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Anket Sharma
Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar - 143005, Punjab, India.

Vinod Kumar
Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, 143005, India.

Ravinder Singh
Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, 143005, India.

Ashwani Kumar Thukral
Professor, Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, 143005, India.

Renu Bhardwaj
Professor, Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, 143005, India.

Correspondence:
Renu Bhardwaj
Professor, Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, 143005, India.

24-Epibrassinolide induces the synthesis of phytochemicals effected by imidacloprid pesticide stress in *Brassica juncea* L.

Anket Sharma, Vinod Kumar, Ravinder Singh, Ashwani Kumar Thukral, Renu Bhardwaj

Abstract
The present study was designed to observe the effect of 24-epibrassinolide (EBL) on active phytochemicals present in *Brassica juncea* L. plants under imidacloprid (IMI) stress. GC-MS analysis of methanolic extracts of 30 days old leaves revealed the stress protective role of 24-EBL by inducing the synthesis of various phytochemicals in *B. juncea* L. plants under the pesticide stress. Major compounds detected were3-n-butylthiolane, 8-methyl-alpha-ionone, 3,5,6-Tetrafluoroanisole, oxalic acid, ethyl 2-isopropylphenyl ester, nonylphenol isomers, linoleic acid, benzenepropanoic acid 3,5-bis(1,1-dimethylethyl)-4-hydroxy- methyl ester, (1E)-1-ethylidene-7a-methyloctahydro-1H-indene, palmitic acid, cis-jasmone, stearic acid, hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, clionasterol, 6-octadecenoic acid.

Keywords: GC-MS analysis, *Brassica juncea,* methanolic extract, imidacloprid, brassinosteroids, 24-epibrassinolide.

1. Introduction
Brassica juncea L. commonly known as Indian mustard belongs to family cruciferae and is one of the economically important plants known for its nutritive as well as medicinal value [1]. Leaf mustard is generally consumed as vegetable and contains numerous nutrients as well as vitamin c, antioxidants, beta-carotene and vitamin e, which contribute in healthy cardiovascular system. Leaf mustard, during its growth process is attacked by various diseases and insects like mites, army worms, *Plutella sp.*, cut worms, aphids, leaf roller, leaf rot and soft rot. In order to control these insect pests, different pesticides are applied which include organophosphates and pyrethroid [2].
The frequent use of pesticides on crops is responsible for contamination with harmful residues of pesticides in *Brassica juncea* [3]. Plants treated with pesticides resulted in phytotoxic symptoms like reduction in net photosynthetic rate (Pn), decline in stomatal conductance (Gs) and changes in intercellular CO_2 concentrations [4].
Brassinosteroids (BRs) are a new class of plant polyhydroxysteroids which are present in low levels in pollens, seeds, and young vegetative tissues and are distributed throughout the plant kingdom [5]. BRs have the ability to confer plant resistance against various types of environmental stresses like temperature, salt, drought, ozone, pesticides and herbicides [6]. In cucumber leaves, exogenous applications of BRs have shown to reduce the phytotoxic effects of herbicides, fungicides and insecticides [4, 7]. In rice, BRs have shown the reduction in damage caused by simazine, butachlor, pretilachlo [8], imidacloprid and chlorpyrifos [9, 10]. In tomato, exogenous application of BRs resulted in decrease in phytotoxic effects under pesticide stress [11]. BRs induced stress protection in plants is achieved by induction of several phytochemicals. Keeping in view the protective role of BRs, present experiment was designed to study the effect of 24-EBL on various phytochemicals in *Brassica juncea* L. plants under IMI stress.

2. Material and methods
2.1. Raising of plant material
Plants of *B. juncea* L. were grown in pots treated with 0.025 % IMI concentration. To study the effect of BRs on synthesis of various phytochemicals in *B. juncea* L., seeds were treated with 100 nM concentrations of 24-EBL (pre-soaking for 8 hours) before sowing. Phytochemical analysis was done in leaves of 30 days old plants.

2.2. Sample preparation

5 g of fresh leaves were extracted with methanol and the methanolic extract was then dried using vacuum rotary evaporator at 50 °C and the residue was dissolved in 2.0 ml methanol.

2.3. Analysis using GC-MS

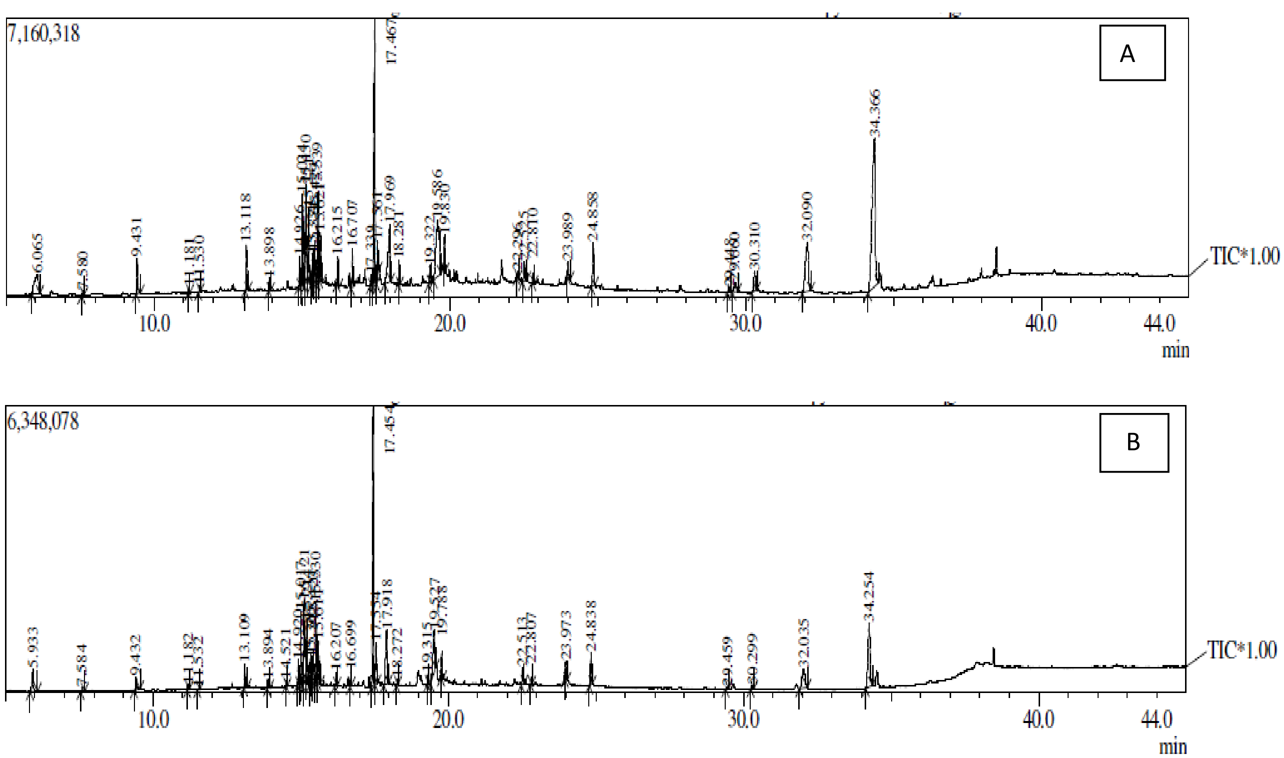
The methanolic extracts of 30 old leaves of *B. juncea* were analysed using Shimadzu GCMS-QP2010 Plus. Helium was used as carrier gas, column oven temperature was initially set at 70 °C and held for 5 minutes, then increased to 250 °C at 10 °C / min and held for 10 minutes, again increased to 300 °C at 10 °C / min and held for 10 minutes. Injection temperature; 280 °C, injection mode; splitless, sampling time; 1 minute, flow control mode; linear, pressure; 110.8 k Pa, total flow 38.9 ml/min, column flow; 1.71 ml/min, linear velocity; 47.9 cm/sec, purge flow; 3 ml/min, sample injection volume; 4 µl, ion source temperature; 250 °C, interface temperature 290 °C, solvent cut time; 3.5 minute and detector gain mode; relative. Analytical column used was DB-5ms with 30 m length and 0.025 mm id.

All the detected compounds were identified by comparing mass spectra with National Institute of Standard and Technology (NIST08s) and Wiley 7 library.

3. Results and discussion

GC-MS analysis of methanolic extracts of 30 day old leaves of *B. juncea* L. plants, resulted in detection of a number of active compounds. Total 35 phytochemicals in the untreated plants were reported. In IMI treated plants, the number was 33, while it was 35 in plants treated with 24-EBL + IMI (Figure 1). The active phytochemicals detected are listed in tables 1, 2 and 3, along with their peak number, retention time and concentration (peak area %) for control, 0.025 % IMI treatment and combined application of 0.025 % IMI + 24-EBL respectively.

The application of 0.025 % IMI resulted in declined levels of active phytochemicals as compared to the control plants but the application of 0.025 % IMI in combination with 24-EBL resulted in recovery of many of these phytochemicals (Table 4). Some compounds like n-Nonylphenol, p-Nonylphenol and (1E)-1-Ethylidene-7a-methyloctahydro-1H-indene, increased under pesticide stress, which were further noticed to increase by the application of 24-EBL. When, only 0.025% IMI treated plants were compared with 100 nM 24-EBL + 0.025% IMI treated plants, the phytochemicals detected with more than 35% recovery were 3-n-butylthiolane, butanoic acid, 2-ethyl-3-hydroxy-4-(1,1-dimethylethoxy) methyl ester, phosphonofluoridic acid, (1-methylethyl)-ethyl ester, 4,7-octadecadiynoic acid, methyl ester, nonylphenol isomer, phenol, 2-methyl-5-(1-methylethyl)-carvacrol, acetic acid, 2-(2,2,6-trimethyl-7-oxa-bicyclo[4.1.0]hept-1-yl)-propenyl ester andlucenin 2 (Table 4). The major compounds detected in combined application of IMI and 24-EBL were 3-n-butylthiolane, nonyphenol isomers, p-tert-amylphenol, longifolenaldehyde, benzenepropanoic acid 3,5-bis(1,1-dimethylethyl)-4-hydroxy-methyl ester, (1E)-1-ethylidene-7a-methyloctahydro-1H-indene, palmitic acid, cis-jasmone and 6-octadecenoic acid (Table 3). GC-MS analysis in *B. juncea* L. revealed the synthesis of many metabolites involved in pesticide stress management. BRs treatment further ameliorated the pesticide stress by enhancing the synthesis of key biomolecules by significant amounts, which indicates their active participation in stress protection. The mechanism by which they are synthesising, needs further studies. Further, the application of BRs on tomato, rice, broccoli, cucumber and strawberry plants has also resulted in lowering the pesticide residues [7, 11]. Our earlier studies done in this direction also indicate the degradation of pesticides and lowering the contents by its residues in *B. juncea* L., when given treatment of BRs.



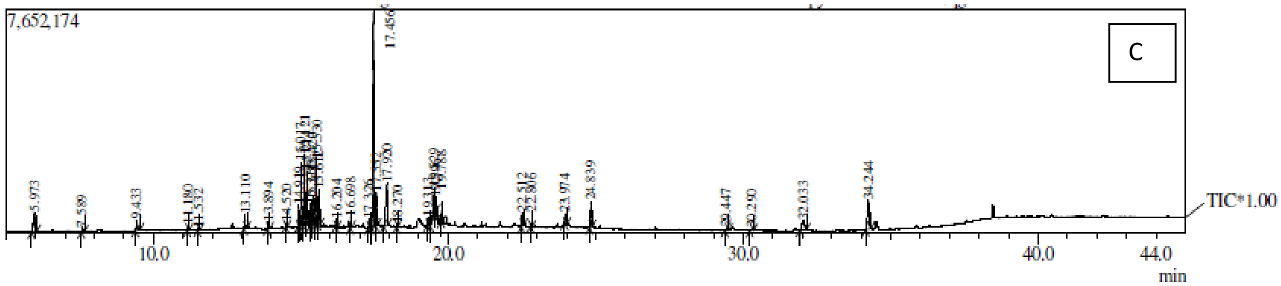


Fig 1: Total ion chromatogram for GC-MS analysis of methanolic extracts of 30 days old plants of *Brassica juncea* L. untreated plants (A), IMI treated plants (B) and 24-EBL + IMI treated plants (C).

Table 1: GC-MS analysis of methanolic extracts of 30 days old plants of *Brassica juncea* L. (Untreated).

Peak No.	Name of compound	Retention time	Area %
1	3-n-Butylthiolane	6.06	3.76
2	Butanoic acid, 2-ethyl-3-hydroxy-4-(1,1-dimethylethoxy)-, methyl ester	7.58	0.19
3	8-Methyl-.alpha.-ionone	9.43	1.74
4	Phosphonofluoridic acid, (1-methylethyl)-, ethyl ester	11.18	0.17
5	4,7-Octadecadiynoic acid, methyl ester	11.53	0.20
6	2,3,5,6-Tetrafluoroanisole	13.11	1.62
7	Pentafluoropropionic acid, hexadecyl ester	13.89	0.50
8	Nonylphenol isomer	14.92	0.97
9	P-((1,1,3,3)-tetramethylbutyl)phenol	15.02	3.22
10	n-Nonylphenol	15.13	2.05
11	Phenol, 4-nonyl- p-Nonylphenol	15.22	2.84
12	Phenol, 2-methyl-5-(1-methylethyl)- Carvacrol	15.33	0.70
13	Tricyclo[3,1,0,0(2,4)]hexane, 3,3,6,6-tetraethyl	15.37	0.77
14	1,4-Methano-1H-cyclohepta[d]pyridazine, 4,4a,5,6,7,8,9,9a-octahydro-10,10-dimethyl-	15.42	1.01
15	p-tert-Amylphenol	15.53	3.15
16	1,2-Epoxy-1,2,5.9,9-pentamethyl-spiro(3.5)non-5-ene	15.62	2.14
17	Stearyl alcohol	16.21	0.99
18	Linoleic acid	16.70	1.34
19	Campherenone	17.33	0.37
20	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	17.46	14.10
21	(1E)-1-Ethylidene-7a-methyloctahydro-1H-indene	17.56	0.97
22	Palmitic acid	17.96	5.84
23	Octadecyl trifluoroacetate	18.28	0.77
24	Acetic acid, 2-(2,2,6-trimethyl-7-oxa-bicyclo[4.1.0]hept-1-yl)-propenyl ester	19.32	0.67
25	2,6,10,15,19,23-Hexamethyl-tetracosa-2,10,14,18,22-pentaene-6,7-diol	19.58	9.22
26	Stearic acid	19.83	1.32
27	[2-(5-hydroxy-pent-2-ynyl)-3-oxo-cyclopentyl]-thioacetic acid s-tert-butyl ester	22.29	0.24
28	Alpha.-18o-1,25-dihydroxycholecalciferol	22.52	1.01
29	Lucenin 2	22.81	0.30
30	Ergost-5-en-3-ol, 22, 23-dimethyl-, (3.beta.)	23.98	0.69
31	2,6-Dodecadienoic acid, 10-bromo-11-hydroxy-3,7,11-trimethyl-, methyl ester	24.85	2.32
32	Cholesterol	29.66	0.93
33	Vitamin E acetate	30.31	1.72
34	Dihydrobrassicasterol	32.09	7.65
35	Clionasterol	34.36	24.15

Table 2: GC-MS analysis of methanolic extracts of 30 days old plants of *Brassica juncea* L. grown in imidacloprid amended soil.

Peak No.	Name of compound	Retention time	Area %
1	3-n-Butylthiolane	5.93	3.00
2	Butanoic acid, 2-ethyl-3-hydroxy-4-(1,1-dimethylethoxy)-, methyl ester	7.58	0.33
3	8-Methyl-.alpha.-ionone	9.43	1.17
4	Phosphonofluoridic acid, (1-methylethyl)-, ethyl ester	11.18	0.32
5	2,6-Dodecadienoic acid, 10-bromo-11-hydroxy-3,7,11-trimethyl-, methyl ester	11.53	0.08
6	2,3,5,6-Tetrafluoroanisole	13.10	1.54
7	Tricosyl pentafluoropropionate	13.89	0.59
8	Phenol, 2-methyl-5-(1-methylethyl)-Carvacrol	14.52	0.27
9	Nonylphenol isomer	14.92	1.35

10	P-((1,1,3,3)-tetramethylbutyl)phenol	15.01	4.52
11	Phenol, 4-nonyl- (CAS) p-Nonylphenol	15.12	6.62
12	n-Nonylphenol	15.21	4.20
13	4-Dodecylphenol	15.32	0.84
14	Tricyclo[3.1.0.0(2,4)]hexane, 3,3,6,6-tetraethyl-,trans	15.36	1.13
15	1,4-Methano-1H-cyclohepta[d]pyridazine, 4,4a,5,6,7,8,9,9a-octahydro-10,10-dimethyl	15.42	1.40
16	p-tert-Amylphenol	15.53	4.46
17	1,2-Epoxy-1,2,5.9,9-pentamethyl-spiro(3.5)non-5-ene	15.61	3.40
18	Stearyl alcohol	16.20	0.90
19	Linoleic acid	16.69	0.84
20	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	17.45	22.28
21	(1E)-1-Ethylidene-7a-methyloctahydro-1H-indene	17.55	2.94
22	Palmitic acid	17.91	6.66
23	Acetic acid, 2-(2,2,6-trimethyl-7-oxa-bicyclo[4.1.0]hept-1-yl)-propenyl ester	18.27	0.29
24	1.Alpha.-18o-1,25-dihydroxycholecalciferol	19.31	0.58
25	cis-Jasmone	19.52	6.53
26	Stearic acid	19.78	1.62
27	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	22.51	1.18
28	Lucenin 2	22.80	0.33
29	Ergost-5-en-3-ol, 22, 23-dimethyl-, (3.beta.)	23.97	1.23
30	2, 6-Dodecadienoic acid, 10-bromo-11-hydroxy-3, 7, 11-trimethyl-, methyl ester.	24.83	2.66
31	Vitamin E acetate	30.29	0.54
32	Dihydrobrassicasterol	32.03	4.74
33	Clionasterol	34.25	11.24

Table 3: GC-MS analysis of methanolic extracts of 30 days old plants of *Brassica juncea* L. raised from 24-EBL treated seeds grown in imidacloprid amended soil.

Peak No.	Name of compound	Retention time	Area %
1	3-n-Butylthiolane	5.97	3.66
2	Butanoic acid, 2-ethyl-3-hydroxy-4-(1,1-dimethylethoxy)-, methyl ester	7.58	0.39
3	8-Methyl-.alpha.-ionone	9.43	1.24
4	Phosphonofluoridic acid, (1-methylethyl)-, ethyl ester	11.18	0.41
5	4,7-Octadecadiynoic acid, methyl ester	11.53	0.12
6	2,3,5,6-Tetrafluoroanisole	13.11	1.24
7	Tricosyl pentafluoropropionate	13.89	0.59
8	Phenol, 2-methyl-5-(1-methylethyl)-Carvacrol	14.52	0.31
9	Oxalic acid, ethyl 2-isopropylphenyl ester	14.91	1.57
10	Nonylphenol isomer	15.01	5.28
11	Phenol, 4-nonyl- (CAS) p-Nonylphenol	15.12	7.28
12	n-Nonylphenol	15.21	4.63
13	4-Dodecylphenol	15.32	1.13
14	Tricyclo[3,1,0,0(2,4)]hexane, 3,3,6,6-tetraethyl	15.36	1.18
15	1,4-Methano-1H-cyclohepta[d]pyridazine, 4,4a,5,6,7,8,9,9a-octahydro-10,10-dimethyl-	15.42	1.61
16	p-tert-Amyl phenol	15.53	5.03
17	1,2-Epoxy-1,2,5.9,9-pentamethyl-spiro(3.5)non-5-ene	15.61	3.66
18	Tricyclo[7.2.0.0(2,6)]undecan-5-ol, 2,6,10,10-tetramethyl- (isomer 3)	16.20	0.72
19	Linoleic acid	16.69	0.83
20	Longifolenaldehyde	17.32	0.62
21	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	17.45	23.76
22	(1E)-1-Ethylidene-7a-methyloctahydro-1H-indene	17.55	3.26
23	Palmitic acid	17.92	7.45
24	Alpha.-18o-1,25-dihydroxycholecalciferol	18.27	0.17
25	Acetic acid, 2-(2,2,6-trimethyl-7-oxa-bicyclo[4.1.0]hept-1-yl)-propenyl ester	19.31	0.62
26	cis-Jasmone	19.52	5.09
27	Stearic acid	19.59	1.47
28	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	19.78	1.58
29	Lucenin 2	22.51	1.33
30	2,6-Dodecadienoic acid, 10-bromo-11-hydroxy-3,7,11-trimethyl-, methyl ester	22.80	0.20
31	3.alpha.,4.alpha.,9.beta.,11-Diepoxymuurolan-10-ol	23.97	0.89
32	Vitamin E acetate	29.44	0.33
33	Dihydrobrassicasterol	30.29	0.31
34	Clionasterol	32.03	2.85
35	6-Octadecenoic acid	34.24	6.40

Table 4: GC-MS analysis showing the effect of 24-EBL on various phytochemicals present in 30 days old plants of *Brassica juncea* L. under imidacloprid stress (CN = control, T1 = 0.025 % IMI, T2 = 0.025 % IMI + 100 nM 24-EBL, (-) = decrease and (+) = increase %).

S. No.	Name of the compound	Peak area (Treatments)			Comparison of peak area (in %)	
		CN	T1	T2	CN vsT1	T1 vs T2
1	3-n-Butylthiolane	4514248	1786348	2512205	(-) 60.43	(+)40.63
2	Butanoic acid, 2-ethyl-3-hydroxy-4-(1,1-dimethylethoxy)-, methyl ester	224975	196448	269216	(-) 12.68	(+) 37.04
3	8-Methyl-.alpha.-ionone	2090086	695077	851778	(-) 66.74	(+) 22.54
4	Phosphonofluoridic acid, (1-methylethyl)-, ethyl ester	200270	188848	283378	(-) 05.70	(+) 50.05
5	4,7-Octadecadiynoic acid, methyl ester	242336	47159	80058	(-) 80.54	(+) 69.76
7	Pentafluoropropionic acid, hexadecyl ester	605749	352547	407573	(-) 41.80	(+) 15.60
8	Nonylphenol isomer	1165217	805152	3622073	(-) 30.90	(+)349.86
9	n-Nonylphenol	2456993	2497690	3181363	(+) 01.66	(+) 27.37
10	Phenol, 4-nonyl- p-Nonylphenol	3403154	3937508	4998894	(+) 15.70	(+) 26.95
11	Phenol, 2-methyl-5-(1-methylethyl)-Carvacrol	840216	159770	214994	(-) 80.98	(+) 34.56
12	Tricyclo[3,1,0,0(2,4)]hexane, 3,3,6,6-tetraethyl	920567	671040	811179	(-) 27.11	(+) 20.88
13	1,4-Methano-1H-cyclohepta[d]pyridazine, 4,4a,5,6,7,8,9,9a-octahydro-10,10-dimethyl	1212662	830377	1108519	(-) 31.52	(+) 33.49
14	p-tert-Amyl phenol	3783256	2653980	3453194	(-) 29.85	(+) 30.11
15	1,2-Epoxy-1,2,5.9,9-pentamethyl-spiro(3.5)non-5-ene	2565575	2025836	2510582	(-) 21.04	(+) 23.92
16	Linoleic acid	1606891	501145	567894	(-) 68.81	(+) 13.31
17	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	16918525	13255456	16312678	(-) 21.65	(+) 23.06
18	(1E)-1-Ethylidene-7a-methyloctahydro-1H-indene	1167356	1751856	2237279	(+) 50.07	(+) 27.70
19	Palmitic acid	7012031	3960537	5114622	(-) 43.52	(+) 29.13
20	Acetic acid, 2-(2,2,6-trimethyl-7-oxa-bicyclo[4.1.0]hept-1-yl)-propenyl ester	805831	170290	425167	(-) 78.87	(+)149.67
21	Stearic acid	1578269	962779	1007884	(-) 39.00	(+) 0 4.68
23	Lucenin 2	359163	198236	912949	(-) 44.81	(+)360.53

4. Conclusion
B. juncea L. finds its significance from nutritive and medicinal value and its bioactive compounds play a major role for this. Extensive use of pesticides for protection against insect pests, resulted in decreased concentrations of active phytochemicals leading to pesticide contamination in vegetables. Present study showed the protective role of 24-EBL in *B. juncea* L. plants under IMI stress.

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