Bioactive Metabolites from Germinated *Cajanus cajan* (L.) Millsp. Seeds after Treating with Different Concentrations of Salicylic Acid

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Salicylic acid (SA) is an effective elicitor for enhancing product formation in various agricultural practices. This study examined the diverse responses to physiological metabolites and the pathway modifications in broad-spectrum resistance-1 (BSR1) of *Cajanus cajan* after treatment with SA using a metabolomics technique. The significance of the SA function at the metabolite level was examined by treating *C. cajan* with various concentrations of SA and germinated by soaking in water for different time periods. The secondary metabolites were recovered and investigated by gas chromatography-mass spectrometry for all the periodic conditions. Chemometric analysis of the collected samples showed that the seeds responded to the SA treatment. Acetic acid increased in germinated seeds after the SA treatment. In addition, the up-regulated metabolite production was downregulated in the *C. cajan* seeds before germination. The levels of metabolites, including hyacinthin, furaneol, citramalic acid, palmitate, stearate, linoleate, tocopherol, glucobrassicin, syringol, and hydroxy acetophenone, were increased after the SA treatment compared to control. Hence, the SA-treated seedling is a potential bio-factory for nutraceutical products to provide significant health benefits to the human population.

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INTRODUCTION

Cajanus cajan (pigeon pea) is one of the most important human dietary nutrient sources in several countries (Talari and Shakappa 2018). It is cultivated in tropical and subtropical countries worldwide, notably in South Asia, Eastern and Southern Africa, Latin America, the Caribbean, and Australia (Suresh and Chandrakanth 2016). Pigeon pea is the second most common protein source and ranks sixth in terms of area and production among grain legumes in India (Chakraborty *et al*. 2007; Fu *et al*. 2006, 2007). Approximately 76% of the total global area planted with *C. cajan* falls within India, which corresponds to approximately 73% of world production (Chakraborty *et al*. 2007).

C. cajan extracts are commonly used worldwide to treat diabetes, dysentery, hepatitis, measles, and as a febrifuge to stabilize the menstrual cycle (Amalraj and Ignacimuthu 1998). *C. cajan* leaves have long been used in traditional Chinese medicine to cure pain, wounds, bedsores, malaria, diet-induced hypercholesterolemia, and anthelmintic activity (Grover *et al.* 2002). The leaf extracts are effective against alcohol-induced liver damage and hypoxic-ischemic brain damage (Huang *et al.* 2006). Chemical composition analysis showed that *C. cajan* leaves are enriched with flavonoids and stilbenes, which are crucial for the therapeutic effects of these leaves on humans (Duker-Eshun *et al*. 2007; Zheng *et al.* 2007).

Vanillic acid or *p*-coumaric acid is a phenolic derivative detected in *C. cajan.* Anthocyanins were detected in the leaves and seeds of *C. cajan*. The leaves were reported to comprise more bioactive compounds than the seeds, indicating the potential use of different parts of the *C. cajan* plant for treating various diseases (Ade-Omowaye *et al*. 2015). Phytochemical analysis of leaves, seeds, and stem extracts of *C. cajan* revealed the presence of alkaloids, saponins, flavonoids, tannins, anthraquinones, alkaloids, and reducing sugars. On the other hand, terpenoids and cardiac glycosides were absent in some parts of *C. cajan* (Hassan *et al.* 2016).

The bioavailability of amino acids plays a significant role in determining the nutritional value of plant proteins, and *C. cajan* has been reported to contain the lowest amount of sulfur-containing amino acids, *e.g.*, methionine and cysteine (Hassan *et al.* 2016; Nwaogy and Emejulu 2010). Red gram contains a few anti-nutritional factors, such as phytolectins, polyphenols (tannins and phenols) (Wu *et al.* 2009; Balasubramanian *et al.* 2018), and a few enzyme inhibitors (trypsin, chymotrypsin, and amylase). These inhibitors were in higher concentration in some wild varieties of *C. cajan* (Singh 1981). Among the commercial varieties, such as BSR1, APK1, CO7, and VBN3 available across the country, there are large variations in the levels of these protease inhibitors. Apart from trypsin inhibitors, several compounds adversely affect human digestive enzyme activity (Singh 1984). The levels of anti-nutritional factors could be decreased by processing methods, such as germination, cooking, and chemical soaking. Chemical soaking is a process that involves soaking dry seeds in salt solutions (1% sodium bicarbonate solution) at different concentrations (Devindra and Aruna 2017). Seed germination is a crucial stage in plant development. Physiological, morphological, and biochemical changes occur during seed development, which is strongly related to the survival rate and quality of seedlings (Ohanenye *et al.* 2020).

Salicylic acid (2-hydroxybenzoic acid) (SA) is an effective extracellular signaling compound of plant cells that can trigger or initiate plant defense responses (An and Mou 2011) which is a signal as opposed to a physiological effector. A single elicitor signal triggers a series of events, which may be aided by various secondary signals. Several elicitor signal transduction fields have shown significant advancements: elicitor signal detection by diverse plant receptors; avirulence determinants and associated plant R proteins that contribute to the generation of secondary plant metabolites (jasmonate, ethylene, and abscisic acid signaling); other lipid messengers, such as phosphatidic acid, diacylglycerol, and lysophosphatidylcholine. Elicitors use these signal elements, either directly or indirectly, to induce the buildup of secondary plant metabolites (Zhao *et al.* 2010). A study was conducted to understand the effects of SA on the germination and early seedling growth of *C. cajan*. At concentrations up to 20 mg/L, SA positively regulates germination and seedling growth. Plants with low SA concentration produced higher number of leaves. At concentrations below 10 mg/L, however, the growth-promoting

impact of SA was significantly lower (Ikhajiagbe 2020).

The product of the phenylpropanoid pathway, salicylic acid, is traditionally a signal in systemic acquired resistance (SAR), and transgenic plants bearing the bacterial nahG gene, which encodes for SA hydroxylase, have a reduced SAR (Dettmer *et al*. 2007). The phenylpropanoid pathway begins with the transformation of *l*-phenylalanine to trans-cinnamic acid. PAL (*l*-phenylalanine ammonia-lyase) (EC 4.3.1.5) is a critical regulatory enzyme in the synthesis of phenolics, catalyzing the ammonia elimination process (Jones 1984). The phenylpropanoid route yields flavonoids, such as anthocyanins and condensed tannins, which are produced from *p*-coumaric acid. Another type of tannin is hydrolyzable tannins, which are gallic acid ester derivatives that can be produced by the shikimate or phenylpropanoid pathways (Ishikura *et al.* 1984). This study was designed to examine the metabolic changes in *C. cajan* during germination upon treatment with the elicitor, SA.

EXPERIMENTAL

Sample Preparation

The *C. cajan* BSR1 variety was collected from Agricultural Research Station, Bhavani Sagar, Tamil Nadu, India. A voucher specimen was deposited in SRM Institute of Science and Technology, Herbarium collection, Kattankulathur, Tamil Nadu, India. The sample was surface sterilized with 0.4 % sodium hypochlorite for 10 mins, followed by placing the seeds in 70 % ethanol for 30 s, and washed three times with sterile distilled water (Sauer and Burroughs 1986). The seeds were soaked in the dark for approximately 4 h with different SA concentrations (0.5, 1, and 1.5 mM). The SA treated seeds were placed in petri dishes lined with layers of filter paper to maintain the moisture during germination. The samples were collected at different incubation times, that is, 8, 16, and 24 h, and stored at –20 °C. All the stored samples were dried completely in an incubator at 50 °C for 6 h until the original weight was obtained. The samples were ground finely and stored at –20 °C until further analysis.

Metabolite Extraction

The powdered samples of different SA concentrations were extracted with HPLC grade methanol in a ratio of 1:6 (w/v) for 72 h in shaking condition (Remi, India) at 100 rpm. The samples were then subjected to sonication at 50 Hz for 2 minutes and again kept in the shaker for 24 h. The methanolic extracts were collected, concentrated to a 1:1 (w/v) ratio, and used for further analysis (Sudjaroen *et al*. 2005).

Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

Samples were analyzed on a gas chromatograph (7890 B GC, Agilent) coupled to a mass spectrometer (5977A MSD, Santa Clara, CA) and operated using the Agilent MSD ChemStation software (Santa Clara, CA). A HP-5 MS 5% phenyl methyl silox capillary column with dimensions 30 m × 250 µm × 0.25 µm was used for separation. Helium was used as the carrier gas with a flow rate of 1 mL/min. A 2 µL sample was injected through an autosampler (G4513A) in splitless mode. The temperatures of the GC oven throughout the run were as follows: initial temperature of 50 °C, held for 2 min, a ramp in temperature of 10 °C per min to 270 °C for 10 min, and a total run time of 34 min. The injector temperature was set to 250 °C. The temperature of the transfer line from the GC column to the MS was 280 °C. The source and quadrupole temperatures were 230 and 150 °C,

respectively. Source fragmentation was achieved by electron ionization (EI) at 70 eV, with a scan range of 40 to 600 *m/z* and a scan rate of 2.60 scans per second. The data were visualized using an Agilent MSD Chemstation data analysis software. The MS spectra obtained were then compared with the reference spectra present in the NIST Lib 2011 (Kartikeyan *et al.* 2022).

Statistical analysis

All the analyses were done in triplicates. The results were analyzed by ANOVA and t-test using Microsoft Excel 2013 version. The significant differences ($P < 0.5$, $P < 0.05$, $P < 0.005$) among individual means were calculated.

RESULTS AND DISCUSSION

Most of the food legumes accumulate anti-metabolic constituents during seed development. Germination helps to enhance the digestion process by simplifying the dietary protein content. On the other hand, the SA treatment during germination elucidated certain types of organic compounds, which are required for seed protection and speeding up the digestion process. *C. cajan* seeds were germinated under SA elicitation (Fig. 1). Numerous metabolites are produced under these conditions (Raymaekers *et al.* 2020). The variation in metabolites among different SA concentrations was studied by GC–MS.

Fig. 1. *Cajanus cajan* stages of seed germination after the imbibition

GC–MS Analysis

The effects of SA treatment during germination of *C. cajan* were studied. Figures 2, 3, and 4 represents the GC–MS chromatograms for each incubation time (*i.e.*, 8, 16, and 24 h) of the *C. cajan* seeds with SA, where the vertical (y) axes correspond to abundance and the horizontal (x) axes correspond to the retention time (min). Since abundance is the number of ions that is detected by monitoring the stream of electrons produced when the ions strike the detector for each *m*/*z*, it is devoid of any unit. Tables 1, 2, and Supp. Table 1 list the secondary metabolites detected, which were segregated based on the incubation hours. Most of the compounds detected were aldehydes, ketones, alcohols, furan derivatives, and heterocyclic compounds. Supp. Table 1 (see Appendix) lists the total secondary metabolites identified. The same pattern of metabolites was detected among three incubation periods during germination, so 24 h incubation was taken for the comparison.

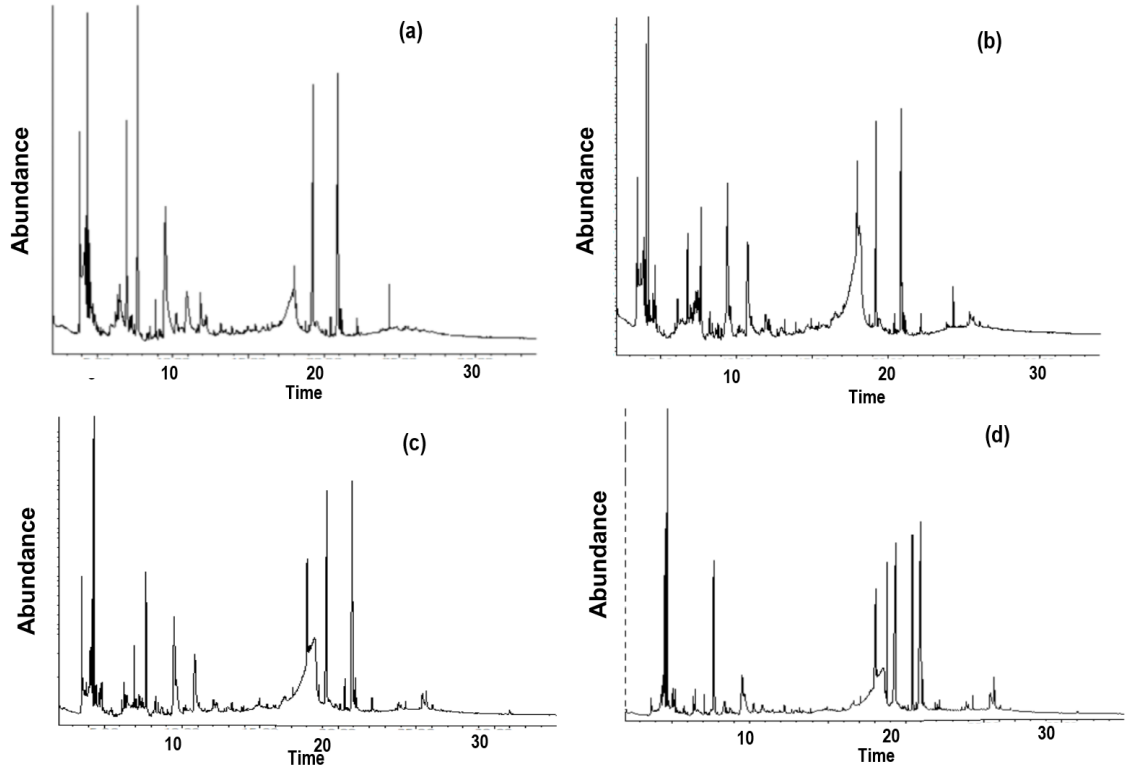

Fig. 2. GC-MS chromatogram of metabolites after 8 h incubation of germinated *C. cajan* seeds with different concentration of salicylic acid treatment: (a) Control, (b) 0.5 mM, (c) 1 mM, (d) 1.5 mM

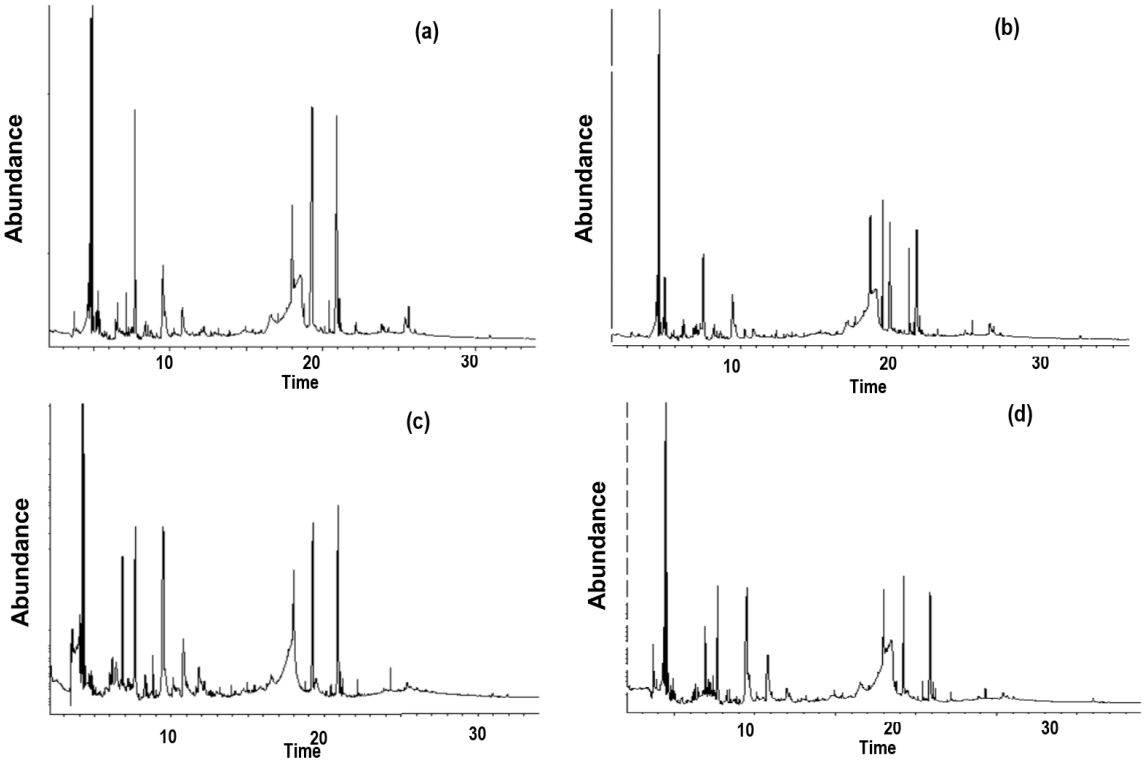

Fig. 3. GC–MS chromatogram of metabolites after 16 h incubation of germinated *C. cajan* seeds with different concentration of salicylic acid treatment: (a) Control, (b) 0.5 mM, (c) 1 mM, (d) 1.5 mM

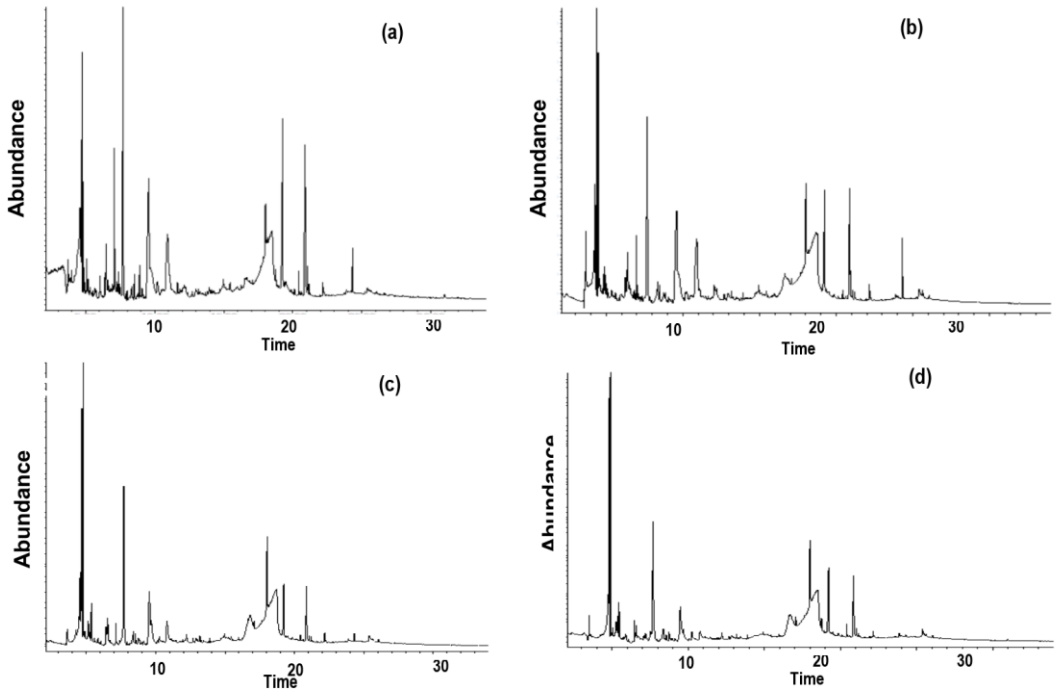

Fig. 4. GC–MS chromatogram of metabolites after 24 h incubation of germinated *C. cajan* seeds with different concentration of salicylic acid treatment: (a) Control, (b) 0.5 mM, (c) 1 mM, (d) 1.5 mM

Approximately 144 metabolites were detected after the SA treatment. Most of the metabolites were steroids, terpenoids, and saturated fatty acids. Among those, acetic acid was a major component in the control and treated samples, which was down-regulated in a SA concentration-dependent manner compared to the control sample. Approximately 42% reduction of acetic acid was found in the 1.5 mM SA-treated sample. Studies have shown that acetic acid alters the organic acid composition in the plasma membrane during germination to promote the reduction of poly-unsaturated acids, which leads to a loss of selectivity (Tunes *et al.* 2012). Acetic acid is an important precursor in synthesizing natural hormones in plants, such as indole-3-acetic acid (Ghosh and Basu 2006). The hypolipidemic effects of acetic acid on the triglyceride levels could be attributed to the inhibition of the metabolic pathways of lipogenesis in the liver by activating AMP-activated protein kinase (AMPK) (Yamashita *et al.* 2007). Acetate and acetol are intermediate substances produced during germination. These components were used when acetic acid was metabolized to produce the ketone bodies, such as glycogen and cholesterol synthesis and fatty acid degradation (Pravasi 2014).

The γ-Tocopherol concentration increased gradually due to the treatment and was not detected in un-germinated seeds. This suggests that the SA treatment enhanced the production of steroid and terpenoid biosynthesis. Studies strongly suggest that γ-tocopherol, which is the most prevalent type of vitamin E in the American diet, is vital for human health and requires more attention (Jiang and Ames 2003). The phenolic compound 2,4-bis(1,1-dimethylethyl) phenol in the 16 h-incubated samples of germinated *C. cajan* seeds has attracted considerable interest because of its anti-oxidative and possible anti-carcinogenic activities (Alghamdi *et al.* 2018).

Table 1. Comparison of Metabolites after 8 h Incubation of Germinated *C. cajan* Seeds with Different Salicylic Acid Concentrations

S.NO	Name	Retention time (min)	Area % covered by metabolites at different concentration of Salicylic acid treatment			
			Control	0.5 mM	1 mM	1.5 mM
1	n-Hexadecanoic acid	19.44	19.067±1.067	23.111±0.964*	18.535±1.103***	13.915±0.876**
2	9,12-Octadecadienoic acid (Z,Z)-	21.43	18.8±0.935	22.174±0.879**	0.298±0.943*	17.204±1.543***
3	Acetic acid	3.63	5.429±0.876	4.939±0.965***	3.1±0.2345*	8.343±0.879**
4	Octadecanoic acid	21.26	5.514±0.77	3.603±0.89**	5.303±0.053***	2.229±1.034*
5	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	20.59	4.941±0.945	3.297±0.923**	6.207±0.879*	-
6	Acetol	3.73	3.715±0.1234	0.871±0.778*	2.065±0.045**	-
7	Hyacinthin	7.77	3.098±0.089	5.301±0.665**	-	0.252±0.059*
8	.β-Monolinolein	25.88	3.21±0.045	-	-	4.059±1.457**
9	Hexadecanoic acid, methyl ester	18.92	2.612±1.032	1.65±0.891***	3.481±0.964**	0.986±0.035*
10	Glycerol β-palmitate	24.20	2.742±0.968	1.886±0.879*	2.315±0.036**	2.657±0.879***
11	γ-Tocopherol	31.54	2.779±0.0984	1.063±0.074*	3.014±1.237***	-
12	Pyruvic acid, methyl ester	4.42	1.039±0.879	0.721±0.041*	0.953±0.964**	1.164±0.879***
13	L-Proline, 5-oxo-, methyl ester	12.99	1.331±0.73	-	0.86±0.032*	-
14	trans-13-Octadecenoic acid, methyl ester	20.64	1.287±0.04	-	-	-
15	Pyrrolidine, 1-(1-oxo-7,10-hexadecadienyl)-	22.57	1.845±0.879	-	1.115±0.013**	0.095±0.021*
16	9,12-Octadecadienoic acid, ethyl ester	21.22	1.006±0.059	-	1.07±0.879**	0.373±0.964*
17	γ-Sitosterol	26.55	1.291±0.964	1.169±0.096**	-	-
18	Pyranone	9.59	0.731±0.769	3.336±0.124*	1.581±0.063***	2.93±0.045**
19	1-Monolinolein	25.91	-	5.183±0.879	-	-
20	Pentadecanoic acid	18.33	0.335±0.032	1.925±0.045*	0.389±0.015***	1.173±0.579**
21	Acetamide, N-(4-ethoxy-3-hydroxyphenyl)-	18.19	0.736±0.029	1.041±0.680**	0.921±0.039***	2.957±1.063*
22	5-Hydroxymethylfurfural	10.89	0.153±0.879	2.008±1.067*	0.837±0.042**	0.779±0.294*
23	Furfuryl alcohol	5.06	-	0.809±0.096*	0.864±0.045**	0.121±0.004***
24	Dioxanediol	3.69	0.317±0.045	-	-	-
25	Benzeneacetaldehyde α-(2-methylpropylidene	12.62	-	-	1.29±0.359*	-
26	Glucobrassicin	22.32	-	-	0.105±0.024*	-
27	2-Furanmethanol	5.26	0.493±0.024	0.141±0.014*	0.218±0.059**	0.593±0.045***
28	Furaneol	8.45	0.327±0.045	0.363±0.068***	0.44±0.879**	0.77±0.001*
29	Butanoic acid, 3-oxo-, 2-hydroxyethyl ester	10.32	-	-	-	1.015±0.274*
30	Trilinolein	25.64	-	0.593±0.045*	-	-

Each value represents mean ± standard error of three triplicates. The analysis was performed in triplicate, and the significance was determined using a t-test. Significance at: *p < 0.5, ** p < 0.05, *** p < 0.005

Hyacinthin belongs to the flavonoid-3-O-glycosides and is involved in phenyl-propanoid biosynthesis induced by the SA treatment at 0.5 mM and reduced at 1.5 mM. The pyruvic acid methyl ester level was reduced to 55% at 1.5 mM, playing a crucial role in intermediary metabolism. In addition, it also acts as a substrate for the tricarboxylic acid (TCA) cycle. Pyruvic acid methyl ester also acts as an antioxidant and stimulates insulin secretion in rats in a dose-dependent manner (Zawalich and Zawalich 1997). The level of phenacetin, a fever reducer, was reduced to half its original content during the SA treatment. The steroid metabolite, γ-sitosterol, was present in the treated samples up to 1mM at approximately 1.5%, and it is utilized completely at 1.5 mM. Syringol was reduced to 27.2% because of the treatment.

Table 2. Comparison of Metabolites after 16 h Incubation of Germinated *C. cajan* Seeds with Different Salicylic Acid Concentrations

S.NO	Name	Retention time (min)	Area % covered by metabolites at different concentration of Salicylic acid treatment			
			Control	0.5 mM	1 mM	1.5 mM
1	Acetic acid	3.63	9.02±0.045	4.485±1.067**	2.148±0.035*	8.683±1.067***
2	Acetol	3.73	3.357±0.378	1.216±0.85*	4.566±0.923**	-
3	Pyruvic acid, methyl ester	4.42	3.04±0.538	0.177±0.684*	1.162±0.035**	1.939±0.923*
4	Benzeneacetaldehyde α-(2-methylpropylidene	12.62	6.02±0.923	-	3.439±0.923*	-
5	1,2,3-Butanetriol	8.77	1.004±0.359	-	-	-
6	Pyranone	9.59	4.404±0.019	2.447±0.247*	2.908±0.045***	2.479±0.014**
7	2-Cyclopenten-1-one, 2-hydroxy-	6.24	1.214±1.067	-	-	-
8	n-Hexadecanoic acid	19.44	16.271±0.037	19.384±0.014**	18.401±0.045***	4.55±0.004*
9	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	20.59	3.116±0.359	5.651±0.690*	5.651±0.963**	-
10	9,12-Octadecadienoic acid (Z,Z)-	21.43	17.848±0.584	-	20.862±0.064*	16.917±0.014***
11	Octadecanoic acid	21.26	3.44±0.085	4.324±0.045**	4.015±0.035*	-
12	1-Monolinolein	25.91	4.048±0.359	-	4.132±0.014***	0.732±0.049*
13	Glycerol β-palmitate	24.20	3.364±0.014	2.63±0.37**	2.777±0.923***	0.816±0.359*
14	n-Hexadecanoic acid, pentamethyldisilyl ester	24.05	16.271±1.340	19.384±0.579*	18.401±1.067**	15.87±0.035***
15	Acetamide, N-(4-ethoxy-3-hydroxyphenyl)-	18.19	2.006±0.579	2.13±0.035***	0.915±0.046*	2.208±0.045**
16	γ-Tocopherol	31.54	0.558±0.045	2.132±0.129*	1.2±0.475**	3.678±1.067***
17	Pyrogallol 1,3-dimethyl ether	12.36	0.945±0.233	0.514±0.359*	0.894±0.035***	1.261±0.014**
18	2,6-Diphenylpyridine	22.11	0.271±0.014	0.221±0.024***	0.351±0.923**	3.537±0.035*
19	Heptadecanoic acid	20.31	0.228±0.024	0.265±0.035*	0.277±0.128**	0.243±0.359***
20	Phenol, 2,4-bis(1,1-dimethylethyl)-	14.40	0.384±0.013	0.33±0.045***	0.504±0.024*	0.474±0.053**

Each value represents mean ± standard error of three triplicates
The analysis was performed in triplicate, and the significance was determined using a t-test.
Significance at: *p < 0.5, ** p < 0.05, *** p < 0.005

The metabolite, 9,12-octadecadienoic acid-(*Z,Z*), was found in all samples with various peak area percentage and possessed cancer-preventive, anti-arthritic, hepatoprotective, anti-acne, anti-histaminic, and anti-eczemic properties (Malik *et al.* 2016). Some furan derivatives, 5-hydroxymethyl furfural, 2-furan methanol, and furaneol, exhibited antimicrobial efficacy towards gram-positive and gram-negative bacteria (Pachaiappan *et al.* 2022). These derivatives are also involved in terpenoid biosynthesis. Furaneol was reduced at 1 mM concentration. In contrast, the treatment increased the methyl furfural and furfuryl alcohol levels by 25%, which undergoes an aminomethylation reaction. A slightly increased concentration was observed in 2,6-diphenylpyridine and 2-phenylpyridine ketone derivatives, which exhibit herbicidal activity.

In plants, two routes for SA biosynthesis have been proposed. Plants generate SA from cinnamate, synthesized because of the activity of phenylalanine ammonia-lyase (PAL), according to the biochemical investigations using isotope feeding. Pathogen-induced SA buildup is reduced when the PAL genes are silenced in tobacco or PAL activity is chemically inhibited in Arabidopsis, cucumber, and potato. Genetic research, however, showed that isochorismate produces the majority of SA. Isochorismate synthase (ICS) and isochorismate pyruvate lyase catalyze two processes in bacteria to produce SA from chorismate (IPL). PBS3 and EPS1, recently discovered that two Arabidopsis genes are critical for pathogen-induced SA buildup. The acyl-adenylate/thioester-forming enzyme family is encoded by PBS3, while the BAHD acyltransferase superfamily is encoded by

EPS1. The PBS3 and EPS1 may play a role in forming the key precursor or regulatory molecule for SA biosynthesis (Rivas and Plasencia 2011). Salicylic acid (SA) accumulates in infected and healthy leaves in response to pathogen infection, mediating the plant defenses against pathogens. In plants, SA is required for pathogenesis-related gene expression and the manufacture of defensive chemicals linked with local and systemic acquired resistance (LAR and SAR). SA is produced from chorismate by the ICS pathway, and the SA produced by this pathway is essential for the LAR and SAR responses (Wildermuth *et al.* 2001). Salicylic acid elicits the plant defense-signaling process, such as butyrolactone synthesized from 2,3-butanediol. This has an antimicrobial effect on phytopathogen *Erwinia carotovora* and the fungus, *Botrytis cinerea* during seed germination and seedling growth, and it also inhibits the acetylcholinesterase activity (Cazar *et al.* 2005; Parthasarathy *et al.* 2022). No changes were observed in the 2,3-butanediol (dimethylene glycol) and 1-monolinolein-involved glycerol metabolism with salicylic acid. 4,6-Dimethyl-pyrimidine is an antifungal agent that controls the spot disease caused by *Alternaria alternata* in *Solanum melongena* (Balai and Kumar 2022). Glycerol β-palmitate (also known as β-palmitate) regulates fatty acid metabolism, promotes calcium absorption, enhances bone matrix strength and stool texture, and has a beneficial influence on the growth of the gut microbiome (Havliekova *et al*. 2015).

The octadecanoic and hexadecenoic acid contents were increased by approximately 50% in a concentration-dependent manner, and methyl linoleate was increased by approximately 90% at a 1 mM concentration. The results also indicate that 9-12-octadecadienoic acid (linoleic acid), glycerol-β-palmitate, and pyranone were increased gradually to 45%, 59%, and 72%, respectively, at 1.5 mM.

The oxidative cleavage of carotenoids in seeds produces dehydro-β-ionone and α-Ionene, which are crucial for herbivore plant communication. These are parts of the induced defense released by wounds from herbivores in Canola, *Brassica napus*, and are a strong repellent towards flea beetle and spider mite (Caceres *et al.* 2016). The defense sequence reduces the cardiac irregularities and is unaffected by the SA treatment. 2,5-Dimethyl-4-(3-amino-4-methyl phenyl) pyridine, 2-benzyl piperidine, and glycolic acid methyl ester are involved in regulating plant growth and are maintained at the same level at all treated concentrations. On the other hand, there was no change in the production of defense metabolites. The glycerol derivative α-monoacetin, quinolizine derivative, 2,4-bis(1,1-dimethyl ethyl)-phenol, α-methyl propargyl alcohol, heptane, 4-propylalkylphenyl ketone compound, 2-acetyl-resorcinol, pyridine 1-acetyl-1,2,3,4-tetrahydro-, benzene acetaldehyde, and 1,2-cyclopentanedione were used during the germination process involving different metabolisms. The ketone derivatives 1-nitro-2-propanone and formic acid are reportedly involved in photosynthetic CO_2 fixation metabolism in barley leaves (Tolbet 1955).

Figure 5 represents the total number of metabolites detected after 8, 16, and 24 h incubation of germinated *C. cajan* seeds with different SA concentrations (Control, 0.5, 1, and 1.5 mM). A drastic increase in the number of metabolites was observed after incubating the *C. cajan* germinated seeds for 24 h with a 0.5 mM concentration of salicylic acid. The lowest number of metabolites was detected in 16 h of incubation. Figures 6 (a), (b), and (c) show the secondary metabolites obtained at an incubation period of 8, 16, and 24 h, respectively, after treating the germinated seeds with 0.5, 1, or 1.5 mM of SA. This was compared with the control samples for all incubation periods (8, 16, and 24 h).

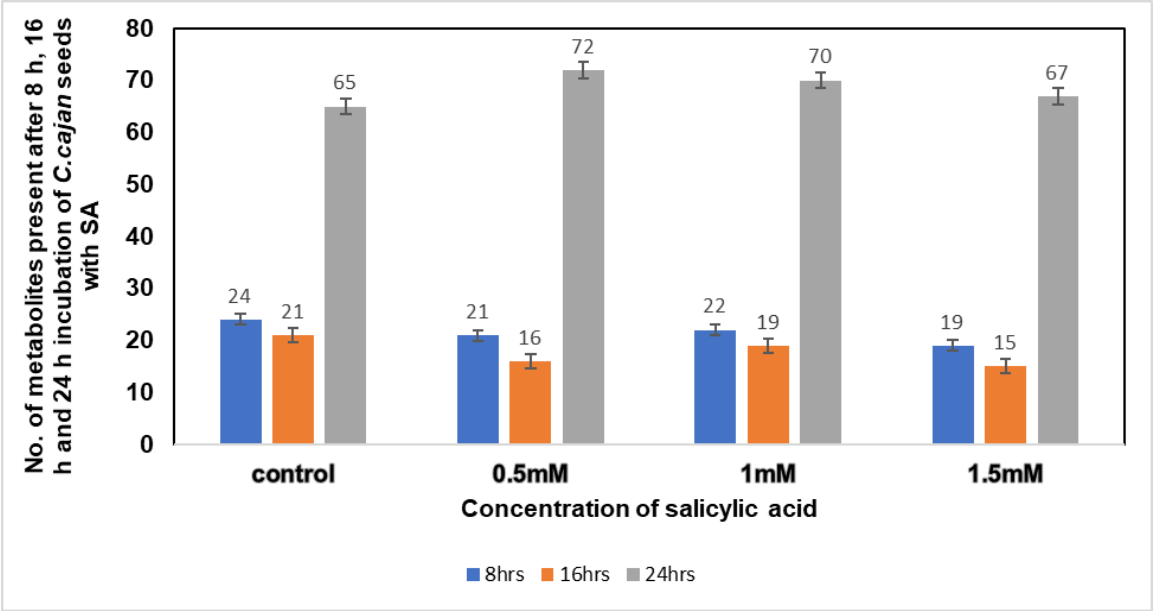

Fig. 5. The total number of metabolites present after 8 h, 16 h, and 24 h incubation of germinated *C. cajan* seeds with different concentrations of salicylic acid treatment (Control, 0.5, 1, 1.5 mM)

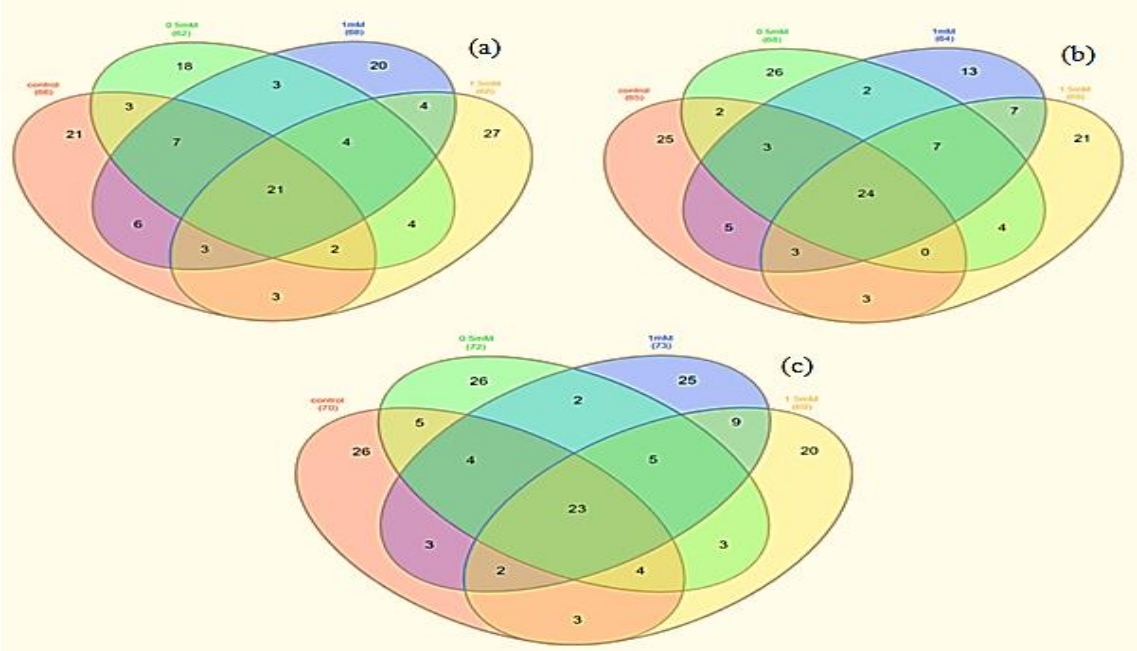

Fig. 6. Venn diagram representing the metabolites present in *C. cajan* seeds after incubation for (a) 8 h, (b) 16 h, (c) 24 h with different concentrations of Salicylic acid treatment (Control, 0.5, 1, 1.5 mM)

Some of the common secondary metabolites identified in all concentrations of the salicylic acid-treated *C. cajan* seeds were acetic acid, acetol, pyranone, (*Z*,*Z*)-9,12-octadecadienoic acid, 2,6-diphenylpyridine, pyruvic acid, methyl ester, 5-methylfurfural, furaneol, pyrogallol 1,3-dimethyl ether, pentadecanoic acid, heptadecanoic acid, hexadecanoic acid, pentamethyldisilyl ester, octadecanoic acid, pyrrolidine, 1-(1-oxo-7,10-hexadecadienyl)-, phenol, 2,4-bis(1,1-dimethylethyl)-, glycerol β-palmitate, *N*-(4-ethoxy-

3-hydroxyphenyl)- acetamide, 2-furanmethanol, and hexadecanoic acid methyl ester (Fig. 6 a). At 16 h of incubation, the SA-treated *C. cajan seeds* contained the following compounds: γ-tocopherol, 7,10-hexadecanoic acid, methyl ester, pyruvic acid, and its methyl ester. In the samples treated with 1.5 mM SA and the control, *1*-monolinolein was identified, whereas γ-sitosterol was present in 0.5 mM and 1mM SA-treated seeds. Certain compounds, such as *l*-proline, 5-oxo-, methyl ester, trans-(2-chlorovinyl) dimethylethoxy-silane, (+)-3-carene, 10-(acetylmethyl)-, α-ionene, and 5-hydroxymethyl furfural were detected at 24 h of incubation in all the SA-treated *C. cajan* seed samples.

Figure 7 shows the number of common and different compounds present in the seeds of the SA-treated *C. cajan* at an incubation period of 8, 16, and 24 h. Some of the common compounds for all the germinated samples were acetic acid, acetol, pyrogallol 1,3-dimethyl ether, methyl ester, hexadecanoic acid, glycerol β-palmitate, acetamide, *N*-(4-ethoxy-3-hydroxyphenyl)-, 2,6-diphenylpyridine, pyranone, pyruvic acid methyl ester, (*Z*,*Z*)-9,12-octadecadienoic acid, and furaneol. Most of the components detected in the treated samples, including trilinolein (alinoleic acid triglyceride), ethyl linoleate, methyl palmitate, methyl-10-octadecenoate, nicotinic acid, 2,6-dimethoxy-4-methylcitramalic acid, 3-methoxymethoxybutyric acid, butane-1,2,3,4 diepoxy, cyclopropylcarbinol, 4-propyl heptane, 1,2-cyclopentanedione, furaneol, ethylene glycol diacetate, 2-hydroxy ethyl acrylate, and glucobrassicin are natural pesticides normally found in many cruciferous plants. These compounds are involved in glucosinolate biosynthesis and acetoin-induced systemic resistance to *Arabidopsis thaliana* (Pachaiappan *et al.* 2022). Nicotinic acid is crucial in supplementing the human body with vitamin B3 or niacin. Studies have shown that it helps elevate HDL cholesterol and reduce LDL cholesterol and triglycerides levels, thereby treating dyslipidemia (Bogan and Brenner 2008; Pachaiappan *et al.* 2018). These results suggest that 1.5 mM salicylic acid is an excellent candidate as a lead compound that can be used in agricultural practices. Various secondary metabolites were obtained from the SA-treated seedlings of *C. cajan*, and research is currently under progress.

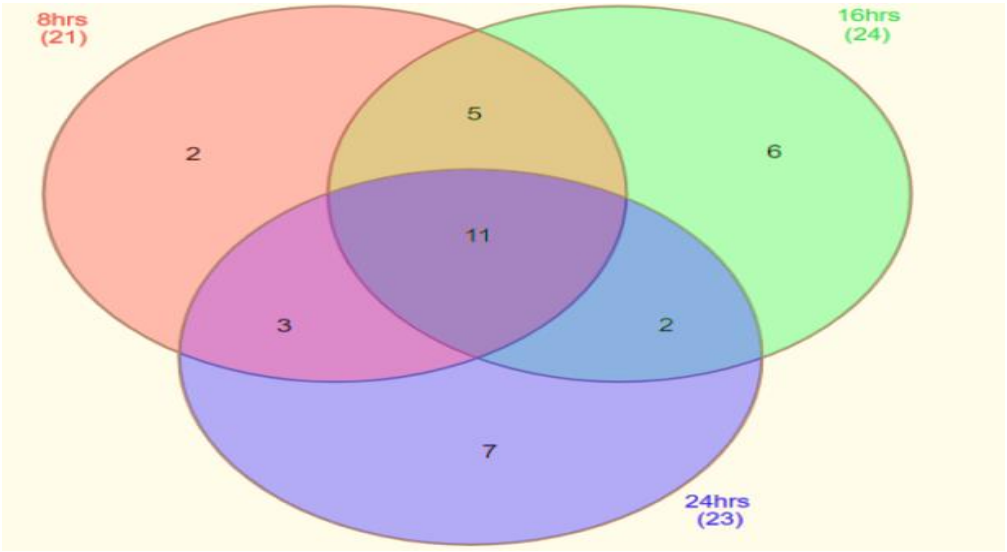

Fig. 7. The number of common and different compounds present in the seeds of the SA-treated *C. cajan* at an incubation period of 8, 16, and 24 h

CONCLUSIONS

Using a metabolomics approach, this work investigated the various responses to physiological metabolites and the pathway changes in *Cajanus cajan*'s broad-spectrum resistance-1 (BSR1) following treatment with salicylic acid, which is an elicitor. By using GC-MS, the secondary metabolites were recovered and studied. The gathered samples' chemometric examination revealed that the seeds responded to the SA treatment. Germinated seeds of *C. cajan* under a SA treatment produced numerous metabolites. The variation in metabolite production was examined by gas chromatography-mass spectrometry (GC–MS). Acetic acid, acetol, pyrogallol 1,3-dimethyl ether, glycerol β-palmitate, acetamide, hexadecanoic acid, methyl ester, pyranone, pyruvic acid, (Z,Z)-9,12-octadecadienoic acid, and furaneol were detected at all the incubation times. The treatment caused a slow rise in tocopherol concentration, which was absent in seeds that had not germinated. This indicates that the SA treatment increased the production of steroids and terpenoids. All samples contained the metabolite (Z,Z)-9,12-octadecadienoic acid, which was documented to prevent cancer and treat arthritis, hepatitis, acne, and eczema. The antibacterial activity of several furan derivatives was reported against both gram-positive and gram-negative bacteria. These derivatives contribute to the production of terpenoid compounds. The 2,6-diphenylpyridine and 2-phenylpyridine ketone derivatives, which have herbicidal action, were found to have a marginally higher concentration. This experimental evidence indicates the positive impact of SA on the production of various defense as well as other nutritional metabolites during the germination of *C. cajan* seeds.

REFERENCES CITED

Ade-Omowaye, B. I. O., Tucker, G. A., and Smetanska, I. (2015). "Nutritional potential of nine underexploited legumes in Southwest Nigeria," *International Food Research Journal* 22(2), 798.

Alghamdi, S. S., Khan, M. A., El-Harty, E. H., Ammar, M. H., Farooq, M., and Migdadi, H. M. (2018). "Comparative phytochemical profiling of different soybean (*Glycine max* (L.) Merr) genotypes using GC–MS," *Saudi Journal of Biological Sciences* 25(1), 15-21. DOI: 10.1016/j.sjbs.2017.10.014

Amalraj, T., and Ignacimuthu, S. (1998). "Hypoglycemic activity of *Cajanus cajan* (seeds) in mice," *Indian Journal of Experimental Biology* 36(10), 1032-1033.

An, C., and Mou, Z. (2011). "Salicylic acid and its function in plant immunity F," *J. Integrative Plant Biology* 53(6), 412-428. DOI: 10.1111/j.1744-7909.2011.01043.x

Balai, L. P., and Kumar, S. (2022). "Integrated disease management of leaf spot of brinjal caused by *Alternaria alternata*: A review," *Environment Ecology* 40(2A), 440-450.

Balasubramanian, A., Bhattacharjee, M., Sakthivel, M., Thirumavalavan, M., Madhavan, T., Nagarajan, S. K., Palaniyandi, V., and Raman, P. (2018). "Isolation, purification and characterization of proteinaceous fungal α-amylase inhibitor from rhizome of *Cheilocostus speciosus* (J. Koenig) CD Specht," *International Journal of Biological Macromolecules* 111, 39-51. DOI: 10.1016/j.ijbiomac.2017.12.158

Bogan, K. L., and Brenner, C. (2008). "Nicotinic acid, nicotinamide, and nicotinamide riboside: A molecular evaluation of NAD+ precursor vitamins in human nutrition," *Annual Review of Nutrition* 28, 115-130. DOI: 10.1146/annurev.nutr.28.061807.155443

Cáceres, L. A., Lakshminarayan, S., Yeung, K. K. C., McGarvey, B. D., Hannoufa, A., Sumarah, M. W., Benitez, X., and Scott, I. M. (2016). "Repellent and attractive effects of α-, β-, and dihydro-β-ionone to generalist and specialist herbivores," *Journal of Chemical Ecology* 42, 107-117. DOI: 10.1007/s10886-016-0669-z

Cazar, M. E., Schmeda-Hirschmann, G., and Astudillo, L. (2005). "Antimicrobial butyrolactone I derivatives from the Ecuadorian soil fungus *Aspergillus terreus* Thorn. var terreus," *World Journal of Microbiology and Biotechnology* 21, 1067-1075. DOI: 10.1007/s11274-004-8150-5

Chakraborty, S. K., Kumbhar, B. K., and Sarkar, B. C. (2007). "Process parameter optimization for instant pigeonpea dhal using response surface methodology," *Journal of Food Engineering* 81(1), 171-178. DOI: 10.1016/j.jfoodeng.2006.10.019

Dettmer, K., Aronov, P. A., and Hammock, B. D. (2007). "Mass spectrometry-based metabolomics," *Mass spectrometry Reviews* 26(1), 51-78. DOI: 10.1002/mas.20108

Devindra, S., and Aruna, T. (2017). "Effect of chemical soaking, toasting and crude α-galactosidase enzyme treatment on the oligosaccharide content of red gram flour," *J. Food Processing and Preservation* 41(3), e12922. DOI: 10.1111/jfpp.12922

Duker-Eshun, G., Jaroszewski, J. W., Asomaning, W. A., Oppong-Boachie, F., and Brøgger Christensen, S. (2004). "Antiplasmodial constituents of *Cajanus cajan*," *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives* 18(2), 128-130. DOI: 10.1002/ptr.1375

Fu, Y., Zu, Y., Liu, W., Efferth, T., Zhang, N., Liu, X., and Kong, Y. (2006). "Optimization of luteolin separation from pigeon pea [*Cajanus cajan* (L.) Millsp.] leaves by macroporous resins," *Journal of Chromatography A* 1137(2), 145-152. DOI: 10.1016/j.chroma.2006.08.067

Fu, Y., Zu, Y., Liu, W., Hou, C., Chen, L., Li, S., Shi, X., and Tong, M. (2007). "Preparative separation of vitexin and isovitexin from pigeon pea extracts with macroporous resins," *Journal of Chromatography A* 1139(2), 206-213. DOI: 10.1016/j.chroma.2006.11.015

Ghosh, S., and Basu, P. S. (2006). "Production and metabolism of indole acetic acid in roots and root nodules of *Phaseolus mungo*," *Microbiological Research* 161(4), 362-366. DOI: 10.1016/j.micres.2006.01.001

Grover, J. K., Yadav, S., and Vats, V. (2002). "Medicinal plants of India with anti-diabetic potential," *Journal of Ethnopharmacology* 81(1), 81-100. DOI: 10.1016/S0378-8741(02)00059-4

Hassan, E. M., Matloub, A. A., Aboutabl, M. E., Ibrahim, N. A., and Mohamed, S. M. (2016). "Assessment of anti-inflammatory, antinociceptive, immunomodulatory, and antioxidant activities of *Cajanus cajan* L. seeds cultivated in Egypt and its phytochemical composition," *Pharmaceutical Biology* 54(8), 1380-1391. DOI: 10.3109/13880209.2015.1078383

Havlicekova, Z., Jesenak, M., Banovcin, P., and Kuchta, M. (2015). "Beta-palmitate – A natural component of human milk in supplemental milk formulas," *Nutrition Journal* 15, 1-8. DOI: 10.1186/s12937-016-0145-1

Huang, G. Y., Liao, X. Z., Liao, H. F., Deng, S. J., Tan, Y. H., and Zhou, J. Y. (2006). "Studies on water-soluble extracts from *Cajanus cajan* leaf against hypoxic-ischemic brain damage," *Traditional Chinese Drug Res. Clinical Pharmacology* 17, 172-174.

Ikhajiagbe, B. (2020). "The effects of salicylic acid on the germination and early seedling growth of pigeon pea (*Cajanus cajan*)," *Notulae Scientia Biologicae* 12(3), 683-692.

Ishikura, N., Hayashida, S., and Tazaki, K. (1984). "Biosynthesis of gallic and ellagic acids with 14 C-labeled compounds in Acer and Rhus leaves," *The Botanical Magazine = Shokubutsu-gaku-zasshi* 97, 355-367. DOI: 10.15835/nsb12310777

Jiang, Q., and Ames, B. N. (2003). "γ-Tocopherol, but not α-tocopherol, decreases proinflammatory eicosanoids and inflammation damage in rats," *The FASEB Journal* 17(8), 816-822. DOI: 10.1096/fj.02-0877com

Jones, D. H. (1984). "Phenylalanine ammonia-lyase: Regulation of its induction, and its role in plant development," *Phytochemistry* 23, 1349-1359. DOI: 10.1016/S0031-9422(00)80465-3

Kartikeyan, A., Vasudevan, V., Peter, A. J., Krishnan, N., Velmurugan, D., Velusamy, P., Anbu, P., Palani, P., and Raman, P. (2022). "Effect of incubation period on the glycosylated protein content in germinated and ungerminated seeds of mung bean (*Vigna radiata* (L.) Wilczek)," *International Journal of Biological Macromolecules* 217, 633-651. DOI: 10.1016/j.ijbiomac.2022.07.036

Malik, B., Pirzadah, T. B., Tahir, I., Abdin, M. Z., and Rehman, U. I. R. (2016). "Phytochemical studies on *Cichorium intybus* L.(CHICORY) from Kashmir Himalaya using GC-MS," *Journal of Pharmacy Research* 10(11), 715-726.

Ohanenye, I. C., Tsopmo, A., Ejike, C. E., and Udenigwe, C. C. (2020). "Germination as a bioprocess for enhancing the quality and nutritional prospects of legume proteins," *Trends Food Sci. Technol.* 101, 213-222. DOI: 10.1016/j.tifs.2020.05.003

Pachaiappan, R., Tamboli, E., Acharya, A., Su, C. H., Gopinath, S. C., Chen, Y., and Velusamy, P. (2018). "Separation and identification of bioactive peptides from stem of *Tinospora cordifolia* (Willd.) Miers," *Plos One* 13(3), e0193717. DOI: 10.1371/journal.pone.0193717

Pachaiappan, R., Rajamuthu, T. P., Sarkar, A., Natrajan, P., Krishnan, N., Sakthivelu, M., Velusamy, P., Ramasamy, P., and Gopinath, S. C. (2022). "N-acyl-homoserine lactone mediated virulence factor (s) of *Pseudomonas aeruginosa* inhibited by flavonoids and isoflavonoids," *Process Biochemistry* 116, 84-93. DOI: 10.1016/j.procbio.2022.02.024

Pachaiappan, R., Nagasathiya, K., Singh, P. K., Gopalakrishnan, A. V., Velusamy, P., Ramasamy, K., Velmurugan, D., Kandasamy, R., Ramasamy, P., and Gopinath, S. C. (2022). "Phytochemical profile of black cumin (*Nigella sativa* L.) seed oil: identification of bioactive anti-pathogenic compounds for traditional Siddha formulation," *Biomass Conversion and Biorefinery* In press. DOI: 10.1007/s13399-022-02951-x

Parthasarathy, S., Soundararajan, P., Krishnan, N., Karuppiah, K. M., Devadasan, V., Prabhu, D., Rajamanikandan, S., Velusamy, P., Gopinath, S. C. B., and Raman, P. (2022). "Detection of adulterants from common edible oils by GC–MS," *Biomass Conversion and Biorefinery* In press. DOI: 10.1007/s13399-022-02913-3

Pravasi, S. D. (2014). "Acetic acid," *Encyclopedia of Toxicology*, pp. 33-35. DOI: 10.1016/B978-0-12-386454-3.00216-5

Raymaekers, K., Ponet, L., Holtappels, D., Berckmans, B., and Cammue, B. P. (2020).

"Screening for novel biocontrol agents applicable in plant disease management – A review," *Biological Control* 144, article 104240. DOI: 10.1016/j.biocontrol.2020.104240

Rivas-San Vicente, M., and Plasencia, J. (2011). "Salicylic acid beyond defense: Its role in plant growth and development," *Journal of Experimental Botany* 62(10), 3321-3338. DOI: 10.1093/jxb/err031

Sauer, D. B., and Burroughs, R. (1986). "Disinfection of seed surfaces with sodium hypochlorite," *Phytopathology* 76, 745-749. DOI: 10.1094/Phyto-76-745

Singh, U. (1981). "Protease inhibitors of pigeon pea (*Cajanus cajan*) and its wild relatives," *Physiology Plantarum* 198, 845-851.

Singh, U., and Eggum, B. O. (1984). "Factors affecting the protein quality of pigeonpea (*Cajanus cajan* L.)," *Plant Foods for Human Nutrition* 34, 273-283. DOI: 10.1007/BF01126556

Sudjaroen, Y., Haubner, R., Würtele, G., Hull, W. E., Erben, G., Spiegelhalder, B., Changbumrumg, S., Bartsch, H., and Owen, R. W. (2005). "Isolation and structure elucidation of phenolic antioxidants from Tamarind (*Tamarindus indica* L.) seeds and pericarp," *Food and Chemical Toxicology* 43, 1673-1682. DOI: 10.1016/j.fct.2005.05.013

Suresh, K., and Chandrakanth, M. G. (2016). "Economic efficiency of improved red gram variety (BRG-2) in Karnataka: A DEA analysis," *Economic Affairs*, 61, 81-88. DOI: 10.5958/0976-4666.2016.00012.7

Talari, A., and Shakappa, D. (2018). "Role of pigeon pea (*Cajanus cajan* L.) in human nutrition and health: A review," *Asian Journal of Dairy and Food Research* 37, 212-220. DOI: 10.18805/ajdfr.DR-1379

Tolbert, N. E. (1955). "Formic acid metabolism in barley leaves," *Journal of Biological Chemistry* 215, 27-34. DOI: 10.1016/S0021-9258(18)66013-2

Tunes, L. M. D., Avelar, S. A. G., Barros, A. C. S. A., Pedroso, D. C., Muniz, M. F. B., and Menezes, N. L. D. (2012). "Critical levels of organic acids on seed germination and seedling growth of wheat," *Revista Brasileira de Sementes* 34, 366-372. DOI: 10.1590/S0101-31222012000300002

Wildermuth, M. C., Dewdney, J., Wu, G., and Ausubel, F. M. (2001). "Isochorismate synthase is required to synthesize salicylic acid for plant defense," *Nature* 414, 562-565. DOI: 10.1038/35107108

Wu, N., Fu, K., Fu, Y. J., Zu, Y. G., Chang, F. R., Chen, Y. H., Liu, X-.L., Kong, Y., Liu, W., and Gu, C.-B. (2009). "Antioxidant activities of extracts and main components of pigeon pea [*Cajanus cajan* (L.) Millsp.] leaves," *Molecules* 14(3), 1032-1043. DOI: 10.3390/molecules14031032

Yamashita, H., Fujisawa, K., Ito, E., Idei, S., Kawaguchi, N., Kimoto, M., Hiemori, M., and Tsuji, H. (2007). "Improvement of obesity and glucose tolerance by acetate in Type 2 diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats," *Bioscience, Biotechnology, and Biochemistry* 71(5), 1236-1243. DOI: 10.1271/bbb.60668

Zawalich, W. S., and Zawalich, K. C. (1997). "Influence of pyruvic acid methyl ester on rat pancreatic islets: effects on insulin secretion, phosphoinositide hydrolysis, and sensitization of the beta cell," *Journal of Biological Chemistry* 272, 3527-3531. DOI: 10.1074/jbc.272.6.3527

Zhao, J. L., Zhou, L. G., and Wu, J. Y. (2010). "Effects of biotic and abiotic elicitors on cell growth and tanshinone accumulation in *Salvia miltiorrhiza* cell cultures," *Applied Microbiology and Biotechnology* 87, 137-144. DOI: 10.1007/s00253-010-2443-4

Zheng, Y. Y., Yang, J., Chen, D. H., and Sun, L. (2007). "Effects of the stilbene extracts from *Cajanus cajan* L. on ovariectomy-induced bone loss in rats," *Yao Xue Xue Bao = Acta Pharmaceutica Sinica* 42(5), 562-565.

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APPENDIX

Supplementary Table 1. Comparison of Metabolites after 24 h Incubation of Germinated *C. cajan* seeds with Different Salicylic Acid Concentrations Treated

S.NO	Name	Retention time (min)	Area % covered by metabolites at different concentration of Salicylic acid treatment			
			Control	0.5 mM	1 mM	1.5 mM
1	p-Dioxin, 2,3-dihydro	3.12	-	-	0.268±0.021	-
2	2-Methyl-1-butyl acetate	3.28	-	0.133±0.049	-	-
3	Formic acid	3.40	0.803±0.038	-	-	-
4	Acetic acid	3.63	15.451±1.793	13.87±1.395	6.773±0.483	6.21±0.371
5	Acetol	3.73	8.118±1.931	4.227±1.246	5.47±0.053	3.42±0.027
6	Glycolic acid, methyl ester	3.89	0.158±0.058	0.375±0.003	-	0.25±0.45
7	Valine	3.95	-	-	0.605±0.036	-
8	α-Methylpropargyl alcohol	4.24	0.537±0.068	-	-	0.41±0.038
9	Butane, 1,2:3,4-diepoxy-, (±)-	4.18	-	0.189±0.037	0.474±0.129	-
10	2-Butanone, 1-hydroxy-, acetate	4.31	-	-	0.419±0.114	0.19±0.04
11	2-Propenoic acid, 2-hydroxyethyl ester	4.29	-	0.77±0.084	-	-
12	1-Nitro-2-propanone	4.35	0.81±0.148	-	-	-
13	Glycol monoacetate	4.39	-	0.766±0.047	-	-
14	Pyruvic acid, methyl ester	4.42	3.031±1.496	0.841±0.974	2.381±0.385	1.67±0.05
15	2,3-Butanediol, [R-(R*,R*)]-	4.61	2.205±0.976	1.988±0.575	2.252±0.483	2.57±0.025
16	4-(2-Hydroxyethyl)-2,2-dimethyl-1,3-dioxolane	4.91	-	-	0.077±0.035	-
17	6-Methoxypiperidin-2-one	5.00	-	-	-	0.12±0.003
18	Furfuryl alcohol	5.06	0.075±0.035	1.585±0.003	0.214±0.48	1.43±0.258
19	2-Furanmethanol	5.26	1.021±0.04	-	1.534±0.035	-
20	4-Cyclopentene-1,3-dione	5.25	-	0.17±0.024	-	-
21	Ethylene glycol, diacetate	5.23	-	0.555±0.038	-	-
22	Acetol acetate	5.36	0.483±0.024	-	-	-
23	2-Propanone, 1-(acetyloxy)-	5.40	-	0.225±0.003	-	-
24	3-Methyl-3-butenoic acid	5.60	-	-	-	0.12±0.483
25	2,5-Dimethylpyrazine	5.65	-	-	0.348±0.038	-
26	Pyrimidine, 4,6-dimethyl-	5.68	1.095±0.483	0.816±0.39	-	-
27	Pyrazine, 2,5-dimethyl-	5.69	-	-	-	0.49±0.024
28	3-Pyridinamine, 2-methyl-	5.79	-	-	-	0.17±0.476
29	3-Penten-1-ol, 4-methyl-	5.83	-	-	0.15±0.024	-
30	2-Butenoic acid, methyl ester	5.86	0.106±0.324	-	-	-
31	2-Butenoic acid, methyl ester, (E)-	5.89	-	0.096±0.174	-	-
32	Butyrolactone	6.03	1.208±0.035	1.033±0.794	1.042±0.174	1.13±0.284
33	2,4-Pentadienoic acid	6.05	-	-	-	0.23±0.038
34	2-Cyclopenten-1-one, 2-hydroxy-	6.24	-	0.943±0.003	1.359±0.481	0.79±0.046
35	1,2-Cyclopentanedione	6.21	1.69±0.038	-	-	-
36	β-Angelica lactone	6.35	0.121±0.024	-	0.093±0.004	-
37	5-Methylfurfural	6.45	0.267±0.394	0.929±0.483	0.268±0.003	0.76±0.483
38	2-Furanmethanol, 5-methyl-	6.58	-	0.179±0.046	-	-
39	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	6.87	0.358±0.003	0.732±0.024	0.343±0.038	0.37±0.067
40	Pyrazine, 2-ethyl-6-methyl-	7.00	-	-	-	0.13±0.483
41	2-Furanacrolein	7.05	-	-	0.113±0.067	-
42	Benzeneethanamine	7.10	-	0.391±0.035	-	-
43	Trimethylpyrazine	7.13	-	-	0.188±0.035	0.26±0.024
44	Piperidine, 2-(tetrahydro-2-furanyl)-	7.14	0.137±0.038	-	-	-
45	2-Furanone, 2,5-dihydro-3,5-dimethyl	7.20	-	-	0.113±0.483	-
46	1-Cyclohexyl-2-phenylacetylamino-2-phenylethane	7.23	-	0.184±0.038	-	-
47	6-Methylene bicyclo [3.2.0]hept-3-en-2-one	7.34	-	0.075±0.046	-	-
48	Corylon	7.65	-	-	0.138±0.024	0.12±0.003
49	Benzene acetaldehyde	7.74	9.843±0.003	-	-	-
50	Hyacinthin	7.77	-	8.82±0.046	6.282±0.038	3.66±0.483
51	2-Ethyl-3,6-dimethylpyrazine	8.29	-	-	-	0.19±0.248
52	2,5-Dimethyl-4-hydroxy-3(2H)-furanone	8.43	-	-	1.429±0.024	-
53	Furaneol	8.45	1.559±0.038	1.961±0.483	-	0.16±0.035
54	Aziridine, 2-(1,1-dimethylethyl)-3-methyl-, trans-	8.69	-	-	-	0.6±0.038
55	Heptane, 4-propyl-	8.73	0.528±0.035	-	-	-

56	4-Propylheptane	8.74	-	-	0.418±0.038	-
57	Cyclopropylcarbinol	8.75	-	0.319±0.690	-	-
58	Phenylethyl Alcohol	8.95	-	-	0.181±0.549	0.22±0.38
59	γ-Decalactone	9.18	-	0.227±0.483	-	-
60	Dicyclobutylidene oxide	9.38	0.089±0.035	-	-	-
61	Pyranone	9.59	5.39±0.539	7.005±0.038	3.956±0.483	3.81±0.024
62	trans-(2-Chlorovinyl) dimethylethoxysilane	10.07	0.107±0.003	0.123±0.067	0.125±0.029	0.15±0.038
63	Pyridine, 1-acetyl-1,2,3,4-tetrahydro-	10.11	0.075±0.067	-	-	-
64	Pyrazine, 2,5-dimethyl-3-(2-methylpropyl)-	10.16	0.087±0.038	0.143±0.247	-	-
65	3,5-Dimethyl-2-isobutylpyrazine	10.17	-	-	0.109±0.594	-
66	Pentane-1,1-diol diacetate	10.32	-	-	-	0.47±0.067
67	α-Monoacetin	10.34	1.01±0.263	-	-	-
68	3-Methoxymethoxybutyric acid	10.34	-	-	0.89±0.469	-
69	Butanedioic acid, 2-hydroxy-2-methyl-, (S)-	10.35	-	0.845±0.1425	-	-
70	5-Hydroxymethylfurfural	10.89	0.589±0.569	1.161±0.035	0.9±0.003	0.39±0.483
71	Benzenepentanol	11.23	0.091±0.024	-	-	-
72	Resorcinol, 2-acetyl-	11.35	0.097±0.035	-	-	0.13±0.046
73	Acetophenone, 2',5'-dihydroxy-	11.36	-	0.125±0.024	-	-
74	2,2-Dimethylpropanoic acid, undecyl ester	11.54	-	-	0.078±0.035	-
75	Butanoic acid, 4-(1,1-dimethylethoxy)-3-hydroxy-,	11.58	-	0.256±0.038	-	-
76	3-Isoamyl-2,5-dimethylpyrazine	11.79	0.221±0.753	-	-	-
77	Pyrazine, 2,5-dimethyl-3-(3-methylbutyl)-	11.80	-	0.176±0.422	-	0.17±0.345
78	Pyrazine, 3-isopentyl-2,5-dimethyl-	11.81	-	-	0.198±0.690	-
79	1,1,3,3,5,5-Hexamethyl-1,5-bis(2-methylpropoxy) trisiloxane	11.92	-	-	-	0.16±0.038
80	Megastigma-4,6(E),8(Z)-triene	12.06	0.191±0.038	0.179±0.240	-	-
81	4,2,7-Ethanylylidenecyclopenta[b]pyran-9-one, 2,3,4,4a,7,7a-hexahydro-7a-methyl	12.08	-	-	0.117±0.757	-
82	α-Ionene	0.14	0.144±0.690	0.302±0.754	0.187±0.642	0.1±0.178
83	Pyrogallol 1,3-dimethyl ether	12.36	1.394±0.187	1.029±0.657	0.994±0.024	0.96±0.684
84	4-Methyl-2-phenyl-2-pentenal	12.61	0.131±0.543	-	-	-
85	Benzeneacetaldehyde, α-(2-methylpropylidene	12.62	-	0.128±0.545	-	-
86	Piperidine, 2-(phenylmethyl)-	12.75	0.08±0.690	-	0.08±0.3532	0.13±0.435
87	(+)-3-Carene, 10-(acetylmethyl)-	12.83	0.226±0.142	0.163±0.135	0.177±0.067	0.15±0.242
88	L-Proline, 5-oxo-, methyl ester	12.99	1.39±0.243	0.783±0.690	1.006±0.978	0.92±0.690
89	4-(2,4,4-Trimethyl-cyclohexa-1,5-dienyl)-but-3-en-2-one	13.15	-	0.171±0.249	-	0.14±0.067
90	Dehydro-β-ionone	13.16	0.514±0.135	0.235±0.246	0.172±0.038	-
91	Homoserine lactone 1-(dimethylamino)naphthalene-5-sulfonamide	13.57	0.112±0.046	0.091±0.425	-	-
92	1H-Pyrido[3,4-b]indole, 2,3,4,9-tetrahydro-1-isobutyl-	13.61	-	-	0.112±0.136	-
93	2-Phenylpyridine	13.86	0.436±0.245	0.379±0.049	-	-
94	Pyridine, 3-phenyl-	13.87	-	-	0.264±0.024	0.37±0.049
95	5-Methyl-2-phenyl-2-hexenal	14.13	0.149±0.035	0.101±0.305	-	-
96	Phenol, 2,4-bis(1,1-dimethylethyl)-	14.40	0.541±0.345	0.341±0.038	0.528±0.003	0.55±0.038
97	2-Hydroxy-1-(1'-pyrrolidiyl)-1-buten-3-one	14.52	0.201±0.067	-	-	-
98	3',5'-Dimethoxyacetophenone	15.08	0.292±0.038	-	0.209±0.647	-
99	Acetophenone, 3',4'-dimethoxy-	15.10	-	0.254±0.305	-	-
100	5,6,6-Trimethyl-5-(3-oxobut-1-enyl)-1-oxaspiro [2.5]-4-one	15.61	-	0.182±0.358	-	-
101	1,7-Trimethylene-2,3-dimethylindole	15.77	0.163±0.038	-	-	0.12±0.038
102	Nicotinic acid, 2,6-dimethoxy-4-methyl-	16.14	-	0.517±0.143	0.339±0.690	-
103	2α,3β-Diacetoxy-2-methoxy-2,3-dihydrobiphenylene	16.21	-	-	0.348±	-
104	Dodecyl acrylate	16.45	-	-	-	0.13±0.315
105	1,2-5,6-Dipyrazinocyclooctane	16.81	0.665±0.024	-	-	-
106	2,5-Dimethyl-4-(3-amino-4-methylphenyl) pyridine	16.84	-	0.448±0.24	0.53±0.038	0.43±0.067
107	(1R,9aS,Z)-1-Methyl-3-propylideneoctahydro-1H-quinolizine	17.28	0.726±0.035	-	0.423±0.004	0.58±0.038
108	1,2-Oxazino[2,3-b]isoquinoline, 2,4a,5,5a,6,7,8,9,9a,10-decahydro	17.31	-	0.372±0.594	-	-
109	Acetamide, N-(4-ethoxy-3-hydroxyphenyl)-	18.19	2.695±0.035	1.525±0.067	1.156±0.125	1.73±0.494

110	Pentadecanoic acid	18.33	-	0.347±0.114	-	2.88±0.690
111	Hexadecanoic acid, methyl ester	18.92	0.34±0.003	0.705±0.038	1.199±0.067	1.88±0.494
112	n-Hexadecanoic acid	19.44	9.451±0.038	12.55±0.125	15.217±1.793	18.7±0.035
113	Hexadecanoic acid, ethyl ester	19.61	-	-	0.288±0.038	0.29±0.067
114	7,10-Hexadecadienoic acid, methyl ester	20.05	0.177±0.358	-	0.451±0.125	0.99±0.024
115	9,12-Hexadecadienoic acid, methyl ester	20.07	-	0.581±0.459	-	-
116	i-Propyl 15-methylhexadecanoate	20.30	-	0.243±0.494	-	-
117	Heptadecanoic acid	20.31	-	-	0.262±0.125	0.33±0.125
118	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	20.59	0.649±0.024	1.407±0.038	2.47±0.067	-
119	Linoleic acid, methyl ester	20.60	-	-	-	3.42±0.690
120	α-Glyceryllinolenate	20.65	0.15±0.547	-	-	-
121	6-Octadecenoic acid, methyl ester, (Z)-	20.71	-	-	-	0.76±0.038
122	10-Octadecenoic acid, methyl ester	20.74	-	-	0.571±0.494	-
123	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	20.79	-	0.342±0.125		-
124	Methyl stearate	20.86	-	-	0.088±0.125	0.18±0.494
125	9,12-Octadecadienoic acid (Z,Z)-	21.43	9.907±0.035	11.52±0.494	15.154±1.793	15.1±0.125
126	9,12-Octadecadienoic acid, ethyl ester	21.22	-	-	0.614±0.024	0.53±0.690
127	Octadecanoic acid	21.26	0.872±0.024	1.767±0.038	2.64±0.067	3.85±0.125
128	2,6-Diphenylpyridine	22.11	0.466±0.067	0.394±0.125	0.403±0.494	0.38±0.038
129	Methyl 15-hydroxy-9,12-octadecadienoate	22.21	0.092±0.024	-	-	-
130	6,9,12,15-Docosatetraenoic acid, methyl ester	22.20	-	0.079±0.035	-	-
131	Cyclopropanebutanoic acid, 2-[[2-[[2-[(2-pentylcyclopropyl)methyl]cyclopropyl]methyl] cyclopropyl]methyl]-, methyl ester	22.21	-	-	-	0.11±0.024
132	E,E,Z-1,3,12-Nonadecatriene-5,14-diol	22.22	-	-	0.11±0.125	-
133	β-Naphthylamine, N-tosylmethyl	22.30	0.11±0.125	-	0.112±0.494	-
134	Glucobrassicin	22.32	-	0.108±0.125	-	-
135	2H-Pyran-2-one, 6-methyl-3-(1-oxopropyl)-4-[(2-phenylethyl) amino]-	22.36	0.25±0.467	0.236±0.494	0.302±0.125	0.15±0.038
136	Pyrrolidine, 1-(1-oxo-7,10-hexadecadienyl)-	22.57	-	-	-	0.41±0.067
137	1-Monolinolein	25.91	3.657±0.038	4.316±0.351	4.083±1.793	3.91±0.494
138	n-Hexadecanoic acid, pentamethyldisilyl ester	24.05	0.598±0.125	0.672±0.494	0.78±0.246	0.99±0.024
139	Glycerol β-palmitate	24.20	2.223±1.793	2.617±0.125	3.181±	3.74±0.465
140	Phthalic acid, di(2-propylpentyl) ester	24.51	-		0.268±0.125	-
141	Trilinolein	25.64	-	0.694±0.038	-	-
142	Linoleic acid trimethylsilyl ester	25.66	-	-	-	0.67±0.125
143	γ-Sitosterol	26.55	1.447±0.024	0.997±0.351	1.205±0.114	-
144	γ-Tocopherol	31.54	-	0.242±0.067	0.469±0.494	1.54±0.038

Each value represents mean ± standard error of three triplicates