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Metabolomic and proteomic signature of *Gloriosa superba* leaves treated with mercuric chloride and phenylalanine, a precursor of colchicine alkaloid

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ABSTRACT

Gloriosa superba is a tropical, medicinally important plant used in the treatment of gout, rheumatism, and other ailments. It produces pharmaceutically important alkaloids like colchicine, gloriosine, thiocolchicoside and others. In the present study, gas chromatography (GC)-mass spectrometry (MS) method has been deployed for the identification of low abundance phytochemicals in mercuric chloride (elicitor)-treated leaves. The analysis revealed nearly 500 molecules including 15 key secondary metabolites like estragole, N-methylloline (alkaloid), aphidocolin, 3-hydroxykynurenine, octyl salicylate, butibufen, anonaine (aporhine alkaloid), bolasterone, austricin, bolandione, octahydrocoumarin, jacaranone, bonducellin, quinacridone, and β-carotene that may have medicinal importance. Liquid chromatography-mass spectrometry (LC-MS) analysis of leaf proteome in the control and phenylalanine (a precursor of colchicine)-treated tissues revealed a total of 982 and 937 proteins respectively. In precursor-treated tissues, 364 differentially expressed proteins were noticed besides others. Key proteins involved in shikimate/chorismate pathway such as phenylalanine ammonia-lyase, chalcone-flavone isomerase (associated with flavonoid biosynthesis), chalcone synthase (involved in the synthesis of bioactive compounds in plants), chorismate synthetase, chorismate mutase, tryptophan synthase, and medium chain triglyceride protein were detected. Importantly, detection of nearly 154 proteins with unknown functions may hold key and play a role in colchicine biosynthetic pathway. These studies suggest that while metabolomic studies help to detect new secondary plant products, proteomic studies assist us in identifying key enzymes implicated in the biosynthetic pathway of alkaloids in *G. superba*.

1. Introduction

Gloriosa superba L. is an important medicinal herb, commonly called as flame lily, climbing lily, or glory lily. Annually, the trade for the seed is around 100–200 million tons with a price range of Indian rupees 600–750/kg. Traditionally, the plant has been used to treat malaria, stomach pain, leprosy, asthma, ulcers, hemorrhoids, dyspepsia, helminthiasis and inflammations. Both *Colchicum autumnale* and *G. superba* from the Liliaceae produce colchicine (N-Deacetyl-N-formylcolchicine) in significant quantities. The yields of colchicine from *G. superba* are high in tubers and seeds (0.15–0.30% and 0.7–0.9% respectively and is cultivated in Southern parts of India (Varsha et al., 2017). Colchicine,

Abbreviations: GC-MS, Gas chromatography-mass spectrometry; LC-MS, Liquid chromatography-mass spectrometry; *G. superba*, *Gloriosa superba*.

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produced by *G. superba* is used to treat gout, rheumatism, and suggested for treating cancer. The tuber is used as an effective antidote against snake poison, insect bites, and scorpion stings (Samy et al., 2008). The plant produces important alkaloids like colchicine, gloriosine (N-Formyldeacetylcolchicine) besides lumicolchicine, 3-demethyl-N-deformyl-N-deacetylcolchicine, 3-demethylcolchicine, and N-formyl deacetylcolchcine (Jana and Shekhawat, 2011). Colchicine has been shown to have antimitotic, anti-inflammatory, anticancer, and antimicrobial activities (Joshi et al., 2010). Colchicine binds with tubulin and prevents the microtubule assembly, thereby prevents division of cells (Herdman et al., 2016). Hence, colchicine has been used in chemotherapy to stop the growth of cancer cells (Johnson et al., 2017). Yokoyama et al. (2017) recorded the effect of colchicine on the enhanced production of interleukin-8, which inhibits the pancreatic cancer cells. But colchicine has limited use as anticancer molecules since high concentrations are necessary for the purpose (Lin et al., 2016). Thus, due to the presence of several bioactive compounds, the plant has gained industrial importance (Sapra et al., 2013).

Since the plant is endangered (Jana and Shekhawat, 2011), and seed set is low (Mamatha et al., 1993), several attempts to propagate the plants using *in vitro* methods were made (Sivakumar and Krishnamurthy, 2004). *In vitro* production of colchicine from *G. superba* has also been attempted (Ghosh et al., 2007; Jawahar et al., 2018; Sivakumar et al., 2019). But, stable and high accumulation of colchicine is still a challenge in the cultivation of *G. superba*. The process of cultivation is labor intensive, time consuming due to the slow growing nature of rhizomes. But the information about the biosynthetic pathway and the genes that encode the enzymes in the pathway have not yet been identified. Therefore, attempts to enhance the content of colchicine, gloriosine and colchicosides (carbotricyclic compounds) in rhizomes and also *in vitro* cultures have met with limited success. Since the demand for naturally occurring colchicine has been substantially increasing year after year (Sivakumar, 2017), it necessitates to unfold the genes that regulate biosynthetic pathway(s) of colchicine, gloriosine, colchicoside and thiocolchicoside (sulfur derivative of colchicoside).

Metabolomics is a promising tool and a key component of plant sciences including natural product chemistry (Verpoorte et al., 2007). Metabolomics help us in identifying the complete set of metabolites (small molecules of <1 kDa) and also in quantitative and qualitative measurement. Metabolites could be important intermediates in the primary and secondary metabolic pathway reactions (Holmes et al., 2008). Metabolomic approach has been effectively used for biomarker identification in *Panax ginseng* (Wang et al., 2017), drug discovery and understanding the mechanism of action of medicinal plants (Tuyiringire et al., 2018), and metabolomic alterations depending on growth stages of *Epimedium pubescens* (Qin et al., 2020). Identification of the secondary metabolites that are produced in low abundance is extremely difficult. Hence, a holistic approach is necessary and perhaps rational in identifying the secondary metabolic pathways. Our previous experiments revealed enhanced colchicine content in morphogenic cultures of *G. superba* treated with mercuric chloride and also phenylalanine (Jawahar et al., 2018). This has prompted us to use mercuric chloride as an elicitor to increase the low-response metabolites of *G. superba*. In fact, different medicinal plant proteomes were analyzed after treating with elicitors. Examples for such elicitor-treated responses are isoflavones-related proteins in *Medicago truncatula* (Lei et al., 2010), chalcone-related proteins in *Boesenbergia rotunda* (Tan et al., 2012), and methyljasmonate treated proteomic signature in fenugreek (Ciura et al., 2017). Elicitation induced proteomics of cultured cells of California poppy, opium poppy, and Madagascar periwinkle showed alkaloid biosynthetic pathway-related enzymes and proteins. In the above cases, differential protein studies helped to create large alkaloid biosynthetic pathway networks (Oldham et al., 2010; Desgagné-Penix et al., 2010; Champagne et al., 2012). Both metabolomic and proteomic profiles of some medicinal plants helped to find out the correlations with the secondary products (Gonulalan et al., 2020). Proteomic analysis of the leaf of *Artemisia annua* by Bryant et al. (2015) lead them to identify the proteins involved in the biosynthesis of bioactive compound artemisinin. Root proteome carried out by Contreras et al. (2019) in *Salvia miltiorrhiza* revealed novel insights into the biosynthesis of tanshinone, an abietane diterpene compound. Likewise, Pedrete et al. (2019) found out the proteins in medicinal plants used for the treatment of diabetes. But till date, metabolomic and proteomic studies have not been carried out in *G. superba* to identify important bioactive compounds and proteins related to the alkaloids colchicine and gloriosine. It is crucial to know the enzymes/proteins that are associated with the biosynthetic pathways of the colchicine, gloriosine, and other secondary metabolites in *G. superba*. Identification of metabolites, enzymes or proteins would aid us to clone the corresponding genes and overexpress them for improving the accumulation of major metabolites. The current study focuses to find out the metabolites using GC-MS and analyse the proteome from leaves of *G. superba* using liquid chromatography, and tandem mass spectrometry (LC-MS) methodologies.

2. Materials and methods

2.1. Analysis of leaf metabolome by GC-MS

Tubers of *G. superba* were collected from Salem, Tamilnadu, India, transplanted into the pots and grown in the net house. Leaves from three-month-old plants were sprayed with 100 μmol of mercuric chloride, and harvested after 48 h of treatment. Foliar absorption is one of the most followed methods to introduce external agents in the form of homogenous solutions. Patra and Sharma (2000) explained foliar absorption of atmospheric mercury and accumulation into plants. Suszcynsky and Shann (1995) used a foliar absorption method to understand the phytotoxicity of soluble mercury in tobacco plants. They were then kept 3-days under shade for drying, powdered by a mortar and pestle and extracted with methanol (100 ml/g dry weight of tissue). Solvent extraction was carried out for 48 h, filtered to remove solid material using Whatman Number 1 filter paper, and subjected to lyophilization. The dried crude extract was dissolved in 80 μl pyridine containing 20 mg/ml methoxyamine hydrochloride, and then kept for 1 h at room temperature. It was derivatized by trimethylsilylimidazole and incubated at 37 °C for 20 min. The chemically modified crude extract was used for GC-MS analysis. Samples were analyzed by LECO GC x GC, combined with the Pegasus HT-C TOFMS (LECO Corporation, USA). Column (Restek, USA) such as 30 m Rxi-5 ms was used in the study with dimensions like 30 mm long, 250 μm internal diameter, and 10 μm film thickness. The injection temperature was set at 250 °C, the oven temperature set at 320 °C and ion source temperature set at 230 °C. Helium gas was used as carrier gas. Active inlet located at back and injected carrier gas were adjusted in split less mode at the flow rate of 1.20 ml/min. Isothermal temperature was maintained at 80 °C for 1 min, then oven temperature ramp to 300 °C at the rate of 15 °C per min (carried out at the University of Hyderabad, India). Total gas chromatography procedure has taken 34 min for completion. For MS analysis, mass range was set between 50 and 550 and spectra acquisition rate was 10/second and the detector worked at −70 volts power supply. In the present study, only foliar spray of mercuric chloride as an elicitor was used, a common practice among physiologists.

2.2. Analysis of leaf proteome by LC-MS

Since precursors stimulate the biosynthetic pathways, phenylalanine, a precursor of colchicine has been sprayed on the leaves of *G. superba* before analysing the proteome. Extraction of proteins from the whole leaves was carried out using urea buffer and a standard protocol (Hari, 1981). Twenty-five μl of the protein sample was used and mixed with 25 μl of Laemmli buffer (Laemmli, 1970) and made it to 100 μl using double distilled water. Samples were incubated in water bath at 100 °C for 15 min and then centrifuged at 6000× *g* for 10 min. Thirty μl

of above sample solution was used to load into the NuPAGE® Novex bis-tris readymade gels (Thermo Fisher Scientific) and used subsequently. The concentration of acrylamide was 4–12% in stacking gel, and the separating gel 4%, and gel thickness was 1.2 mm. Ten-well format was used for the purpose. MES 1 X [(2-(N-Morpholino) ethanesulfonic acid)] was used as running buffer. After sample loading into the wells, the electrophoretic unit was maintained at 200 V for 35 min. Ten kD to 250 kD protein marker was used to identify the desired protein based upon molecular weight. After running the gel, staining was carried out using Coomassie solution for 3 h. The gel was destained using destaining solution, by changing it several times until clear bands appeared on the electrophoretic gel. For mass spectrometric studies, trypsin in gel digestion method was followed (Shevchenko et al., 1996). Protein bands were excised form the gel and chopped into small pieces (1 × 1 mm) and then transferred to 1.5 ml tubes. The Coomassie stain was then washed from the chopped pieces. Eight hundred μl of 70% 50 mM ammonium bicarbonate and 30% acetonitrile was used to wash the gel pieces. Based on the staining concentration in gel slices, washing time varied from 30 to 55 min. The solution was removed, 500 μl acetonitrile was added and allowed for 10 min to shrink the slices. The solution was removed and washed with 50 mM ammonium bicarbonate for 10 min. The solution was again removed and acetonitrile was added to shrink the gel pieces. After removing acetonitrile, the gel pieces were kept in a vacuum centrifuge for 5 min for drying. The dried gel pieces were rehydrated with 200 μl of 10 mM dithiothreitol and 50 mM ammonium bicarbonate. In this rehydration step, temperature was maintained at 56 °C for 45 min. Then tubes were removed from the incubator and kept at room temperature for 30 min. Meanwhile, the excess solution was removed and replaced with 200 μl of 55 mM idoacetamide in 50 mM ammonium bicarbonate. The solution was removed, and the gel pieces were washed with 50 mM ammonium bicarbonate and acetonitrile one after the other, for two times (as mentioned above). Acetonitrile was removed and kept for vacuum drying for 5 min. After performing the step, 15 ng/μl of trypsin (Roche grade) in 25 mM and 1 mM $CaCl_2$ were used to rehydrate the gel pieces at 37 °C for 16 h before digestion. The peptides were extracted from gel slices using 500 μl of 5% formic acid and 30% acetonitrile by vortexing for 1 h. Then the extraction was kept under a vacuum centrifuge for drying and stored at − 20 °C.

Hundred μl of solution was taken in a C18 reverse phase material tips and used for desalting and purifying peptide samples. The dried peptide samples were taken and 100 μl of 1% trifluoroacetic (TFA) acid added and vortexed for 5 min. The C18 tips were washed two times with 100 μl acetonitrile, and three times again with 100 μl of 0.1% TFA. The peptide samples were drawn and released for 15 slow times. After completing this step, C18 tips were washed twice with 100 μl of 0.1% TFA. The peptides were eluted from C18 block by 100 μl of 70% acetonitrile and 1% formic acid. This step was repeated for complete elution of peptides. The eluted sample was dried in a vacuum concentrator and then 20 μl of 5% formic acid added and then subjected to LC-MS analysis. For metabolomic studies, experiments were repeated and technical triplicates were used.

2.3. Analysis of proteomic profile

Proteomic studies were carried out using high performance liquid chromatography (HPLC) coupled mass spectrometry. In the present study, for liquid chromatography of peptides EASY-nLC system (Thermo Fisher Scientific, USA) was used. Peptide mixture was loaded to 15 cm EASY-SPRAY C18 reverse phase column along with buffer A (0.1% formic acid in 5% acetonitrile) and separated by buffer B (0.1% formic acid in 95% acetonitrile) with a back pressure of 480 bars. Gradient ranges were measured in percentage of buffer B and flow rate was adjusted to 300 nl/min. Gradient ranges occurred as 0–25% buffer B in 37 min; 25–40% B in 7 min; 40–90% B in 5 min, and 90% of buffer B holds for 5 min. Total time taken for the gradient run was 60 min. The EASY-nLC system was directly coupled to Q Exactive HF with a Nano-Flex source (Thermo Fisher Scientific). Parameters used for full MS set included were maximum scan resolution at 60,000 and 400–1650 *m/z*, with maximum ion injection time of 100 ms and the automatic gain control (AGC) target was 3e6. Resolution for MS spectra was set to 15,000 at 200–2000 *m/z* with a maximum ion injection time of 100 ms, and the AGC target was 1e5. Normal collision energy (NCE) was 28 and S-Lens RF value set was 55. Ions were excluded with unassigned, 1, 6–8, > 8 charge states. Dynamic exclusion was kept on to remove the sequence repetition. Peaks repeated in 30 s, were excluded for fragmentation. Based on the mass spectrometry data, peaks were produced by Sequest HT software, and then analysed by Proteome Discoverer Software. Full proteome of *Arabidopsis thaliana* in FASTA format was set as a reference database. In the peptide analysis, deamination (+0.984 Da - N, Q) and oxidation (+15.995 Da -M) were kept under dynamic modifications and carbamidomethyl (+57.021 Da -C) was set as static modification. Peptide confidence was set as high, and the minimal number of peptides per protein was set as 1 which counted only high scoring peptides. Only peptide spectrum match (PSM) with delta Cn better than 0.15 was considered and fragment mass tolerance was set as 0.05 Da. Then, the results were extracted to excel file and then proteins were manually examined. Proteomic experiments were repeated with biological replicates.

3. Results

3.1. Metabolomic analysis

A total of nearly 500 metabolites were detected through GC-MS in the leaf tissue and the metabolites identified using Golm Metabolome Database Library. Though a number of metabolites were identified, only key metabolites and their known functions are shown in the Table 1 and the rest as Supplementary material (Supplementary Figures 1 to 8 and Supplementary Table 1). The structures of important metabolites are put up in Supplementary Table 4. Among the important metabolites identified by GC-MS are estragole, N-methylloline (alkaloid), aphidocolin, 3-hydroxykynurenine, octyl salicylate, butibufen, anonaine, bolasterone, austricin, bolandione, octahydrocoumarin, jacaranone, bonducellin, quinacridone, and β-carotene. Some of these metabolites have pharmacological activities and detected for the first time in *G. superba*. The relevant GC-MS chromatogram is shown in the Fig. 1.

3.2. LC-MS analysis of G. superba leaf proteome

LC-MS generated peptide peaks of *G. superba* total leaf proteome was analyzed using *Arabidopsis thaliana* as a reference database and the chromatogram of the total peptide mixture is shown in the Fig. 2. The analysis revealed a total of 982 proteins, that were grouped manually, based on functional annotation (Supplementary Table 2). Annotation revealed 41 proteins which are associated with photosynthesis, 83 carbohydrate metabolism, 34 lipid metabolism, 41 amino acid metabolism, 23 cell structure proteins, 8 cell cycle regulation proteins, 29 chaperons, 57 protein catabolism, 89 translation, 38 transcription, 13 ubiquitin associated pathway, 48 ATP syntheses and ATPases, 46 cell redox homeostasis, 35 cell signaling, 43 Krebs cycle, 25 metal ion binding, 18 transport, 7 mitochondrial respiratory system, 48 nucleotide metabolism, 18 heat and cold shock related, 27 methyl and sulfur transferases, 11 hydrolases (catalyze hydrolysis of several types of compounds), 8 growth and development, 12 RNA processing, 20 vitamin synthesis and binding, 6 ungrouped/other proteins, and 154 with an unknown function. The spectrum of protein groups as detected by LC-MS in untreated leaf of *G. superba* is depicted in a pie chart (Fig. 3). Some of the important proteins recognized in the control leaf include SSCD 1 (A0A178W9V5) related to shikimate pathway and aromatic acid metabolism, MCT (A0A178VW22) related to isoprenoid biosynthetic pathway, quinone oxidoreductase (Q9FKG8) related to quinone

Table 1
Important secondary metabolites identified along with their molecular formula in leaf of *G. superba*.

Key metabolites identified in the leaf tissue	Molecular formula	Nature of compound/activity	Reference
Estragole	$C_{10}H_{12}O$	A phenylpropene compound, gives natural scent.	Vincenzi et al. (2001)
N-Methylloline	$C_9H_{16}N_2O$	It is a loline alkaloid and acts as natural insecticide and induces drought and stress tolerance.	Nagabhyru et al. (2013)
Aphidicolin	$C_{20}H_{34}O_4$	A tetracyclic diterpene, extracted from the fungus *Cephalosporum aphidicola*, used as antibiotic compound and has antiviral and antimitotic properties. Inhibits DNA replication.	Yasuhara and Kitamoto (2014)
3-Hydroxykynurenine (3-HK)	$C_{10}H_{12}N_2O_4$	A metabolite of tryptophan. It is an antioxidant and has scavenging properties. 3-HK is the entry point to the quinolinic acid branch of the kynurenine pathway.	Leipnitz et al. (2007)
Octyl salicylate	$C_{15}H_{22}O_3$	Inhibits carcinoma cells. It is an ingredient in sunscreens and cosmetics to absorb ultraviolet-B (UV-B) from Sun.	Ziklo et al. (2020)
Butibufen	$C_{14}H_{20}O_2$	The anti-inflammatory, analgesic and antipyretic.	Nunes et al. (2020)
Anonaine	$C_{17}H_{15}NO_2$	Bioactive benzylisoquinoline alkaloid. It exhibits anti-cancer, trypanocidal and antiplasmodial activities.	Li et al. (2013)
Bolasterone	$C_{21}H_{32}O_2$	Also known as 7α, 17α,-dimethyltestosterone, it is a 17α-alkylated androgen/anabolic steroid used in veterinary medicine.	Basaria (2010)
Austricin	$C_{15}H_{18}O_4$	Sesquiterpene lactone that reduces inflammation. Austricin improves the melanogenesis by increasing the melanin content and tyrosinase activity of B16 melanoma cells.	Li et al. (2021)
Bolandione	$C_{18}H_{24}O_2$	It is also known 19-norandrostenedione, as well as 19-norandrost-4-en-3,17-dione. Precursor of the anabolic-androgenic steroid nandrolone.	Diel et al. (2008)
Octahydrocoumarin	$C_9H_{14}O_2$	Coumarin, used as a flavor and fragrance agent. Useful in preparing coumarin alternatives and more stable than coumarin. Coumarins are potential anticancer compounds.	Akkol et al. (2020)
Jacaranone	$C_9H_{10}O_4$	It is a sedative. An anti-malarial compound and induces apoptosis in melanoma cells.	Lozada-Lechuga et al. (2009)
Bonducellin	$C_{17}H_{14}O_4$	Flavonoid and anti-viral compound. It also has anti-malarial activity.	Basavaiah et al. (1998)
Quinacridone	$C_{20}H_{12}N_2O_2$	Red pigment. It has industrial colorant applications like outdoor paints, inkjet printer ink, tattoo inks, color laser printer toner.	Suzanne (2005)
β-carotene	$C_{40}H_{56}$	Red-orange pigment found in plants. Used in the food industry, and is a precursor of vitamin A, natural colorant, and an antioxidant.	Tang (2010)

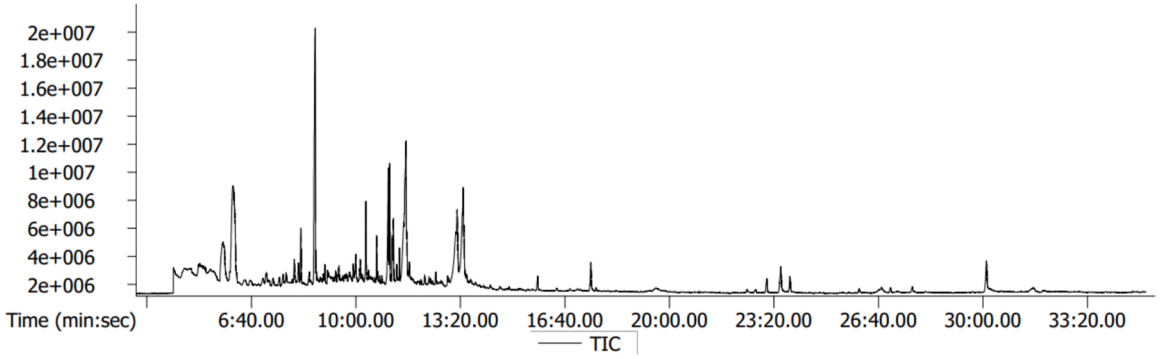

Fig. 1. GC-MS spectra of the leaf sample of *G. superba* showing metabolite peaks.

biosynthetic pathway, STF 1 soybean TGACG-motif binding factor 1 (A0A178UGL7), an important basic leucine zipper protein involved in many plant growth and developmental processes, ISPH (A0A178V3K9) related to isopentenyl diphosphate biosynthetic process, and plastid-lipid associated protein (PAP) (A0A178V8A7) related to carotenoid biosynthesis.

Precursor (phenylalanine) feeding to the leaf tissues stimulated the colchicine accumulation considerably in our previous experiments. Hence, untreated and phenylalanine-treated leaf proteome analysis was carried out to find out the differentially expressed proteins. Leaf proteome profile revealed a total of 982 proteins in the control tissues, in phenylalanine-treated tissues, and 937 proteins were detected (Supplementary Tables 2 and 3). Out of 982 proteins, 573 were found common between the control and phenylalanine-treated tissues. Eight proteins were exclusively noticed in untreated leaf proteome, in comparison with seven proteins observed in phenylalanine-treated samples (Table 2). While 409 proteins were differentially expressed in untreated leaf tissues, 364 in phenylalanine-treated tissues (Supplementary Tables 2 and 3). The differentially expressed proteins in phenylalanine-treated tissues include a serine hydroxymethyltransferase -involved in nematode resistance in plants, a pentapeptide repeat-containing protein (necessary for providing surface to mediate protein-protein interactions), X-chromosome associated protein5 (XAP5) CIRCADIAN TIME KEEPER (XCT), medium chain triglyceride protein (may play a role in colchicine biosynthesis), and acyl-CoA-binding protein (associated in the intracellular transport, and pool formation of acyl-CoA esters. These are important intermediates and regulators in lipid metabolism and cellular signaling. Similarly, phenylalanine ammonia-lyase that converts phenylalanine into trans-cinnamic acid, an important intermediate in the biosynthetic pathway of shikimate and colchicine, chalcone-flavone isomerase, involved in the biosynthesis of flavonoids, chalcone synthase (decarboxylative condensations of malonyl units with a Co-A linked starter molecule and produce important bioactive compounds in plants), chorismate synthase, tryptophan synthase, chorismate mutase (many aromatic secondary plant products are synthesized from chorismate pathway) were identified. Apart from the above, other important proteins such as methylenetetrahydrofolate reductase, associated with methionine biosynthesis as well as infection-related morphogenesis, *O*-methyltransferase-like protein that methylates oxygen atom of many secondary metabolites including phenylpropanoids, flavonoids and alkaloids, methylthioribose kinase1 (MTK1), a phloem-specific expression of the Yang cycle gene *5-METHYLTHIORIBOSE KINASE1* in *Plantago* and *Arabidopsis*, and recycles in leaves, tetratricopeptide thioredoxin-like (TTL) proteins, positive regulators of brassinosteroid signaling

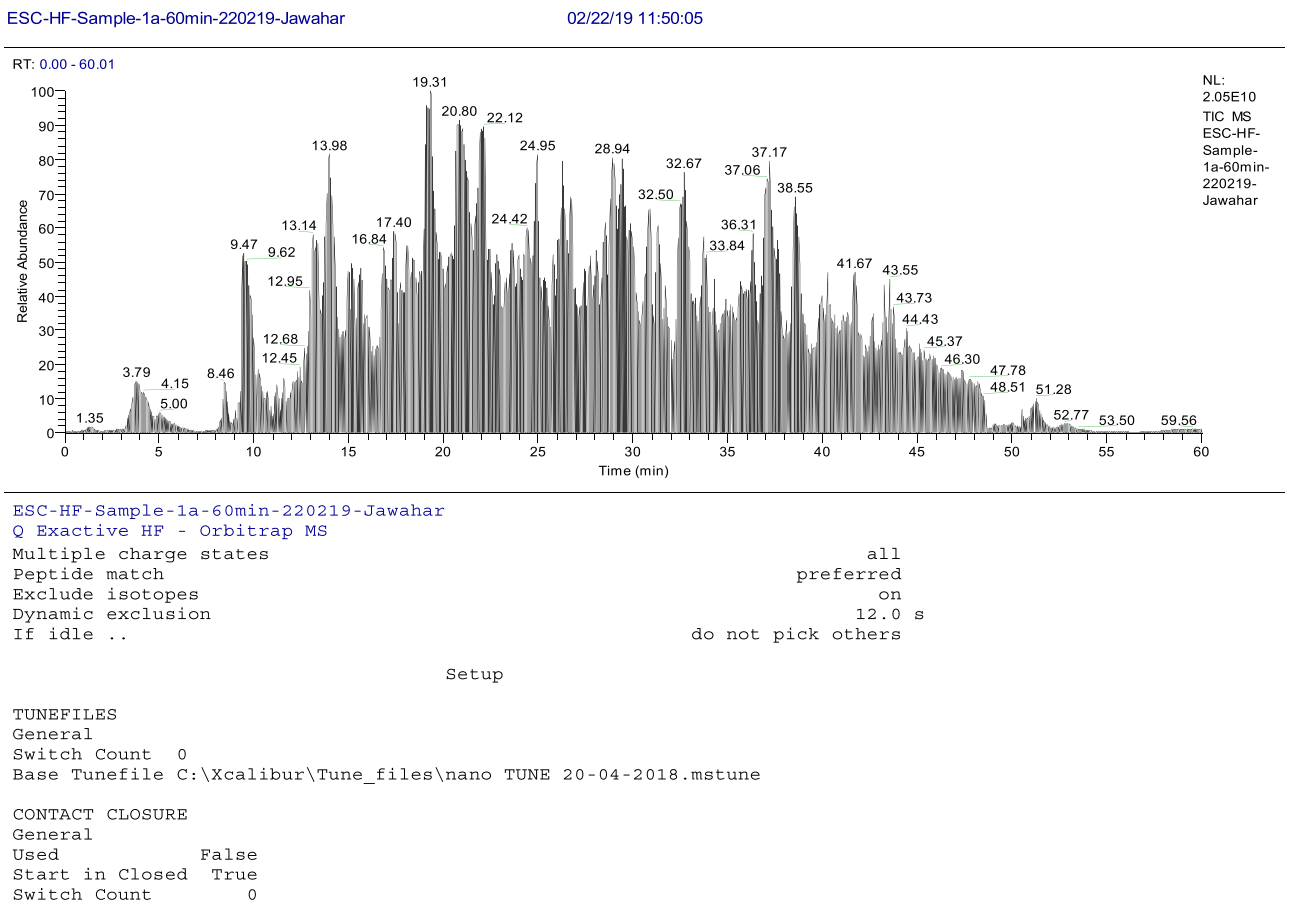

Fig. 2. LC-MS generated chromatogram of the total peptide mixture of *G. superba* leaf.

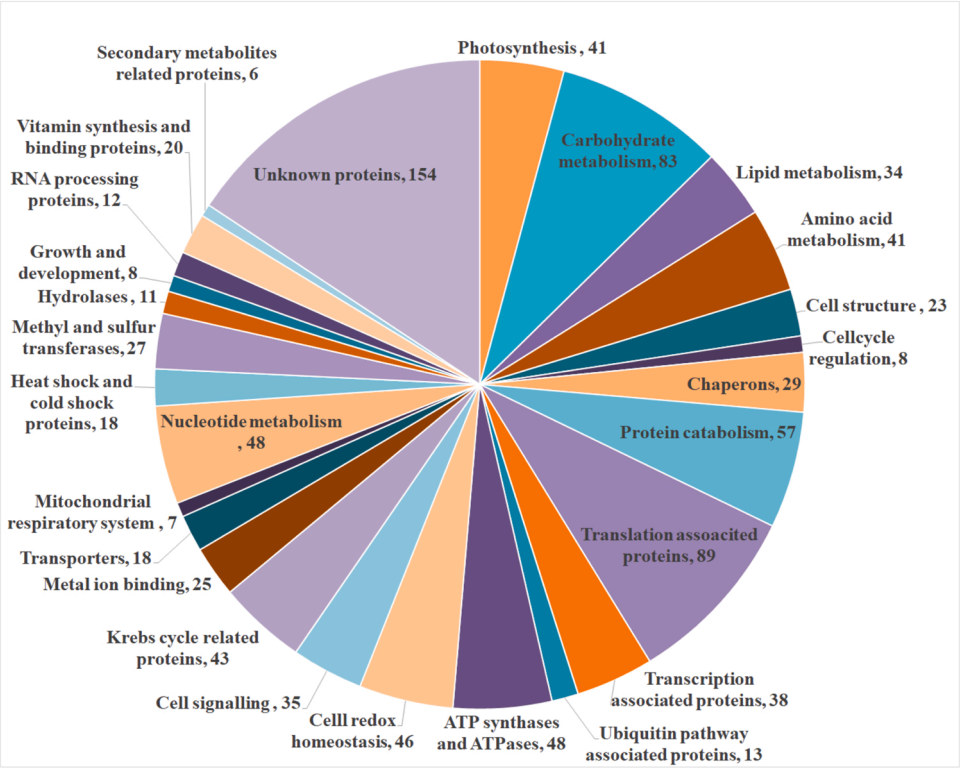

Fig. 3. Pie chart showing the protein groups in untreated (control) leaf of *G. superba* as identified by LC-MS, and categorization carried by functional annotation of proteins.

Table 2
Unique proteins identified in both control leaf and phenylalanine-treated leaf tissues. The cutoff value for high cumulative peptide-spectrum match (ΣPSM) is 50.

S. No.	Proteins expressed in control leaf only	
	Protein ID	**Protein name**
1.	Q7×8A GI: 75295946	OSJNBa0036B21.24 protein (Os04g0459500 protein, 402 aa protein)
2.	Q10NA9 GI: 122247218	Heat shock cognate 70 kDa protein, putative, expressed (Os03g0276500 protein, 650 aa protein)
3.	Q0DI00 GI: 122247218	Os05g0427800 protein (148 aa protein)
4.	Q53NW9 GI: 75320207	Pyridoxine biosynthesis protein, putative (Os11g0708500 protein, 363 aa protein)
5.	Q6Z8F4 GI: 75325498	Phosphoribulokinase (Os02g0698000 protein, 403 aa protein)
6.	Q2QTJ1 GI: 122248610	Ribulose bisphosphate carboxylase small chain C, chloroplast, putative, expressed (Os12g0292400 protein, 175 aa protein)
7.	Q9SNK3 GI: 75313656	Glyceraldehyde-3-phosphate dehydrogenase (444 aa protein)
8.	Q7XN85 GI: 75232875	OSJNBa0011F23.7 protein 441 aa protein
S. No	**Proteins expressed in phenylalanine-treated leaf only**	
	Protein ID	**Protein name**
1.	Q570E5 GI:75283569	Glyceraldehyde 3-phosphate dehydrogenase A subunit 161 aa protein
2.	Q651T8 GI:75288752	Putative vacuolar proton-ATPase (Os06g0662000 protein) 620 aa protein
3.	Q6EUQ9 GI:75290257	Putative vacuolar proton-ATPase (Os02g0175400 protein) 621 aa protein
4.	Q6L509 GI:75291272	Putative hsp70 (Os05g0460000 protein) 646 aa protein
5.	Q93W07 GI:75305734	Vacuolar ATPase B subunit (Os06g0568200 protein), 488 aa protein
6.	Q0WLF6 GI:122236074	Vacuolar-type H^+-ATPase subunit A 623 aa protein
7.	Q10RP0 GI:122247523	Cell division cycle protein 48, putative, expressed (Os03g0151800 protein), 809 aa protein

pathway which may be associated with colchicine biosynthesis *via* brassinosteroid signaling, protein argonaute 9 (AGO9), required to specify cell fate in ovule, and involved in the formation of female gamete formation, CCCH-zinc finger protein, associated with mRNA metabolism were identified as differentially expressed (Supplementary Tables 2 and 3). Our LC-MS analysis thus revealed the presence of several key proteins involved in shikimate/chorismate pathway such as 1-Deoxy-D-xylulose-5-phosphate reductoisomerase, aldolase-type TIM barrel family protein, 5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase, phenylalanine ammonia-lyase, chalcone-flavone isomerase, chalcone synthase, chorismate synthetase, chorismate mutase, O-methyltransferase and tryptophan synthase in phenylalanine-treated tissues (Table 2). These are the key enzymes in the phenylpropanoid pathway and may be involved in the biosynthesis of secondary metabolites in *G. superba*.

4. Discussion

4.1. Profile of leaf metabolome

Medicinal plants are a source of a wide spectrum of secondary plant products synthesized from different metabolic pathways by enzyme catalyzed reactions (Lu et al., 2016). Metabolomics deals with the large-scale study of molecules such as primary and secondary metabolites in any part of the cell, tissue or an organism. Together, the study of these molecules and their interactions in different species is commonly known as metabolomics. Mercuric chloride has been used as a good elicitor and it induces so many plant secondary metabolites. Tomiyama and Fukaya (1975) observed that significant quantities of rishitin accumulate in tuber cultures when treated with mercuric chloride. Gustine and Moyer (1981) reported 3.15 mM $HgCl_2$ induces phytoalexins in callus tissue cultures of *Canavalia ensiformis*, *Medicago sativa*, and various species of *Trifolium*. Kasparová et al. (2007) reported that 1 μmol concentration mercury chloride showed strong elicitation on *Trifolium pratense* L. suspension cultures. Mercuric chloride accumulates 1300% more yield of daidzein, 375% more yield of genistein, and 300% more yield of genistein in cultured cells in comparison with control cultures of *Trifolium*. Thus, mercuric chloride has been used as a good elicitor to induce many plant secondary metabolites including our study on colchicine accumulation in morphogenic cultures of *Gloriosa superba* (Jawahar et al., 2018). This has prompted us to use it as an elicitor to find out the metabolites especially bioactive compounds in this plant. Foliar spray of mercuric chloride is usually transported to the roots and other parts of the plants through phloem tissues (Beauford et al., 1977; Suszcynsky and Shann, 1995; Patra and Sharma, 2000; Millhollen et al., 2006). In the present study, though a large number of leaf metabolites were noticed belonging to different pathways, only some of the key secondary metabolites that appear to be important have been listed. Metabolite profiling is a much-needed tool to authenticate the medicinal plants for screening, identification and subsequent validation and commercialization (Roessner et al., 2001). Using GC-MS method, important metabolites were identified in medicinal plants earlier. These metabolites are produced in ultra-low quantities in plants. Further, isolation of such compounds in pure form is laborious and cumbersome. Accordingly, only few compounds like colchicine, 3-demethylcolcine, gloriosine, 3-demethylgloriosine were purified and their spectra studied (data not shown since the methods are to be patented), but not all. Casuga et al. (2016) identified anticancer compounds such as propanetriol, α-sitosterol in leaves of *Broussonetia luzonica* by GC-MS analysis. Quercetin 7,3′,4′-trimethoxy and ergost-5-en-3-olthese are the two important anti-microbial compounds identified by GC-MS in the plant *Drynaria quercifolia* (Rajesh et al., 2017). In the present study, many functional and important secondary metabolites have been identified using GC-MS. Estragole (1-allyl-4-methoxybenzene) is a natural scent, mostly found in fennel herbal teas and a constituent of basil oil (Gori et al., 2012). Raffo et al. (2011) quantified estragole in fennel by GC-MS method. N-Methylloline is a loline alkaloid and acts as natural insecticide and also associated with herbivore stress tolerance (Schardl et al., 2007; Pan et al., 2014). Since symbiotic fungi are known to produce loline alkaloids, it is not clear whether this alkaloid is of plant or symbiotic fungal origin (Schardl et al., 2007). *G. suberba* harbors endophytic fungi (Shobha et al., 2019). Mesitylene, a precursor to diverse fine chemicals has been discerned in the present study. Anonaine is another alkaloid found in leaves of *G. superba*. It has been detected in several

species of Magnoliaceae, Annonaceae and found to have antiplasmodial, antifungal, anticancer, antidepression, antiinflammatory and vasorelaxant activity (Li et al., 2013). Another intriguing, toxic metabolite known as 3-hydroxykynurenine has been identified which can exert dual actions in the central nervous system. It is a tryptophan derivative, and induces oxidative damage and cell death, hence it has been hypothesized to play a vital role in neurological and psychiatric disorders (Colin-Gonzalez et al., 2013). Aphidicolin has been unearthed in the present study, which is a tetracyclic diterpene compound used as antibiotic and also has antiviral and antimitotic properties (Dalziel et al., 1973). Octyl salicylate is another good metabolite noticed in the present study. It is a derivative of salicylic acid and is used in cosmetics as a UV absorbent (Klessig et al., 2016). In GC-MS analysis, two pigment compounds quinacridone and β-carotene were also discovered. Butibufen is an important non-steroidal agent detected (Table 1). It is an anti-inflammatory, analgesic and antipyretic compound, which displayed activity within the potency range of ibuprofen. Hence, butibufen appears to be a potential compound and can be used in the treatment of rheumatic diseases (Aparicio, 1977). Austricin, is a sesquiterpene lactone compound, first isolated by Zitterl-Eglseer et al. (1991) in *Achillea setacea*. These authors and also Benedek et al. (2007) found that austricin has antiphlogistic (anti-inflammatory) activity. Octahydrocoumarin (mixture of *cis* and *trans*) is another important compound identified in leaves of *G. superba* in the present study for the first time, and it serves as a replacement for toxic coumarin. An antimalarial compound, jacaranone has also been noticed in the leaves (Table 1). Gachet et al. (2010) identified jacaronone in the extracts of *Jacaranda glabra* and identified jacaranone-derived glucosidic ester activity against *Plasmodium falciparum*. Bonducellin was first isolated from *Caesalpinia bonducella* and *Caesalpinia pulcherrima* which acts as an inhibitor of multidrug resistance efflux pump (McPherson et al., 1985). It is a flavonoid compound detected in the present study which has excellent anti-viral property (Asadipour et al., 2017). Identification of all these metabolites from leaves of *G. superba* helps us in validating these compounds against many diseases and make great progress in pharmaceutical industry. The study may help to produce some of these bioactive compounds on an industrial scale. However, quantification of these metabolites and their toxicological effects need to be studied.

4.2. Analysis of leaf proteome of G. superba using LC-MS

To enhance the colchicine and gloriosine contents, it is vital for us to find out the proteins or enzymes implicated in the biosynthetic pathway (s). The knowledge about the biosynthetic pathway of colchicine, gloriosine and colchicosides is incomplete. Also, genomic sequence information in *G. superba* is scarce or totally absent. So, the effective strategy to dissect out or to find out the proteins involved in the colchicine biosynthetic pathway is to feed the plant with a remote precursor like that of phenylalanine and sort out the differentially expressed proteins between the control and precursor-treated tissues. The aromatic amino acid phenylalanine was used to improve colchicine content in morphogenic cultures of *G. superba* (Jawahar et al., 2018). This has prompted us to use it as a foliar spray for activating the biosynthesis of biologically active compounds in *G. superba*. The use of biostimulants is common in tea, basil, and rosemary (Thomas et al., 2009; Azza and Yousef, 2015; Nia et al., 2016). Phenylalanine plays an important role in shikimate pathway and also secondary metabolites. There are general amino acid transporters that transport amino acids from leaves. Else, amino acids can be transported *via* phloem into the root tissues. Though such experiments were not performed in the present study, such a possibility cannot be ruled out. A comparative proteome analysis between control and precursor-treated tissues certainly reveals the critical information involved in the proteome. Such an analysis associated with *Alternaria alternata* infection in *Withania somnifera* leaf was earlier carried out by Singh et al. (2017) and it revealed the differential expression of many proteins. It has been noticed that an understanding of the protein network and the differentially expressed proteins in medicinal plants and their controlled regulation is highly crucial for the identification of the pathway enzymes involved in bioactive compound synthesis (Hashiguchi et al., 2017). Based on the functional annotation, the proteins were categorized into different groups.

Control leaves showed higher number of proteins compared to phenylalanine-treated tissues. This could be because that precursor enhances the activities of the specific pathway enzymes which requires allocation of energy and metabolic resources. Therefore, the number of proteins detected could be less in precursor-treated tissues compared to controls. Some of the proteins noticed in control leaves are serine hydroxymethyltransferase, involved in nematode resistance in plants (Kandoth et al., 2017), pentapeptide repeat-containing protein necessary for providing surface to mediate protein-protein interactions for carrying out many functions (Sharma and Pandey, 2016), XCT, a circadian time keeper (Xu et al., 2017), GALK protein, a mevalonate/-galactokinase family protein, medium chain triglyceride proteins which may play a role in colchicine biosynthesis, acyl-CoA-binding protein associated not only in the intracellular transport, but also in protection, and pool formation of acyl-CoA esters (Lung and Chye, 2016), and tetratricopeptide repeat (TPR-like) superfamily protein associated with hormone signaling (Schapire et al., 2006). These also act as intermediates and regulators in lipid metabolism and cellular signaling. Some of these proteins may be associated with colchicine or gloriosine biosynthetic pathway. An isoprenoid biosynthetic pathway associated 2-C-methyl-D-erythritol-4-phosphate cytidyltransferase (MCT) enzyme that influences the pathway and a quinone oxidoreductase related to quinone biosynthetic pathway have been noticed. Quinone oxidoreductase reduces free radical load in cells and stabilizes a number of cellular regulators including p53 (Pey et al., 2019). Soybean TGACG-motif binding factor 1 (STF 1), an important basic leucine zipper protein involved in plant growth and developmental processes (Shin et al., 2016), (E)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase (LytB/IspH) related to isopentenyl diphosphate biosynthetic process, and plastid-lipid associated protein (PAP) related to carotenoid biosynthesis have been detected. These proteins appear to play crucial roles in several of the biosynthetic pathways associated with secondary metabolites.

Unique proteins have been observed in the leaf proteome. Interestingly, several of them have been found involved in the shikimate biosynthetic pathway, involved in secondary plant product biosynthesis. Phenylalanine ammonia-lyase, an enzyme that converts phenylalanine into trans-cinnamic acid, an important enzyme in the biosynthetic pathway of shikimate and crucial in defense response in microbial pathogens (Kim and Hwang, 2014), chalcone-flavone isomerase involved in the biosynthesis of flavonoids (Cheng et al., 2018), chalcone synthase that helps in iterative decarboxylative condensations of malonyl units with a Co-A linked starter molecule and produce a wide spectrum of important bioactive compounds in plants (Abe and Morita, 2010), and chorismate synthase which catalyzes the conversion of 5-enolpyruvylshikilate 3-phosphate to chorismate, the common precursor in the biosynthesis of aromatic compounds including p-aminobenzoic acid. Chorismate gives rise to tryptophan from which the hormone IAA is synthesized (Mano and Nemoto, 2012). Besides hormones, many secondary plant products with multiple biological functions are synthesized from aromatic amino acids and chorismate (Bartel, 1997; Vogt, 2010). Such important enzymes have been detected in leaf proteome, indicating that leaf is able to synthesize the bioactive compounds. Similarly, TETRATRICOPEPTIDE THIOREDOXIN-LIKE (TTL) proteins are positive regulators of brassinosteroid signaling pathway (Amorim-Silva et al., 2018), and may be associated with colchicine biosynthesis *via* brassinosteroid signaling. AGO9 protein, linked with plant development (Schmidt et al., 2015), a methylenetetrahydrofolate reductase connected with methionine biosynthesis as well as infection-related morphogenesis, especially when rice is infected by blast fungus (Yan et al., 2013), *O*-methyltransferase-like protein which

methylates oxygen atom of many secondary metabolites including phenylpropanoids, flavonoids and alkaloids (Lam et al., 2007), zinc finger C-x8-C-x5-C-x3-H type family protein especially cysteine-3-histidine (CCCH)-type zinc finger proteins play vital roles in mRNA metabolism, growth and development (Bogamuwa and Jang, 2014). Similarly, methylthioribose kinase1 (MTK1), a phloem-specific expression of the Yang cycle gene *5-METHYLTHIORIBOSE KINASE1* in *Plantago* and *Arabidopsis*, and recycles in leaves (Pommerrenig et al., 2011) has been detected. Several of the proteins induced by phenylalanine in the leaves of *G. superba* appear to be specific and may be critical for the biosynthesis of secondary plant products including colchicine, gloriosine and colchicosides. It is predicted that these proteins may play a vital role in enhancing the colchicine content. Thus, the application of proteomic approach is certainly crucial where genomic and proteomic sequence information is scarce in the medicinal plants. Present study identifies some of the important enzymes involved in the colchicine/-secondary plant product biosynthetic pathway. Corresponding genes if cloned and overexpressed in *G. superba*, it might help in intensifying our efforts to increase the secondary metabolite accumulation especially colchicine, lumicolchicine, 3-demethylcolchicine, gloriosine, 3-demethylgloriosine and others. Surprisingly, a large number of proteins have been recognized with unknown functions. Some of them may be associated with the biosynthesis of secondary metabolites (listed in the Supplementary Tables 2 and 3). The dataset reported here helps us in better understanding the leaf proteome of *G. superba*. Such a comparative proteomic analysis reveals the association of many key proteins in the pathway of colchicine as well as other secondary metabolites. However, further investigations of both identified and unidentified proteins, cloning the corresponding genes help us in unraveling the biosynthetic pathways and subsequently for augmenting the bioactive compounds such as colchicine and gloriosine in the medicinally important *G. superba* plant.

5. Conclusions

G. superba is a tropical medicinal plant that produces alkaloids of medicinal importance like colchicine, gloriosine, colchicosides and others, but their biosynthetic pathways are little known. Also, if this plant also produces other important bioactive compounds or not is unknown. In the present study, several bioactive compounds have been detected for the first time from this plant using GC-MS analysis. Also, proteins implicated in the biosynthetic pathway of colchicine/secondary metabolites have been detected by proteomic analysis. Unknown proteins detected in the pathway may hold key in detecting the enzymes implicated in the colchicine biosynthetic pathway. The work can pave the way for characterization of the proteins, cloning of the corresponding genes and their overexpression for enhanced production of some of the key bioactive compounds.

Author contribution statement

PBK has conceived and conceptualized the experiments. JG has performed the experiments under the supervision of PBK. JG and PBK have analyzed the data. JG, HKP, SAK, JN, DM, PS, JCh, PB, RP, PBK have prepared the manuscript. JG, HKP, YD, JCh, PS, RP, PBK have reviewed, edited and refined the manuscript. PS, JG and PBK have revised and others have approved the MS.

CRediT authorship contribution statement

PBK has conceived and conceptualized the experiments. JG has performed the experiments under the supervision of PBK. JG and PBK have analyzed the data. JG, HKP, SAK, JN, DM, PS, JCh, PB, RP, PBK have prepared the manuscript. JG, HKP, YD, JCh, PS, RP, PBK have reviewed, edited and refined the manuscript.

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Declaration of competing interest

All authors declare that they have no known competing interests that can appear to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2022.114557.

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