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

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(Article begins on next page)

16

17 **Abstract**

18

19 *Ambrosia artemisiifolia* L. (common ragweed) is an invasive plant which allelopathic properties
20 have been suggested by its field behaviour and demonstrated through phytotoxicity bioassays.
21 However, the nature of the molecules responsible for the allelopathic activity of common ragweed
22 has not been explored. The main objective of this study was to identify the phytotoxic molecules
23 produced by *A. artemisiifolia*. A preliminary investigation has indicated that a methanol extract of
24 *A. artemisiifolia* completely inhibited the germination of cress and radish. Semi-preparative
25 fractionation of the methanol extract allowed separating the phytotoxic fraction which contained a
26 single compound. The structure of this compound was elucidated by LC-MS/MS, HRMS, NMR
27 and FTIR as the sesquiterpene lactone isabelin (C₁₅H₁₆O₄). The effect of pure isabelin was tested on
28 four different weed species, confirming the inhibitory activity of the molecule. The results indicate
29 directions for future studies about herbicidal specific activity of isabelin, as pure molecule or in the
30 crude extract, as potential candidate for biological weed control.

31

32

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

34

35 **Introduction**

36

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

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

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

64

65 **Materials and methods**

66

67 ***Chemicals***

68

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

70

71 ***Plant material***

72

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

81

82 ***Extraction of plant material***

83

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

88

89 ***Germination test***

90

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

94 solution. After evaporation of the solvent, 150 μ L water was added in each dish and 25 seeds were
95 placed on the filter paper in five separated batches per condition. The Petri dishes were closed and
96 germination was conducted in germination chamber at 25 °C for 48 h, with a light/darkness cycle of
97 16/8 hours. Petri dishes moistened with demineralized water alone or pre-treated with extraction
98 solvent were used as control. The germination index (I_g) was calculated by the number of
99 germinated seeds (n) and the mean length of the radicle (m) in sample (s) and control (c), according
100 to equation 1.

101

$$102 \quad I_g = \frac{n_s \cdot m_s}{n_c \cdot m_c} \cdot 100 \quad (1)$$

103

104

105

106 ***Purification of the crude extract***

107

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

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

119 A liquid-liquid purification of the crude extract was also conducted prior to semi-preparative
120 chromatography in order to eliminate the large amounts of polar compounds interfering with the
121 chromatographic separation: 30 mL of methanol crude extract were brought to dryness, re-dissolved
122 in the same volume of H₂O acidified with 0.2% H₃PO₄ and LL extracted with 30 mL of
123 dichloromethane. The dichloromethane fraction was dried by rotavapor and re-dissolved in 3 mL
124 acetonitrile, obtaining a ten-fold concentration of the purified extract.

125

126 *LC-MS/MS analysis*

127

128 LC-MS system was a Varian MS-310 triple quadrupole mass spectrometer equipped with an ESI
129 source, and 212 LC pump (Agilent, Milan, Italy). Separation was performed on a Luna C₁₈ column,
130 5 μm particle size, 50 × 2.0 mm (Phenomenex, Torrance, CA, USA). The mobile phase solvents
131 were water (A) and acetonitrile (B), both containing 0.1% (v/v) acetic acid. The mobile phase
132 gradient was from 90 to 10% A in 10 min with a flow rate of 0.2 mL/min. ESI conditions used in
133 negative polarization were: needle potential -3500V, shield -500V, capillary -30V. Gas conditions
134 were set with 20.0 psi of air as nebulizing gas and 25.0 psi at 300°C N₂ as drying gas. The
135 respective ion transitions were as follows: m/z 259 → 147.1 (collision energy 16.5 V), m/z 259 →
136 170.8 (collision energy 12.0 V), m/z 259 → 214.6 (collision energy 9.5 V). The m/z 214.6 ion was
137 used for quantification..

138

139

140 *LC-HRMS analysis*

141

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

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

149

150 *NMR analysis*

151

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

155

156 *FTIR analysis*

157

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

160

161 *Statistical analysis*

162

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

168

169

170 **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 C₁₈ 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

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

208

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

210

211 Full scan analysis of the fractions allowed correlating the phytotoxicity with the presence of a peak
212 at 5.4 min, in negative polarization mode, relative to the signal of m/z 259 which was the only peak
213 in the 2nd fraction. This fraction was therefore used for further investigation. MS/MS breakdown
214 from parent ion gave two main fragments at m/z 215 and 171, with collision energy of 9.5 and 12.0
215 V respectively. The two consequent losses of 44 Da could be related to two losses of CO₂. The
216 HRMS spectrum (Fig. 1.) indicated an exact mass of 259.0902 Da (M-H ion), suggesting a
217 molecular formula of C₁₅H₁₆O₄ (with an error of 6.834 mDa). HRMS analysis confirmed two
218 consequent losses of 43.9884 Da, leading to the ions m/z 215.1018 and m/z 171.1135 and allowed
219 to calculate that those losses can be related to CO₂ (with an error of 1.429 mDa) confirming the
220 MS/MS data. NMR and FTIR analysis was then performed in order to obtain the chemical structure
221 of the molecule.

222

223 ***NMR analysis***

224

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

235

236 ***FTIR analysis***

237

238 In order to confirm the identification, FTIR was conducted on the purified compound (Fig. 2). The
239 assignment of the main FTIR bands was based on Bellamy^[23] and Lin-Vien et al.^[24]. The two
240 bands at 2927 and 2852 cm^{-1} are typical of the in-phase and out-phase vibrations of the hydrogen
241 atoms of the CH_2 group. The 2927 cm^{-1} band exhibits a shoulder at higher frequency, which should
242 be the asymmetrical stretching mode of the CH_3 group while the symmetrical stretching band,
243 expected at 2872 cm^{-1} , is likely overlapped by the CH_2 stretching. The weak band at 3095 cm^{-1}
244 should correspond to the $=\text{CH}$ stretching vibration. The $\text{C}=\text{O}$ stretching band at 1756 cm^{-1} is in the
245 range of the five-membered ring unsaturated lactones (1795-1740 cm^{-1}). This assignment is
246 confirmed by the IR spectra of some germacranolides exhibiting an absorption close to 1560 cm^{-1} .
247^[25] The FTIR spectrum is in accordance with the supposed structure except for the absorption at

248 3490 cm⁻¹ which is typical of O-H stretching and could be due, besides the 1658 cm⁻¹ band, to the
249 absorption of traces of water.

250

251 ***Identification of the phytotoxic compound***

252

253 The analytical characterization led to the identification of the compound as the sesquiterpenoid
254 isabelin (Fig.3). This is a known germacranolide found in *Ambrosia psilostachia*,^[22] as in *Zexmenia*
255 *valerii*,^[26] and *Mikania cynanchifolia*.^[27] Recent studies have focused on sesquiterpenoids
256 produced by *A. artemisiifolia* for pharmacological applications or for their antimicrobial activity.^{[28-}
257 ^{30]} Isabelin has been reported in *A. artemisiifolia* by Porter and Mabry^[31] and, recently, a study by
258 Tagliatalata-Scafati et al.^[14], about allergenic compounds of pollen, reported high concentration of
259 isabelin in *A. artemisiifolia* samples collected in the Piedmont region. Although isabelin is not a
260 newly discovered molecule, its phytotoxic effect had never been reported. This effect could be
261 related to the microtubules development in cell mitosis, as was observed for other sesquiterpene
262 lactones from *Artemisia annua* by Dayan et al.,^[32] but further physiologic tests are needed to better
263 understand isabelin mechanisms of action.

264

265 ***Quantification and anatomical distribution of isabelin in ragweed***

266

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

274

275 *Assessment of the phytotoxicity of pure isabelin*

276

277 The concentration of isabelin in methanol extract, which provided total inhibition of cress
278 germination, was on average 0.6 mM. In order to confirm the phytotoxicity of isabelin, germination
279 tests were performed using a methanolic solution of pure isabelin at the same concentration.
280 Germination tests at decreasing pure isabelin concentrations were also conducted on cress in order
281 to obtain a dose/effect relationship (Fig. 4). The 0.6 mM isabelin solution, although significantly
282 compromising the germination of cress ($I_g = 16.1\%$), did not inhibit it completely as the crude
283 extract at the same concentration. The curve confirmed the relationship between the concentration
284 of the molecule and the inhibition of the seeds germination, showing a significant effect of
285 inhibition at concentration higher than 0.12 mM pure isabelin in the solution and allowing
286 calculating a 50% reduction of the I_g at 0.27 mM concentration. In order to assess the phytotoxicity
287 of isabelin on other species, methanol extract and 0.6 mM isabelin solution were tested on cress, red
288 clover, rapeseed, darnel and radish. The corresponding germination indexes are reported in table 4.
289 Methanol extract shown a significant inhibition on all the tested seeds, with I_g values ranging from
290 0.0 for radish to 7.8 ± 2.1 for darnel. In all cases the effect of the crude extract was major than that
291 of pure isabelin. The minor effect exerted by pure isabelin as compared with the crude extract could
292 probably be due to a synergistic effect related to other compounds present in the whole extract, as it
293 often happens for the bioactive molecules. Furthermore, germination tests carried out with pure
294 isabelin showed a wider range of effect on different species. Cress showed the most intense
295 response, an intermediate effect was obtained on red clover and rapeseed, while radish and darnel
296 seemed to be the most resistant species. Diverse responses in the inhibitory effect on different
297 species were observed by Vidotto et al.,^[19] on plants grown on soils containing ragweed residues.
298 In germination tests this could be due to mechanisms of action or to different seed size as for
299 specific characters of the species.

300

301 **CONCLUSION**

302

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

311

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313

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316

317 **References**

318

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FIGURE CAPTIONS

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411 Figure 1. HRMS spectrum of the phytotoxic compound..

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413 Figure 2. FTIR spectrum of the phytotoxic compound.

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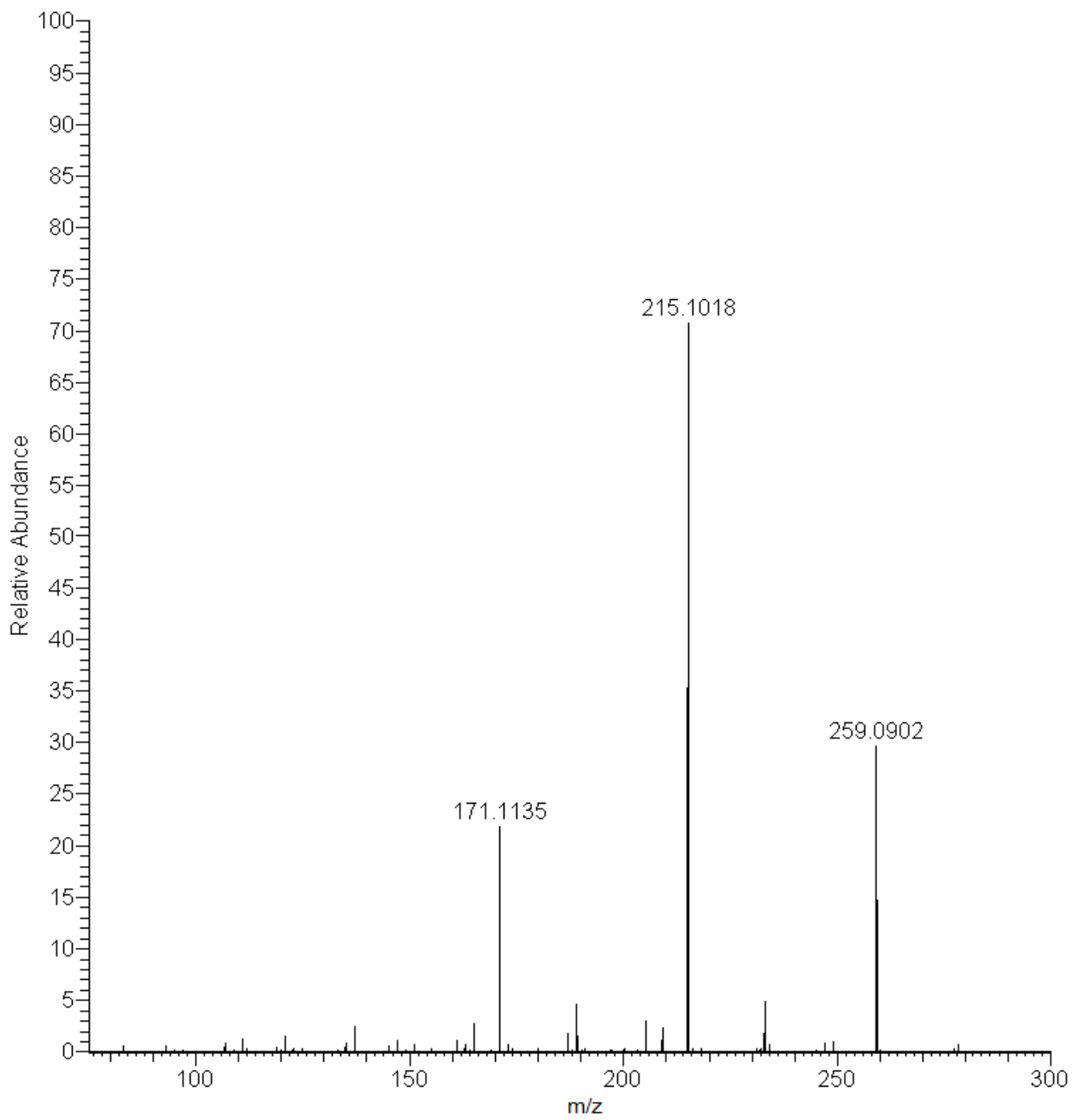
415 Figure 3. Molecular structure of isabelin.

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

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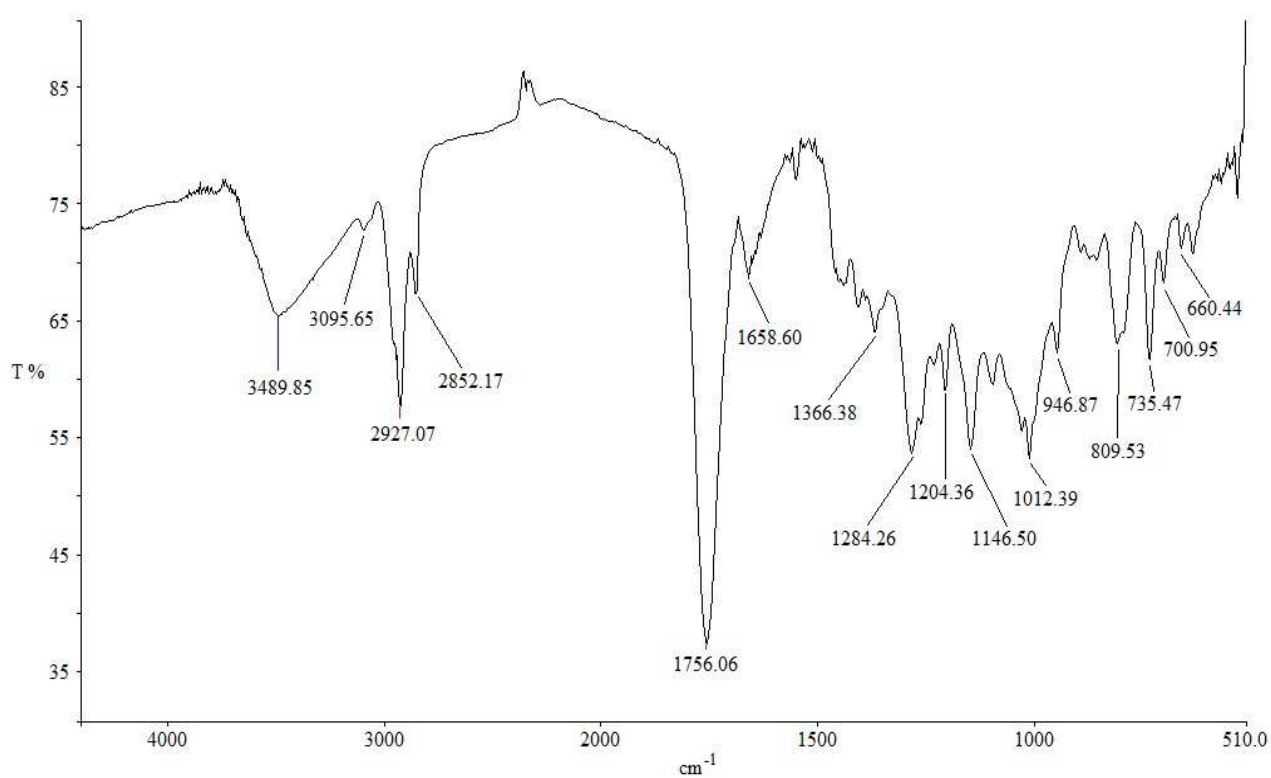
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441 Fig. 2

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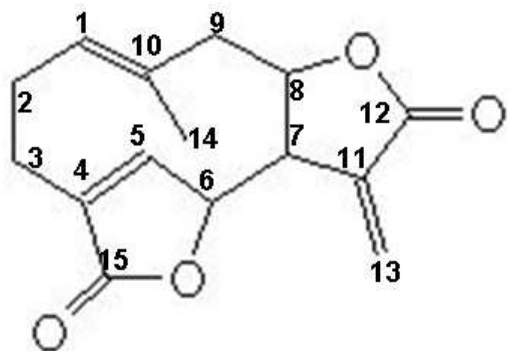
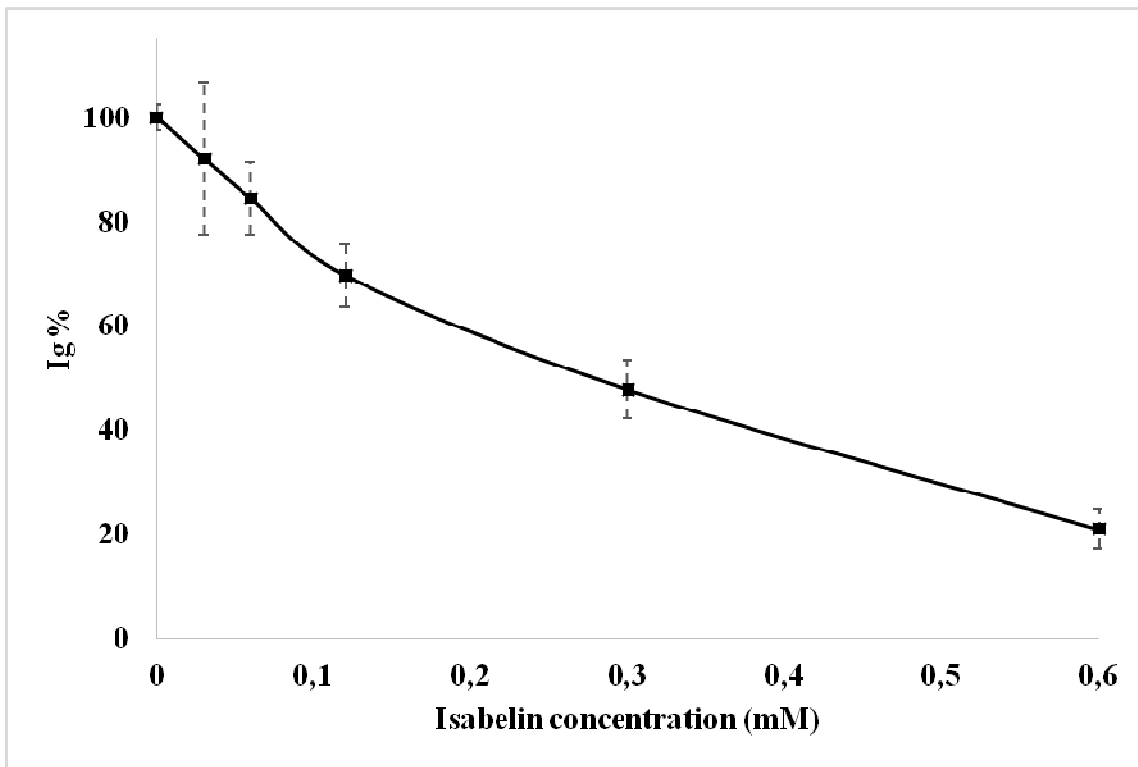


Fig. 3



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483 Fig. 4

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

	<i>Cress</i>	<i>Radish</i>
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).

487 Table 2. ¹³C and ¹H NMR chemical shifts (δ, ppm) and J-coupling constants (Hz) of isabelin for the
 488 two different conformers A and B.
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position	A conformer		B conformer	
	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	---

490
 491 ^a low intensity

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

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

494
 495 All values for 3 replicates on 5 seeds, (+/- S.E.).
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