

1 **Bioactivity-guided isolation of rosmarinic acid as a principle bioactive**  
2 **compound from the butanol extract of *Isodon rugosus* against pea aphid,**  
3 ***Acyrtosiphon pisum***

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## 18 **Abstract**

19 Aphids are agricultural pest insects that transmit viruses and cause feeding damage on a global scale. Current pest  
20 control involving the excessive use of synthetic insecticides over decades has led to multiple forms of aphid  
21 resistance to most classes of insecticides. In nature, plants produce secondary metabolites during their interaction  
22 with insects and these metabolites can act as toxicants, antifeedants, anti-oviposition agents and deterrents towards  
23 the insects. In a previous study, we demonstrated that the butanol fraction from a crude methanolic extract of an  
24 important plant species, *Isodon rugosus* showed strong insecticidal activity against the pea aphid, *Acyrtosiphon*  
25 *pisum*. It was however not known as which compound was responsible for such activity. To further explore this  
26 finding, current study aimed to exploit a bioactivity-guided strategy to isolate and identify the active compound in  
27 the butanol fraction of *I. rugosus*. As such, reversed-phase flash chromatography, acidic extraction and different  
28 spectroscopic techniques were used to isolate and identify the new compound, rosmarinic acid as the bioactive  
29 compound in *I. rugosus*. Insecticidal activity of rosmarinic acid was carried out using standard protocols on *A.*  
30 *pisum*. The data was analyzed using qualitative and quantitative statistical approaches. Considering that a very low  
31 concentration of this compound ( $LC_{50} = 5.4$  ppm) causes significant mortality in *A. pisum* within 24 h, rosmarinic  
32 acid could be exploited as a potent insecticide against this important pest insect. Furthermore, *I. rugosus* is already  
33 used for medicinal purposes and rosmarinic acid is known to reduce genotoxic effects induced by chemicals, hence  
34 it is expected to be safer compared to the current conventional pesticides. While this study highlights the potential of  
35 *I. rugosus* as a possible biopesticide source against *A. pisum*, it also provides the basis for further exploration and  
36 development of formulations for effective field application.

## 37 **Introduction**

38 Aphids are among the most important agricultural pest insects of many crops worldwide. They feed exclusively on  
39 plant phloem sap by inserting their needle-shaped mouthparts into sieve elements, usually resulting to the stunting,  
40 discoloration and deformation of plants, while the growth of sooty molds on honeydew produced by these insects  
41 reduces the economic value of crops [1, 2]. Moreover, aphids are also vectors of many important plant viruses [3-5].  
42 The pea aphid, *Acyrtosiphon pisum* (Hemiptera: Aphididae), adversely affects economically important legume  
43 crops worldwide. It is oligophagous, comprising of a number of biotypes or races living on a number of legume  
44 hosts (red clover, pea and broad bean and alfalfa races) [6-9]. Current aphid control strategies predominantly rely on

45 the use of insecticides such as carbamates, organophosphates, pyrethroids, neonicotinoids and pymetrozine [10].  
46 However, the frequent use of these insecticides over the decades has led to multiple forms of aphid resistance to  
47 most classes of insecticides, making it very difficult to control this insect pest [11].

48 The use of botanical pesticides could present a safe alternative compared to the use of broad spectrum  
49 chemical insecticides in crop protection [12, 13]. In nature, plants produce secondary metabolites during their  
50 interaction with insects and these metabolites can act as toxicants, antifeedants, anti-oviposition agents and  
51 deterrents towards the insects [14-16]. Because of such wide insecticidal properties, the study of secondary  
52 metabolites and the development of new potent formulations based on them has become increasingly important.  
53 Screening of plant extracts followed by bioactivity-guided fractionation, isolation and identification of active  
54 principles is considered to be one of the most successful strategies for the discovery of bioactive natural products  
55 against insect pests [17].

56 *Isodon rugosus* (Wall. ex Benth.) Codd (syn. *Plectranthus rugosus* Wall. ex. Benth.) is an aromatic  
57 branched shrub, belonging to the family Lamiaceae. The plant is used in Pakistani traditional medicine for many  
58 diseases as an antiseptic, hypoglycemic, antidiarrheal and as bronchodilator [18, 19]. Among many other traditional  
59 medicinal uses, the plant extracts and different solvent fractions are known to be effective as antifungal,  
60 antibacterial, phytotoxic and antioxidant agents and are able to show lipoxygenase inhibitory activities [20-23].  
61 Based on phytochemical studies, this plant is known to contain steroids, flavonoids, terpenoids, saponins, tannins,  
62 cardiac glycosides, coumarins, reducing sugars and  $\beta$ -cyanin. Diterpenoids (rugosinin, effusanin-A, effusanin-B,  
63 effusanin-E, lasiokaurin and oridonin) and triterpenoids (plectranthoic acid A and B, acetyl plectranthoic acid and  
64 plectranthadiol) have also been successfully isolated from this plant [24-26]. However, despite several studies on the  
65 bioactivity of *I. rugosus* where most efforts were focused towards human health, none of these have isolated and  
66 assessed the insecticidal activity of compounds from this plant. In a previous study, we evaluated the aphicidal  
67 properties of the hexane, dichloromethane, butanol and ethyl acetate fractions of a crude methanolic extract from *I.*  
68 *rugosus*, and confirmed that the butanol fraction showed the best activity against the pea aphid, *A. pisum* [27]. To  
69 further explore this finding, a bioactivity-guided strategy against *A. pisum* was used to isolate and identify the active  
70 compound in the butanol fraction of *I. rugosus*.

## 71 **Materials and methods**

## 72 **Insects**

73 A continuous colony of *A. pisum* was maintained on faba bean plants (*Vicia faba*) in the Laboratory of Agrozoology  
74 at Ghent University, Belgium at 23–25 °C and 65±5% relative humidity (RH) under a 16:8 h light: dark photoperiod  
75 [28]. All the bioassays were performed under these conditions. Newly born nymphs (< 24 h old) of *A. pisum* were  
76 used for all the bioassays. By gentle probing of the aphids with a brush and also by observing post-mortem color  
77 change of the body, mortality was assessed after 24 h of treatment.

## 78 **Plant collection and extraction**

79 The aerial parts of *I. rugosus* were collected from lower Northern areas of Pakistan in the month of October, 2012.  
80 The plant material was shade-dried for up to 3 months and ground to powder using an electric grinder. 1 kg of the  
81 dried powder was soaked in a glass jar containing 3 L of methanol at room temperature. After two days, the solvent  
82 layer was filtered with Whatman filter paper No. 1 and this process was repeated three times. The resulting filtrate  
83 was concentrated by using a rotary evaporator at 35 °C and the obtained crude methanolic extract was stored at 4 °C  
84 [29, 27]. For fractionation, 90 g dried crude methanolic extract was mixed with five parts of water and then  
85 extracted successively by n-hexane (4 × 150 mL), dichloromethane (4 × 150 mL), ethyl acetate (4 × 150 mL) and n-  
86 butanol (4 × 150 mL) as described by Khan et al. [27]. All the fractions were concentrated using a rotary evaporator  
87 under reduced pressure at 40 °C. The resulting extracts were stored in a refrigerator at 4 °C until further use.

## 88 **Isolation of the bioactive principle**

89 Based on bioassays conducted by Khan et al, [27] the butanol extract presented the best biological activity against *A.*  
90 *pisum* and was hence selected in this study for further bioactivity-guided fractionation and identification of the  
91 active principle. The butanol extract (500 mg) was eluted with a Reveleris automated flash chromatography  
92 instrument on a 12 g C18 pre-packed column (GRACE, Columbia, MD, US) starting with 100% water. The gradient  
93 was ramped to 100% methanol over 60 column volumes (CV) and after collection of 95 fractions, the solid phase  
94 was flushed with 5 CV acetonitrile. The flow rate was set to 30 mL/min (Table 1). Based on the UV spectral data,  
95 the 95 fractions were combined into a total of 14 subfractions. These combined fractions were evaporated under  
96 reduced pressure at 45 °C and finally under a high vacuum resulting in 14 subfractions (1A- 14A) (Table 2). The 14  
97 subfractions were evaluated for their bioactivity against *A. pisum*, of which fraction 3A was selected on the basis of

98 maximum bioactivity for further fractionation through preparative liquid chromatography (prep-LC). A 10%  
99 solution of fraction 3A was prepared in methanol. Two solvents were used, water (solvent A) and acetonitrile  
100 (solvent B). A gradient was set starting with 100% solvent A from 0 to 100 min. From 100 min to 110 min, solvent  
101 B went from 18% to 100% and stayed at 100% until 128 min, and then to 0% at 128.10 min and stayed at 0% until  
102 132.10 min. After concentration under reduced pressure with a rotary evaporator and finally under a high vacuum,  
103 three fractions, 3A-1, 3A-2 and 3A-3 were obtained. Fraction 3A-3 was selected for active compound identification  
104 (NMR and LC-MS) on the basis of the bioactivity against *A. pisum*. This compound was obtained in pure form by  
105 doing a second flash chromatographic separation of 5 g of butanol extract and by using the run conditions as  
106 mentioned in Table 3. From the second flash chromatography, a total of 354 fractions were collected which were  
107 combined into six fractions, 1B, 2B, 3B, 4B, 5B and 6B on the basis of UV spectra and were further analyzed for  
108 their bioactivity after concentration with a rotary evaporator under reduced pressure and high vacuum (Table 4).  
109 Fraction 1B was selected for further purification on the basis of best bioactivity. On the basis of knowledge  
110 regarding the acidic compound present in sub fraction 3A-3 (from <sup>1</sup>H NMR and HPLC-MS analysis), an extraction  
111 under acidic conditions was done to isolate the active compound from sub fraction 1B. For this purpose, 200 mg of  
112 fraction 1B was dissolved in 10 mL of distilled water and acidified with 4 drops of hydrochloric acid (12 M).  
113 Following extraction with ethyl acetate (four times 5 mL), two phases, ethyl acetate and aqueous, were obtained.  
114 Both the ethyl acetate and the aqueous phase were concentrated. The ethyl acetate phase fraction was more bioactive  
115 with lower LC values. Last traces of ethyl acetate were removed azeotropically with toluene and evaporation under  
116 high vacuum of the residues resulted in 60 mg from the ethyl acetate phase and 60 mg from the aqueous phase. The  
117 purified active principle was identified through different spectroscopic techniques.

118 **Table 1. First reversed-phase flash chromatography conditions of butanol fraction (500 mg) from *Isodon***  
119 ***rugosus***

<b>Run Conditions</b>	
<b>Cartridge</b>	Reveleris 12 g C18 40 $\mu$ m
<b>Solvent A</b>	Water
<b>Solvent B</b>	Methanol
<b>Solvent C</b>	Acetonitrile
<b>Flow rate</b>	30 mL/min
<b>Injection type</b>	Dry sample
<b>ELSD Carrier</b>	Isopropanol
<b>Per vial volume</b>	25 mL
<b>UV1 Wavelength</b>	220 nm
<b>UV2 Wavelength</b>	254 nm

121 **Table 2. Subfractions (1A-14A) collected from the first reversed-phase flash chromatography of butanol**  
122 **extract (500 mg)**

<b>Fractions</b>	<b>Weight (mg)</b>
1A	52
2A	11
3A	46
4A	7
5A	20
6A	15
7A	54
8A	58
9A	14
10A	49
11A	18
12A	15
13A	21
14A	18

124 **Table 3. Second reversed-phase flash chromatography conditions of butanol fraction (5 g) of *Isodon rugosus***

<b>Run Conditions</b>	
<b>Cartridge</b>	Reveleris 120 g C18 40 µm
<b>Solvent A</b>	Water
<b>Solvent B</b>	Methanol
<b>Flow rate</b>	85 mL/min
<b>Injection type</b>	Dry sample
<b>ELSD Carrier</b>	Isopropanol
<b>Per vial volume</b>	25 mL
<b>UV1 Wavelength</b>	220 nm
<b>UV2 Wavelength</b>	254 nm

125

126 **Table 4. Subfractions (1B-6B) from the second reversed-phase flash chromatography of butanol extract (5 g)**

<b>Fractions</b>	<b>Weight (mg)</b>
1B	530
2B	830
3B	1523
4B	195
5B	140
6B	128

127

## 128 **Identification of the bioactive compound**

129 Mass spectra were recorded using a HPLC-MS instrument consisting of an Agilent (Waldbronn, Germany) model  
130 1100 liquid chromatograph with a diode array detector coupled with a mass spectrometer with electrospray  
131 ionization geometry (Agilent MSD 1100 series). The prep-LC consisted of an Agilent 1100 Series liquid  
132 chromatograph using a Supelco Ascentis C18 column (I.D. x L 21.2 mm x 150 mm, 5 µm particle size) connected to



133 an UV-VIS variable wavelength detector (VWD) and automatic fraction collector. Flash chromatography was  
134 performed with the Reveleris Flash System (GRACE). <sup>1</sup>H and <sup>13</sup>C NMR spectra were obtained on a BRUKER  
135 Advance III 400 spectrometer. All the solvents and chemicals used were of analytical grade. Optical rotation was  
136 taken with a JASCO P-2000 series polarimeter.

## 137 **Insecticidal bioactivity**

138 For the bioassays, artificial diet test cages were prepared as described by Sadeghi et al. [30] 100 µL of liquid  
139 artificial diet was sealed between two layers of parafilm. Ten neonate aphids were placed on these layers of the  
140 parafilm and to prevent the escape of aphids, the cages were covered with a hollow plastic ring having a ventilated  
141 lid. These cages were placed in an inverted position in six aerated well plates. Five concentrations were used for  
142 each treatment against the aphids. A stock solution of 1% was prepared by adding 1 mg of each fraction in 100 µL  
143 of water. In case of the reversed-phase flash fractions, five concentrations with 50, 25, 12.5, 6.3 and 3.1 ppm and in  
144 case of the prep-LC and acidic extraction fractions, five concentrations of 5, 2.5, 1.3, 0.7 and 0.3 ppm were prepared  
145 by diluting the stock solution with the artificial diet of aphids. For each concentration, a final volume of 300 µL was  
146 made to carry out three replications of each treatment (100 µL for each replication). Pure isolated and identified  
147 active compound was analyzed in eight different concentrations including 50, 25, 12.5, 6.3, 3.1, 1.6, 0.8 and 0.4 ppm  
148 by using a stock solution of 1 mg of compound in 100 µL of water. The untreated artificial diet was used as a control  
149 and three replications were used for each treatment in all the bioassays. Mortality was analyzed after 24 h of each  
150 treatment.

151 Additionally, the growth of the surviving aphids exposed to 0.4 ppm of the active compound for 24 h was  
152 followed for 9 days (on the same treated diet) in comparison to the untreated aphids.

## 153 **Data analysis**

154 For statistical analysis, Probit analysis of mortality vs. concentration using POLO-Plus program version 2  
155 was conducted and the lethal concentrations (LC<sub>50</sub>, LC<sub>90</sub>) and their corresponding 95% confidence intervals (95%  
156 CI) were estimated for each fraction. When the 95% CI's did not overlap, LC's were considered to be significantly  
157 different.

## 158 **Results**

## 159 **Bioactivity of fractions from the butanol extract of *I. rugosus***

160 Bioactivity of the fourteen fractions (1A-14A) obtained through the first reversed-phase flash chromatography of  
161 500 mg of butanol extract of *I. rugosus* was analyzed for 24 h against *A. pisum*. Except fractions 8A, 9A, 11A, 13A  
162 and 14A, all the other fractions showed considerable toxic effects against *A. pisum*. Fraction 3A ( $LC_{50} = 2.1$  ppm and  
163  $LC_{90} = 29.5$  ppm) had the highest activity as compared to all other fractions, followed by fraction 5A ( $LC_{50} = 3.3$   
164 ppm and  $LC_{90} = 50$  ppm). Fraction, 1A ( $LC_{50} = 5.5$  ppm and  $LC_{90} = 66$  ppm), 2A ( $LC_{50} = 8.9$  ppm and  $LC_{90} = 81$   
165 ppm), 4A ( $LC_{50} = 6.8$  ppm and  $LC_{90} = 112$  ppm) and 6A ( $LC_{50} = 17.8$  ppm and  $LC_{90} = 187$  ppm) gave considerable  
166 mortality. Fraction 7A ( $LC_{50} = 74$  ppm and  $LC_{90} = 267$  ppm) showed lower mortality. Moderate toxicity was  
167 observed with fraction 10A ( $LC_{50} = 36$  ppm and  $LC_{90} = 53$  ppm) and 12A ( $LC_{50} = 51$  ppm and  $LC_{90} = 109$  ppm)  
168 (Table 5).

169 **Table 5. Toxicity of subfractions of the butanol fraction from first reversed-phase flash chromatography against newborn (< 24 h old) *Acyrtosiphon***  
 170 ***pisum* nymphs following 24 h exposure to artificial diet containing different concentrations of subfractions**

171

172	Fractions	LC <sub>50</sub> (95% CI) ppm	Ratio	LC <sub>90</sub> (95% CI) ppm	Ratio	Slope ± SE	Chi-Square	HF
173	1A	5.5 (3-8) a	2.6	66 (37-211) a	2.2	1.1 ± 0.3	7.1	0.5
174	2A	8.9 (6.1-12) a	4.2	81 (47-231) a	2.7	1.3 ± 0.3	5.6	0.4
175	3A	2.1 (0.6-3.8) a	1.0	30 (18-85) a	1.0	1.1 ± 0.3	7.5	0.6
176	4A	6.8 (3.8-10) a	3.2	112.2 (54-561) a	3.8	1.1 ± 0.3	4.6	0.4
177	5A	3.3 (1.3-5.4) a	1.6	50 (28-176) a	1.7	1.1 ± 0.3	10.1	0.8
178	6A	18 (13-27) b	8.5	187 (90-808) a	6.3	1.3 ± 0.3	3.8	0.3
179	7A	74 (52-169) c	35.3	267 (131-1651) a	9.1	2.3 ± 0.6	8.1	0.6
180	8A	-	-	-	-	1.7 ± 0.7	7.0	0.5
181	9A	-	-	-	-	2.0 ± 1.3	4.7	0.4
182	10A	36 (33-40) d	17.2	52.5 (46-64) a	1.8	8.0 ± 1.4	2.8	0.2
183	11A	-	-	-	-	1.6 ± 0.6	8.5	0.7
184	12A	51 (43-71) c	24.5	109 (77-241) a	3.7	3.9 ± 1.0	2.2	0.2
185	13A	-	-	-	-	1.5 ± 1.2	6.6	0.5
186	14A	-	-	-	-	2 ± 1.3	4.7	0.4

187 Data is presented as 50% (LC<sub>50</sub>) and 90% (LC<sub>90</sub>) lethal concentration values (both in ppm) together with their respective 95% confidence interval (95% CI), the slope ± SE of the toxicity vs concentration curve, and the Chi-Square and

188 heterogeneity factor HF as accuracy of data fitting to probit analysis in POLO-PlusV2. Different letters in the same column indicate significant differences due to non-overlapping of 95% CI. Ratio, LCx, fraction/LCx, 3A

189 **Bioactivity of subfractions from fraction 3A collected through prep-**

190 **LC**

191 The three collected subfractions (3A-1, 3A-2 and 3A-3) of 3A were analyzed against *A. pisum* for 24 h. Fraction 3A-  
192 1 and fraction 3A-2 gave negligible toxic effects (no LC<sub>50</sub> and LC<sub>90</sub>). Fraction 3A-3 was the most toxic fraction  
193 analyzed against *A. pisum* with low LC's (LC<sub>50</sub> = 1 ppm and LC<sub>90</sub> = 14 ppm) (Table 6).

194 **Table 6. Toxicity of subfractions of fraction 3A against newborn (< 24 h old) *Acyrtosiphon pisum* nymphs following 24 h exposure to artificial diet**  
 195 **containing different concentrations of subfractions**

<b>Fractions</b>	<b>LC<sub>50</sub> (95% CI) ppm</b>	<b>Ratio</b>	<b>LC<sub>90</sub> (95% CI) ppm</b>	<b>Ratio</b>	<b>Slope ± SE</b>	<b>Chi-Square</b>	<b>HF</b>
3A-1	-	-	-	-	2.0 ± 1.3	4.9	0.4
3A-2	-	-	-	-	1.5 ± 1.2	6.6	0.5
3A-3	1 (0.6-1.6) a	1	14 (6.1-97) a	1	1.1 ± 0.3	14.8	1.1

196 Data is presented as 50% (LC50) and 90% (LC90) lethal concentration values (both in ppm) together with their respective 95% confidence interval (95% CI), the slope ± SE of the toxicity vs concentration curve, and the Chi-Square and  
 197 heterogeneity factor HF as accuracy of data fitting to probit analysis in POLO-PlusV2. Different letters in the same column indicate significant differences due to non-overlapping of 95% CI. Ratio, LCx, fraction/LCx, 3A-3

### 198 **Spectroscopic analysis of fraction 3A-3**

199 Out of three subfractions of 3A (3A-1, 3A-2 and 3A-3), fraction 3A-3 was the most bioactive fraction against *A.*  
200 *pisum*. This fraction 3A-3 was analyzed through <sup>1</sup>H NMR which confirmed that the bioactive fraction 3A-3  
201 contained rosmarinic acid. Different gradients were used to purify the compound but during different Prep-LC runs,  
202 the chromatographic behavior, that is, peak shape and position, of this fraction was inconsistent. Therefore, the  
203 reversed-phase flash chromatography was repeated with 5 g of butanol fraction of *I. rugosus* in order to get the most  
204 bioactive compound in pure form.

### 205 **Bioactivity of fractions of butanol extract from the second reversed-** 206 **phase flash chromatography**

207 Six fractions (1B-6B) obtained through second reversed-phase flash chromatography of the butanol extract of *I.*  
208 *rugosus*, were analyzed against *A. pisum* for 24 h. Out of the six fractions analyzed, fraction 4B, 5B and 6B showed  
209 negligible toxicity (no LC<sub>50</sub> and LC<sub>90</sub>). Fraction 1B was more toxic (LC<sub>50</sub> = 2.5 ppm and LC<sub>90</sub> = 28 ppm) and  
210 moderate toxicity was observed for fraction 2B (LC<sub>50</sub> = 7.5 ppm and LC<sub>90</sub> = 71 ppm). Lower toxicity was found for  
211 fraction 3B (LC<sub>50</sub> = 16.3 ppm and LC<sub>90</sub> = 101 ppm) (Table 7).

212 **Table 7. Toxicity of subfractions of the butanol fraction from second reversed-phase flash chromatography against newborn (<24 h old) *Acyrtosiphon***  
 213 ***pisum* nymphs following 24 h exposure to artificial diet containing different concentrations of subfractions**

214

215	<b>Fractions</b>	<b>LC<sub>50</sub> (95% CI) ppm</b>	<b>Ratio</b>	<b>LC<sub>90</sub> (95% CI) ppm</b>	<b>Ratio</b>	<b>Slope ± SE</b>	<b>Chi-Square</b>	<b>HF</b>
216	1B	2.5 (1-4.1) a	1.0	28 (18-69) a	1	1.2 ± 0.3	11.4	0.9
217	2B	7.5 (4.3-11) b	3.0	71 (38-280) a	2.5	1.3 ± 0.3	16.5	1.3
218	3B	16 (11-26) c	6.5	101 (52-417) a	3.6	1.6± 0.3	22.3	1.7
219	4B	-	-	-	-	1.0 ± 0.3	25.3	2.0
220	5B	-	-	-	-	1.5 ± 1.2	6.6	0.5
221	6B	-	-	-	-	1.8 ± 0.7	6.5	0.5

222 Data is presented as 50% (LC50) and 90% (LC90) lethal concentration values (both in ppm) together with their respective 95% confidence interval (95% CI), the slope ± SE of the toxicity vs concentration curve, and the Chi-Square and  
 223 heterogeneity factor HF as accuracy of data fitting to probit analysis in POLO-PlusV2. Different letters in the same column indicate significant differences due to non-overlapping of 95% CI. Ratio, LCx, fraction/LCx, 1B

224 **Bioactivity of the ethyl acetate and aqueous phase of acidic**  
225 **extraction**

226 Both collected phases of acidic extraction were analyzed for their insecticidal potential through bioassay against *A.*  
227 *pisum* for 24 h. The aqueous phase gave negligible toxic effect (no LC<sub>50</sub> and LC<sub>90</sub>) while the ethyl acetate phase  
228 showed more toxicity (LC<sub>50</sub> = 0.2 ppm and LC<sub>90</sub> = 9.2 ppm) (Table 8).



229 **Table 8. Toxicity of ethyl acetate and aqueous phase of acidic extraction against newborn (< 24 h old) *Acyrtosiphon pisum* nymphs following 24 h**  
 230 **exposure to artificial diet containing different concentrations of both phases**

<b>Fractions</b>	<b>LC<sub>50</sub> (95% CI) ppm</b>	<b>Ratio</b>	<b>LC<sub>90</sub> (95% CI) ppm</b>	<b>Ratio</b>	<b>Slope ± SE</b>	<b>Chi-Square</b>	<b>HF</b>
Aqueous	-	-	-	-	1.5 ± 1.2	6.6	0.5
Ethyl acetate	0.2 (0.04-0.5) a	1	9.2 (3.9-13)a	1	0.8 ± 0.3	4.2	0.3

231 Data is presented as 50% (LC50) and 90% (LC90) lethal concentration values (both in ppm) together with their respective 95% confidence interval (95% CI), the slope ± SE of the toxicity vs concentration curve, and the Chi-Square and  
 232 heterogeneity factor HF as accuracy of data fitting to probit analysis in POLO-PlusV2. Different letters in the same column indicate significant differences due to non-overlapping of 95% CI. Ratio, LCx, fraction/LCx, ethyl acetate

## 233 Identification of the most bioactive compound

234 Out of the two phases of acidic extraction, the ethyl acetate phase fraction was the most active. After removing ethyl  
235 acetate azeotropically, this fraction was analyzed and the active compound was identified as rosmarinic acid through  
236 HPLC-MS, optical rotation measurement and  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy.

### 237 HPLC-MS

238 Both isolated and commercial rosmarinic acid (Sigma Aldrich) had the same peak appearance in the HPLC-MS  
239 chromatograms with the same solvent gradient. Both had a pseudo-molecular ion with an  $m/z$  value of 359 with  
240 negative mode electrospray ionization which confirmed that it was rosmarinic acid (Fig 1).

241 **Fig 1. Mass spectra (negative mode electrospray ionization) of rosmarinic acid obtained via HPLC-MS with a**  
242 **pseudo molecular ion at  $m/z$  value of 359** (a) Isolated rosmarinic acid (b) Commercial rosmarinic acid

### 243 Optical rotation and $^1\text{H}$ and $^{13}\text{C}$ NMR

244 Brown crystals;  $[\alpha]_{\text{D}}^{24} +78.0^\circ$  ( $c$  0.233, MeOH);  $^1\text{H}$  NMR (400 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  3.01 (1H, dd,  $J = 8.3, 14.3$  Hz,  
245  $\text{H}^{\text{7a}}$ ), 3.10 (1H, dd,  $J = 4.4, 14.3$  Hz,  $\text{H}^{\text{7b}}$ ), 5.19 (1H, dd,  $J = 4.4, 8.3$  Hz,  $\text{H}^{\text{8}}$ ), 6.27 (1H, d,  $J = 15.9$ ,  $\text{H}^{\text{17}}$ ), 6.61 (1H,  
246 dd,  $J = 2.0, 8.0$  Hz,  $\text{H}^{\text{6}}$ ), 6.70 (1H, d,  $J = 8.0$  Hz,  $\text{H}^{\text{5}}$ ), 6.75 (1H, d,  $J = 2.0$  Hz,  $\text{H}^{\text{2}}$ ), 6.78 (1H, d,  $J = 8.2$  Hz,  $\text{H}^{\text{14}}$ ), 6.95  
247 (1H, dd,  $J = 2.0, 8.2$  Hz,  $\text{H}^{\text{15}}$ ), 7.04 (1H, d,  $J = 2.0$  Hz,  $\text{H}^{\text{11}}$ ), 7.55 (1H, d,  $J = 15.9$  Hz,  $\text{H}^{\text{16}}$ );  $^{13}\text{C}$  NMR (100 MHz,  
248  $\text{CD}_3\text{OD}$ ):  $\delta$  37.9 ( $\text{C}^{\text{7}}$ ), 74.6 ( $\text{C}^{\text{8}}$ ), 114.4 ( $\text{C}^{\text{17}}$ ), 115.2 ( $\text{C}^{\text{11}}$ ), 116.3 ( $\text{C}^{\text{5}}$ ), 116.5 ( $\text{C}^{\text{14}}$ ), 117.6 ( $\text{C}^{\text{2}}$ ), 121.8 ( $\text{C}^{\text{6}}$ ), 123.2  
249 ( $\text{C}^{\text{15}}$ ), 127.7 ( $\text{C}^{\text{10}}$ ), 129.2 ( $\text{C}^{\text{1}}$ ), 145.3 ( $\text{C}^{\text{4}}$ ), 146.2 ( $\text{C}^{\text{3}}$ ), 146.8 ( $\text{C}^{\text{12}}$ ), 147.7 ( $\text{C}^{\text{16}}$ ), 149.7 ( $\text{C}^{\text{13}}$ ), 168.4 ( $\text{C}^{\text{18}}$ ), 173.5 ( $\text{C}^{\text{9}}$ );  
250 ESI-MS:  $m/z$  (%) 359 (M- $\text{H}^+$ , 100). Optical rotation and NMR data were in accordance with the literature (Fig 2)  
251 [31, 32].

252 **Fig 2. Structure of rosmarinic acid isolated from *I. rugosus***

## 253 Bioactivity of *I. rugosus* rosmarinic acid and commercial rosmarinic 254 acid

255 Rosmarinic acid isolated from *I. rugosus* and commercial rosmarinic acid (Sigma Aldrich) were analyzed against *A.*  
256 *pisum* for their pesticidal activity for 24 h. Both *I. rugosus* rosmarinic acid (RA) ( $\text{LC}_{50} = 0.2$  ppm and  $\text{LC}_{90} = 5.4$   
257 ppm) and commercial RA ( $\text{LC}_{50} = 0.2$  ppm and  $\text{LC}_{90} = 14$  ppm) gave similar toxic effects (Table 9).

258 **Table 9. Toxicity of isolated rosmarinic acid (RA) and commercial rosmarinic acid (RA) against newborn (< 24 h old) *Acyrtosiphon pisum* nymphs**  
 259 **following 24 h exposure to artificial diet containing different concentrations of isolated rosmarinic acid and commercial rosmarinic acid**

Compound	LC <sub>50</sub> (95% CI) ppm	Ratio	LC <sub>90</sub> (95% CI) ppm	Ratio	Slope ± SE	Chi-Square	HF
Commercial RA	0.2 (0.05-0.5) a	1	14 (7.4-42) a	2.6	0.7 ± 0.2	15.5	0.7
<i>I. rugosus</i> RA	0.2 (0.04-0.4) a	1	5.4 (3.3-12) a	1	0.8 ± 0.2	10.5	0.5

260 Data is presented as 50% (LC50) and 90% (LC90) lethal concentration values (both in ppm) together with their respective 95% confidence interval (95% CI), the slope ± SE of the toxicity vs concentration curve, and the Chi-Square and  
 261 heterogeneity factor HF as accuracy of data fitting to probit analysis in POLO-PlusV2. Different letters in the same column indicate significant differences due to non-overlapping of 95% CI. Ratio, LCx, compound/LCx, *Isodon rugosus* RA

## 262 **Comparison of the growth of surviving aphids exposed to** 263 **rosmarinic acid-treated and untreated diet after 24 h of bioassay**

264 After incorporating the rosmarinic acid in aphid's diet at 0.4 ppm, its effect on *A. pisum* that survived after 24 h  
265 treatment, was analyzed every day for up to 9 days (on same treated diet). It was confirmed that rosmarinic acid had  
266 a drastic effect on their growth. Firstly, most aphids exposed to treated diet were dead while the survivors did not  
267 grow further to become adults and were thus not able to reproduce further. Fig 3 shows a comparison between  
268 treated and untreated aphids. There was a clear difference between untreated and treated aphids after day 4, and by  
269 day 9 the treated aphids were all dead, while the untreated aphids were still alive.

270 **Fig 3. Comparison between growth of surviving aphids exposed to rosmarinic acid-treated and untreated diet**  
271 **after 24 h of bioassay, (a) to (i) comparison observed for up to 9 days, all treated aphids died by day 9**

## 272 **Discussion**

273 Screening candidate plants, purifying active ingredients, isolating and identifying the active plant constituents is  
274 required to discover new bioactive natural products [33]. We applied this methodology to identify rosmarinic acid as  
275 an active principle from the plant *I. rugosus*. Based on our previous study on the insecticidal activity of botanical  
276 extracts from various plant species, we found that the extract from *I. rugosus* was the most toxic to *A. pisum* [27]  
277 Further fractionation showed that the butanol fraction most likely contained the active principle. In this study we  
278 used the bioactivity-guided strategy to isolate and identify the active compound as rosmarinic acid. This strategy is  
279 interesting and has been used in previous studies to identify bioactive compounds. For example, the butanol fraction  
280 from *Citrullus colocynthis* was reported to be active against the black legume aphid, *Aphis craccivora*, and through  
281 the bioactivity-guided isolation strategy, the active principle, 2-O-β-D-glucopyranosylcucurbitacin E, was  
282 successfully isolated [34]. Similarly, in another study involving bioactivity-guided isolation, the active principle,  
283 aianthone, was isolated from the aqueous fraction of *Ailanthus altissima* against *A. pisum* [35].

284 In this study, the butanol fraction was subfractionated through reversed-phase flash chromatography. After  
285 bioactivity testing of all the resulting subfractions (1A-14A) against *A. pisum*, fraction 3A with lower LC values was  
286 selected for further fractionation. Through prep-LC, fraction 3A was subfractionated and the resulting subfractions  
287 (3A-1, 3A-2 and 3A-3) were analyzed for their bioactivity. Fraction 3A-3 with lower LC values was subjected to

288 spectroscopic analysis. <sup>1</sup>H NMR spectroscopy confirmed that the isolated fraction contained rosmarinic acid.  
289 However, due to the inconsistent chromatographic behavior during prep-LC, not enough compound could be  
290 collected to record <sup>13</sup>C NMR data. The inconsistent chromatographic behavior with peak splitting observed could  
291 have arisen from several causes; a contamination on guard or analytical column inlet, a blocked frit or a small void  
292 at the column inlet (~wear). The problem of peak shifting (variable retention times) could have been due to small  
293 changes in mobile composition, temperature fluctuations, column overloading or a combination of these problems  
294 which could have led to different UV patterns for each run. Due to this problem, the reversed-phase flash  
295 chromatography was repeated with a larger amount of the butanol fraction. Out of all the resulting subfractions (1B-  
296 6B), 1B was selected with lower LC values against *A. pisum*. Fraction 1B was subjected to acidic extraction to get  
297 two phases, aqueous and ethyl acetate. The ethyl acetate phase fraction was more active with lower LC values. After  
298 removing ethyl acetate, the active principle was identified through different spectroscopic techniques as rosmarinic  
299 acid. Similarly in another study, Chakraborty et al, [36] reported the isolation of caffeic acid and rosmarinic acid  
300 from *Basilicum polystachyon* through acidic extraction with HCl followed by partitioning with ethyl acetate and  
301 analyzed their antimicrobial activities.

302 This study reports the isolation and purification of rosmarinic acid (RA) from *I. rugosus* and its bioactivity  
303 against *A. pisum* for the first time. There was no significant difference observed between the bioactivity depicted by  
304 both isolated and commercial rosmarinic acid. *I. rugosus* rosmarinic acid gave LC values of LC<sub>50</sub> = 0.2 ppm and  
305 LC<sub>90</sub> = 5.4 ppm. These are very low LC values depicted after 24 h of bioassay and such low LC values have not  
306 been previously reported in any studies with compounds against *A. pisum* using the same feeding bioassay  
307 methodology [30, 37-39, 28, 40, 41]. This means that a very low amount of rosmarinic acid can cause significant  
308 toxic effects against *A. pisum* in 24 h. Very few insecticidal activities have been reported for rosmarinic acid.  
309 Regnault-Roger et al, [42] investigated the insecticidal activities of polyphenolic compounds, isolated from five  
310 plants belonging to Lamiaceae family against *Acanthoscelides obtectus* (Say) and observed that among all the  
311 polyphenolic compounds, rosmarinic acid and luteolin-7-glucoside were more toxic. An interesting avenue to follow  
312 for future studies will be the analyses of the underlying molecular mechanisms responsible for the cause of mortality  
313 in rosmarinic acid-treated aphids.

314 Additionally, a comparison between the growth of surviving aphids exposed to rosmarinic acid-treated and  
315 untreated diet after 24 h of bioassay was analyzed. It was clearly observed that the growth of surviving *A. pisum*

316 nymphs stopped after 48 h of exposure to rosmarinic acid-treated diet, resulting in a size reduction and ultimately  
317 death as compared to aphids exposed to an untreated diet. A similar observation was made by Sadeghi et al, [30]  
318 who observed that the aphid size was reduced after 48 h of exposure to novel biorational insecticides, flonicamid  
319 and pymetrozine, and mortality was observed after 72 h.

## 320 **Conclusion**

321 In this study, *I. rugosus* was identified as an interesting source for a botanical insecticide against *A. pisum*.  
322 Following bioactivity-guided selection, rosmarinic acid was isolated and identified through spectroscopic analysis as  
323 the bioactive compound in the *I. rugosus* extract. Based on the bioassay results, either the extracts from *I. rugosus* or  
324 the isolated insecticidal compound, rosmarinic acid could be exploited to develop potent aphicides, because of the  
325 high mortality of aphids caused at very low rosmarinic acid concentrations. This potential botanical insecticide may  
326 fit well in integrated pest management programs designed to control aphids. Considering that *I. rugosus* is already  
327 used for medicinal purposes, it is expected to be safer compared to the current conventional pesticides used to  
328 control aphids. Also, rosmarinic acid is known to reduce genotoxic effects induced by chemicals, which is contrary  
329 to some currently used toxic synthetic pesticides that could induce genotoxic effects in consumers. While this study  
330 highlights the potential of *I. rugosus* as a possible biopesticide source against a notorious insect pest such as *A.*  
331 *pisum*, it also provides the basis for further exploration and development of a formulation for effective field  
332 application.

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## 338 **Author Contributions**

339 SK, CT, EB, RA, NF, SM, GS and MS conceived and designed the research. SK conducted the experiments. GS and  
340 SM contributed new reagents and/or analytical tools. SK, CT and EB, MA analyzed the data. SK, CT, EB, SM, GS  
341 and MS wrote the manuscript. All authors read and approved the manuscript.

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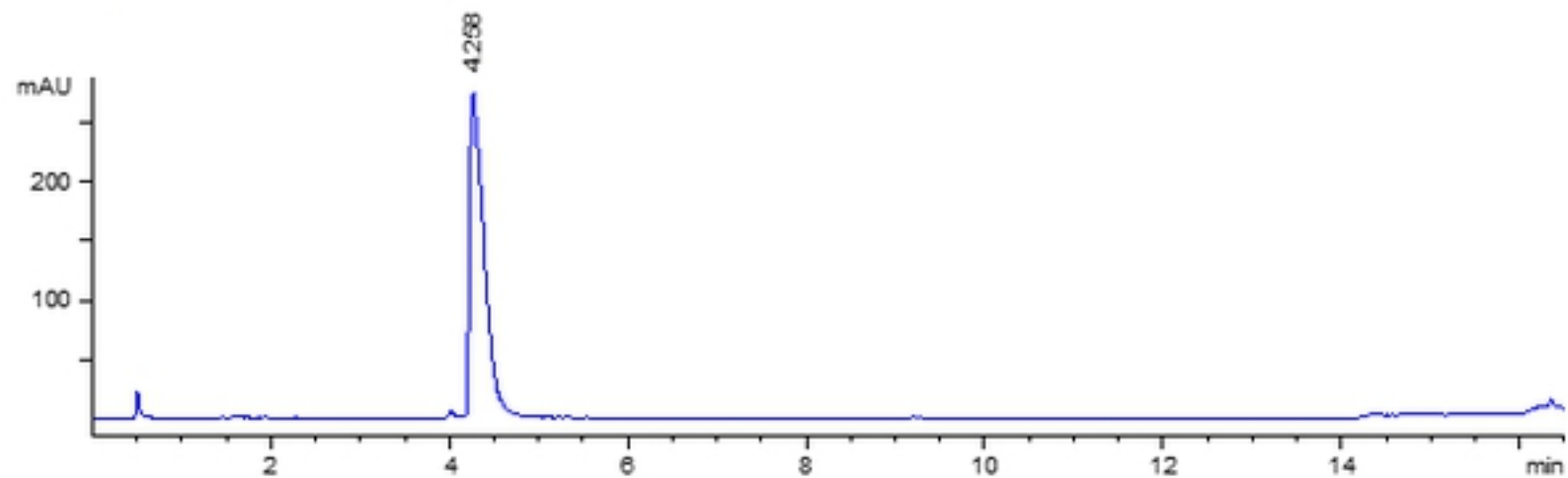


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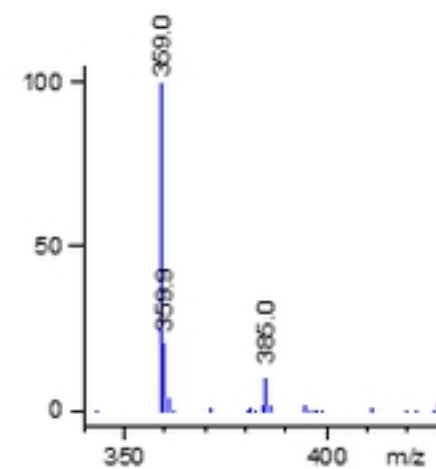
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### a) Isolated rosmarinic acid

UV signal at 280 nm

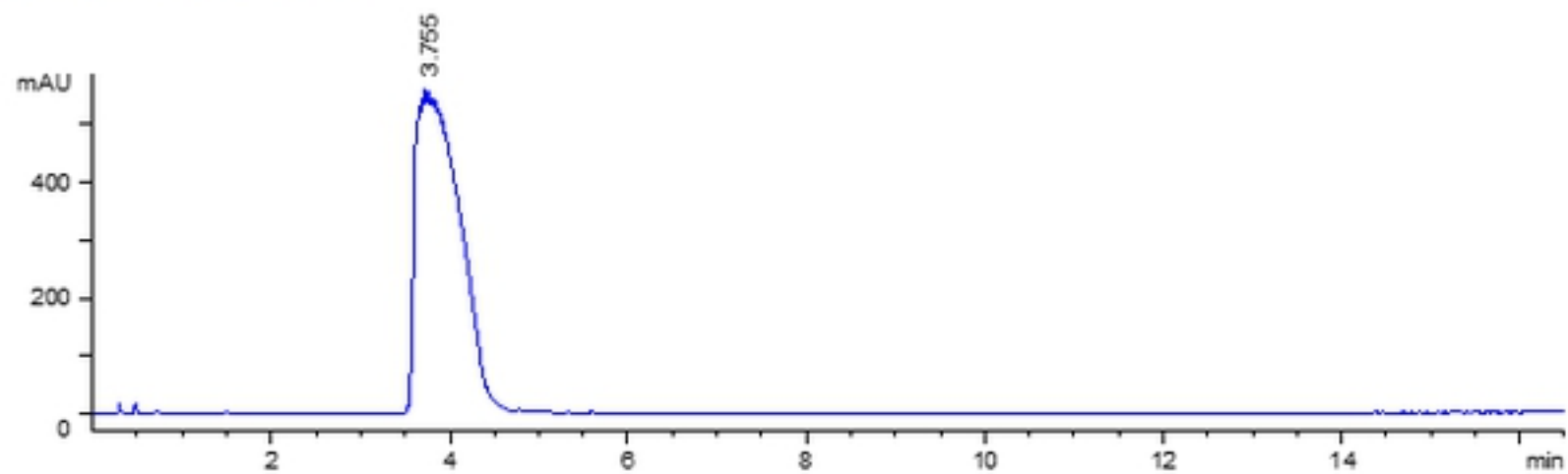


MS signal at 4.3 min



### b) Commercial rosmarinic acid

UV signal at 280 nm



MS signal at 3.8 min

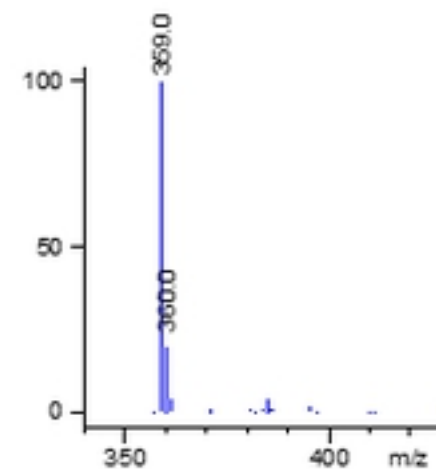


Figure 1

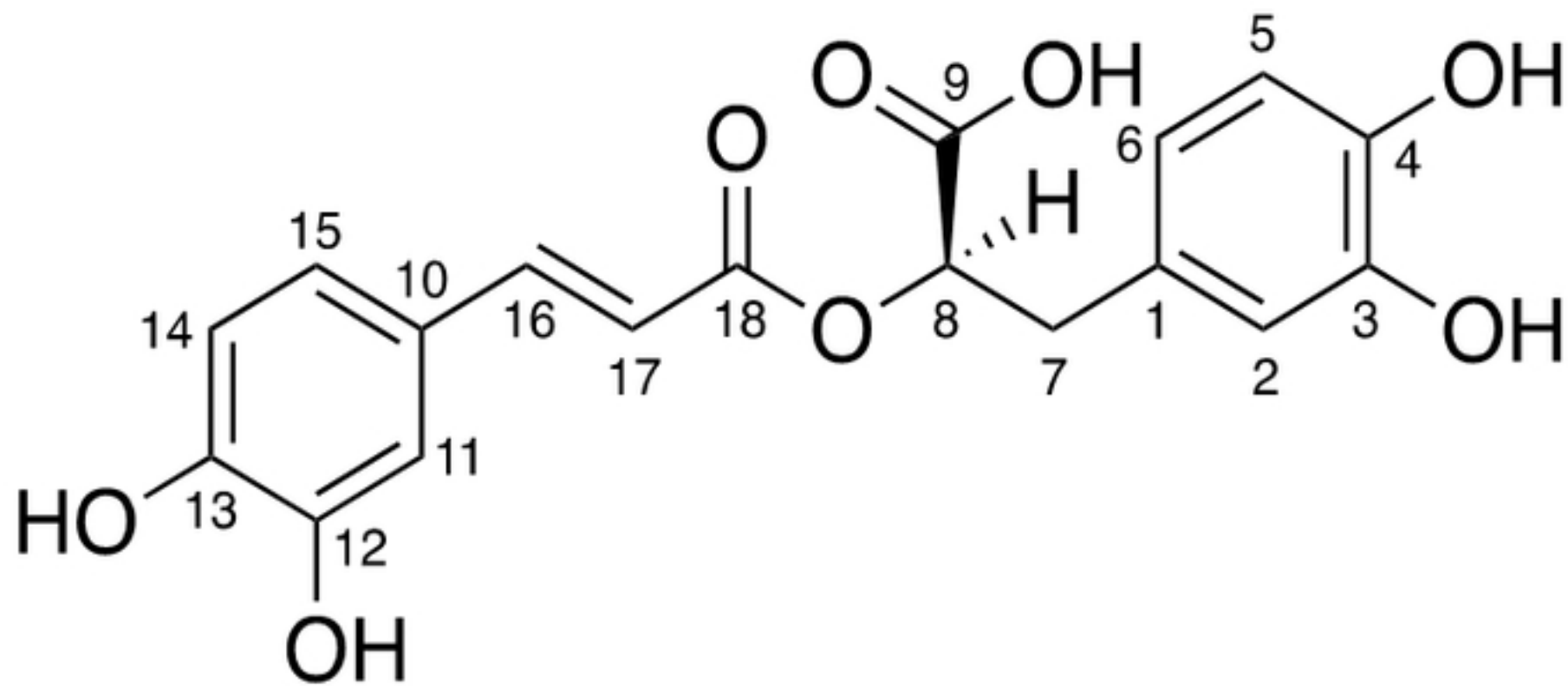


Figure 2

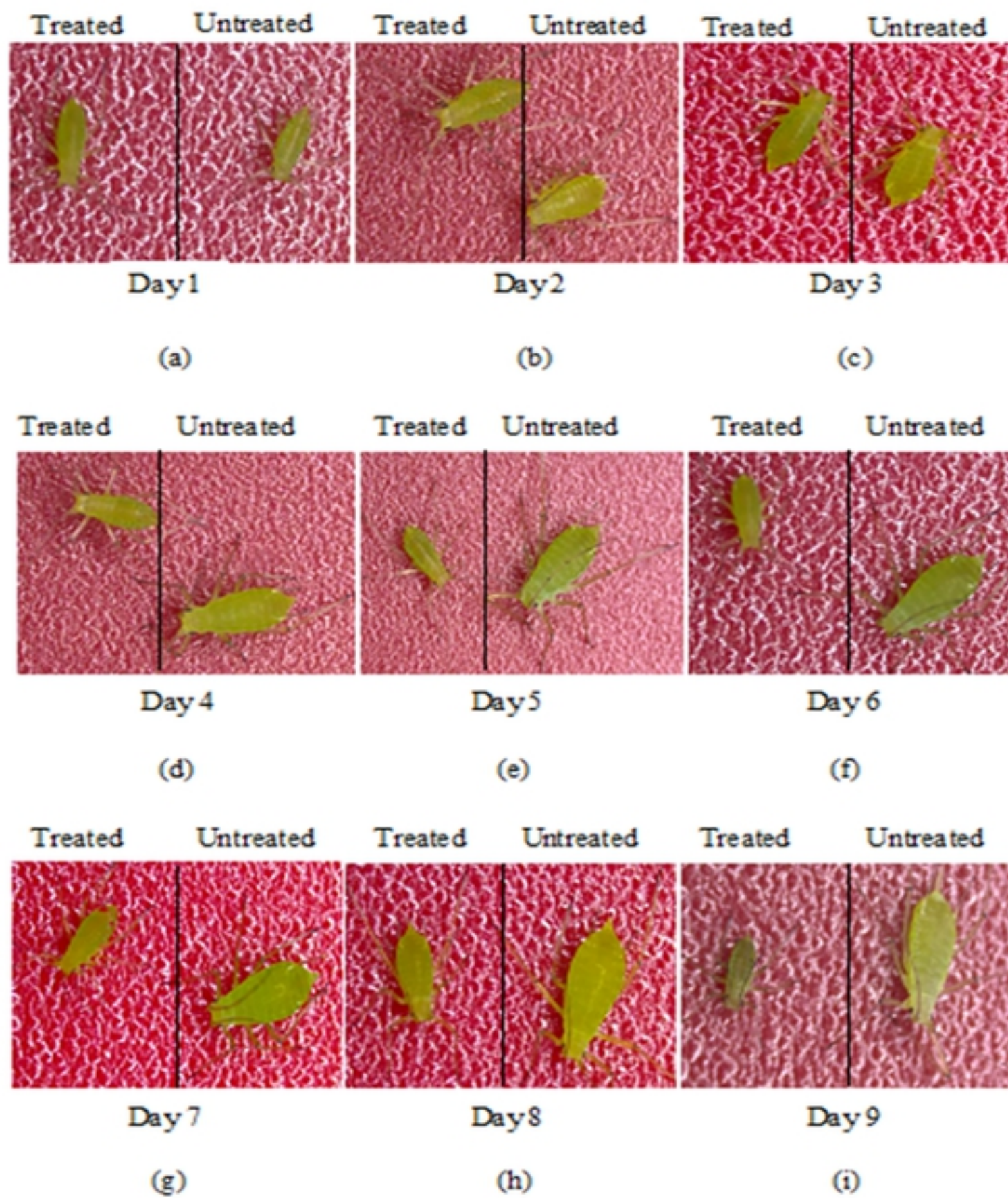


Figure 3