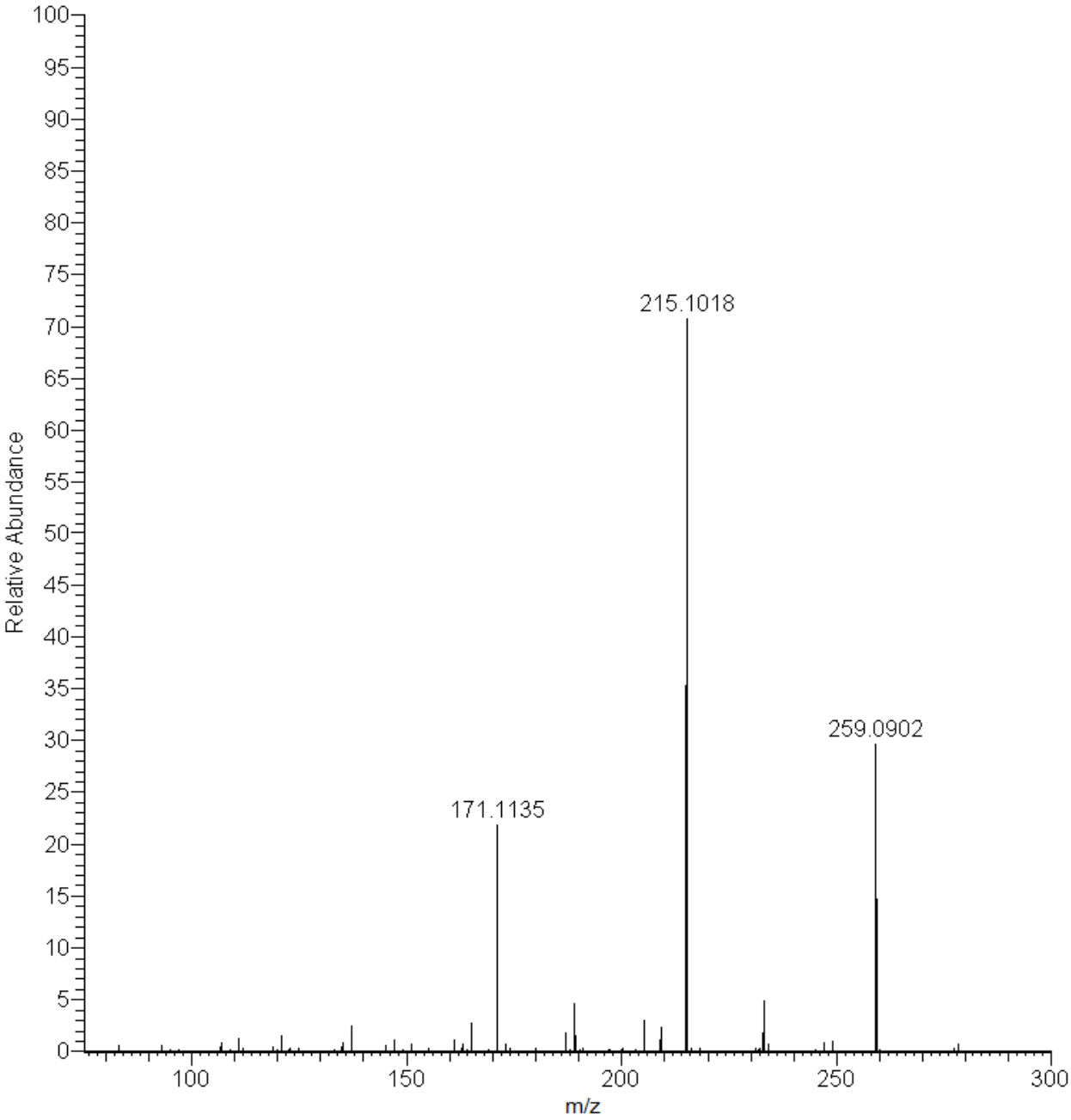
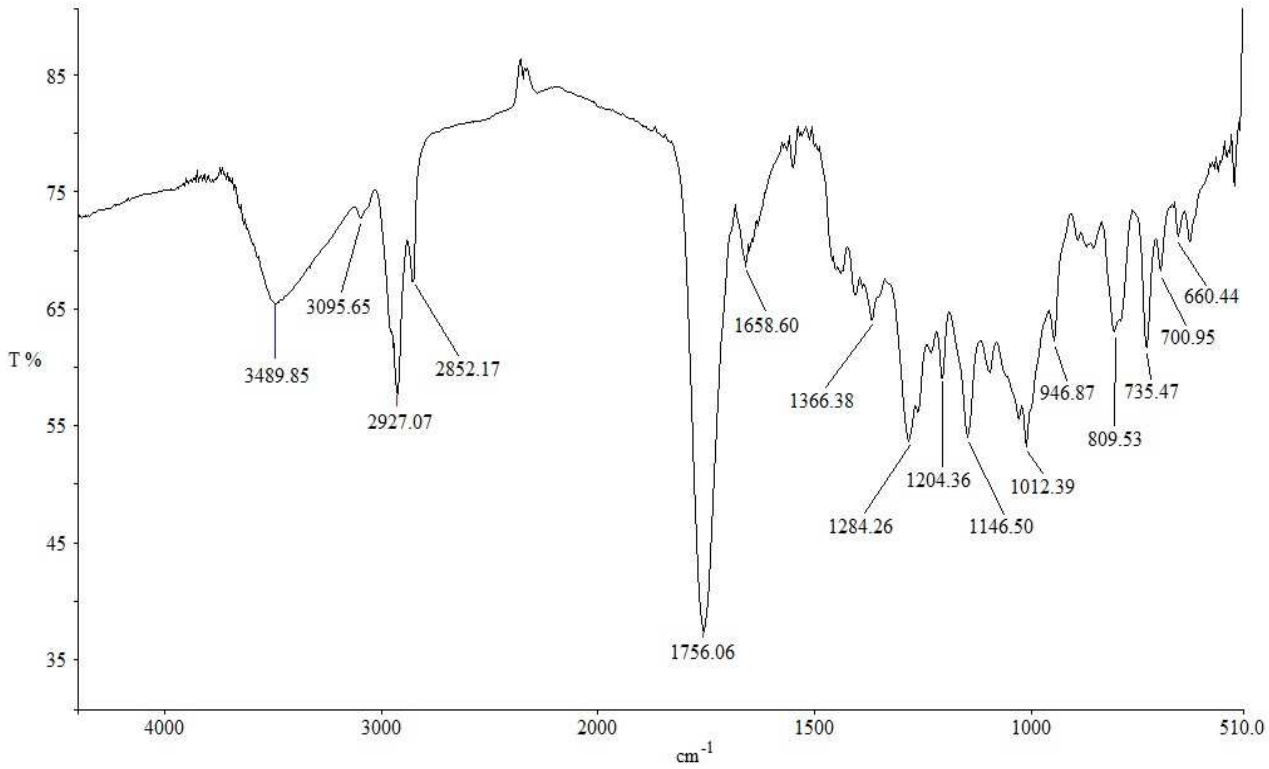

Relative Abundance
m/z
215.1018
259.0902
171.1135
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
100
150
200
250
300

Fig. 1


85
75
65
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45
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T %
3489.85
3095.65
2927.07
2852.17
1756.06
1658.60
1366.38
1284.26
1204.36
1146.50
1012.39
946.87
809.53
735.47
700.95
660.44
4000
3000
2000
1500
1000
510.0
cm-1

Fig. 2

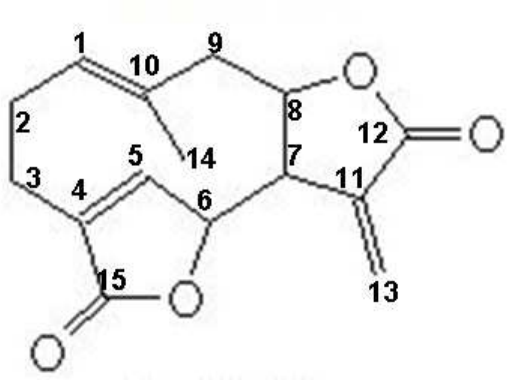

Fig. 3

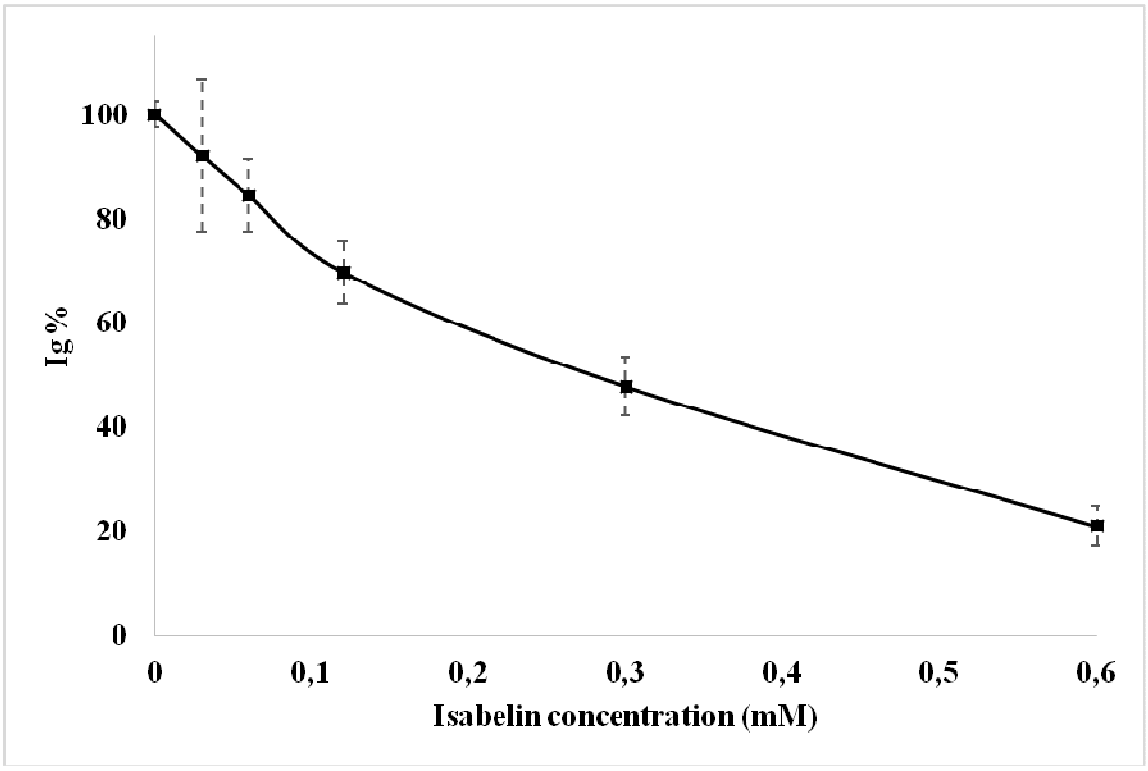
100
80
60
40
20
0
Ig %
0
0,1
0,2
0,3
0,4
0,5
0,6
Isabelin concentration (mM)

Fig. 4

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485 **Table 1**. Effect of ragweed crude extracts on the germination index of cress and radish.

	Cress	*Radish*
Water control	100 ± 5.4 [a]	100 ± 16.2 [a]
Methanol control	86.3 ± 7.5 [a]	102.2 ± 27.3 [a]
Methanol extract	0.0 [b]	0.0 [b]
Ethyl ether control	91.0 ± 25.5 [a]	79.43 ± 7.7 [a]
Ethyl ether extract	0.0 [b]	0.0 [b]
n-hexane control	81.6 ± 12.8 [a]	93.0 ± 15.2 [a]
n-hexane extract	44.7 ± 4.2 [ab]	105.4 ± 4.8 [a]

All values for 3 replicates on 5 seeds, (+/- S.E.).
Within columns, different letters indicate significant differences (R.E.G.W.Q test, P = 0.05).

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Table 2. ^{13}C and ^{1}H NMR chemical shifts (δ, ppm) and J-coupling costants (Hz) of isabelin for the two different conformers A and B.

	A conformer		**B conformer**	
position	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)
1	130.6, CH	4.93, dd (H1-H2α = 15), (H1-H2β = 4)	130.32, CH	4.84, dd (H1-H2α = 6), (H1-H2β = 6)
2	23.64, CH_2	2.18, 2.56, m	24.03, CH_2	2.74, 2.48, m
3	25.55, CH_2	2.75, 2.37, m	22.58, CH_2	2.74, 2.34, m
4	131.88, C	---	134.23, C	---
5	147.23, CH	6.76, d (H5-H6 = 2)	151.8, CH	6.61, d (H1-H6 = 1)
6	81.38, CH	5.21 (H6-H7 = 1)	82.04, CH	5.05 (H6-H7 = 6)
7	50.7, CH	3.25 (H7-H8 = 8)	55.57, CH	2.70 (H7-H8 = 9)
8	81.69, CH	4.44, m	75, CH	4.40, m
9	41.62, CH_2	1.90, 3.07, m	47.18, CH_2	1.92, 2.80, m
10	131.24, C	---	132.12, C	---
11	136.78, C	---	136.93, C	---
12	168.6, C	---	169.89, C	---
13	123.49, CH_2	6.39, 6.16, d (1)	125.12, CH_2	6.43, 5.84, d (1)
14	20.95, CH_3	1.64, s	18.2, CH_3	1.65, s
15	171.39[a], C	---	172.16[a], C	---

[a] low intensity

Table 3. Germination indexes of crude extract and purified isabelin on different species.

	Rapeseed	*Cress*	*Darnel*	*Radish*	*Red clover*
Crude extract	5.5 ± 2.2	1.3 ± 0.6	7.8 ± 2.1	0.0	3.2 ± 1.5
Pure isabelin	43.2 ± 7.3	16.1 ± 1.8	59.5 ± 10.9	65.8 ± 13.8	44.8 ± 4.0

All values for 3 replicates on 5 seeds, (+/- S.E.).