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1 *Research Article for Process Biochemistry*

2 **Enhanced production of lignans and neolignans in chitosan-treated flax**
3 ***(Linum usitatissimum L.)* cell cultures**

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19 **Abstract**

20 *Linum usitatissimum* is a source of pharmacologically active lignans and neolignans. An effective
21 protocol has been established for the enhanced biosynthesis of lignans and neolignans in cell cultures
22 of *Linum usitatissimum* by using chitosan addition. Gene expression analysis of monolignols (*PAL*,
23 *CCR* and *CAD*), lignans (*DIR*, *PLR* and *UGT*) and neolignans (*PCBER*) biosynthetic genes by RT-
24 qPCR as well as monolignol biosynthetic *PAL*, *CCR* and *CAD* enzyme activities evidenced a
25 stimulation following chitosan treatment. Validated reverse phase high-performance liquid
26 chromatography coupled to diode array detection was used to quantify secoisolariciresinol
27 diglucoside (SDG) and lariciresinol diglucoside (LDG), dehydrodiconiferyl alcohol glucoside (DCG)
28 and guaiacylglycerol- β -coniferyl alcohol ether glucoside (GGCG) showed that chitosan treated cell
29 cultures had better accumulation of these metabolites. Maximum enhancements of 7.3-fold (28 mg/g
30 DW) occurred for LDG, 3.5-fold (58.85 mg/g DW) in DCG and while the least enhancement of 2-
31 fold (18.42 mg/g DW) for SDG was observed in 10 mg/L chitosan treated cell cultures than to
32 controls. Furthermore, same concentration of chitosan also resulted in 1.3-fold increase in
33 antioxidant activity. Compared to the lignans and neolignans accumulations observed in wild type
34 and RNAi-PLR transgenic flaxseeds, chitosan-treated cell cultures appeared to be a very effective
35 production system for these compounds.

36 **Keywords:** *Linum usitatissimum*; chitosan; lignans; neolignans; elicitation; cell culture.

37 **1. Introduction**

38 Flax (*Linum usitatissimum* L.) is used for more than 10,000 years in terms of consumption and
39 cultivation [1, 2]. More recently, the detection of polyphenols (lignans, neolignans) has opened
40 new dimensions for flax in pharmaceutical and nutraceutical industries to be used for preventing
41 cancers, cardiovascular disorders, diabetes, lupus nephritis and other disorders [3]. Along with
42 lignans, *L. usitatissimum* is considered a good source for accumulation of neolignans which are
43 potential candidates in anti-inflammatory and antifungal applications [4].

44 Haworth (1942) coined the term lignan for the first time to define a class of dimeric
45 phenylpropanoids in which two units C₆-C₃ are linked by the central C₈ [5], lignans are a group
46 of phytochemicals (polyphenols) which are formed by connection of 2 cinnamyl alcohols [6].
47 Whereas Gottlieb (1978) speculated that molecules having 2 phenylpropanoid parts attached in a
48 different fashion, such as C5-C5' should be called neolignans [7]. Lignans and neolignans are
49 structurally made of two propyl benzene units (Fig 1). In lignans these units are associated by a
50 β-β' bond while this substitution pattern is different in neolignans [8]. Lignans are reported in
51 more than 65 families of vascular plants, and are extracted from almost all parts of a plant [9].

52 Pharmacological activities of lignans have been demonstrated in various experimental models.
53 Lignans induce the synthesis of 2-hydroxy estrogen in females which has a potential role in
54 deterrence of cancer [10]. Lignans are believed to be responsible for inhibiting the growth of
55 different human prostate cancer cell lines [11, 12] According to reported data [13] when a dose
56 of 10 mg/kg of lignans administered subcutaneously in athymic mice, have diminished the
57 proliferation of 201 human colon cancer cell lines. Flax was also reported to abate the elevated
58 blood glucose concentration in post meal duration [14, 15]. A study conducted on a group of
59 females in which they were fed with ground flax seeds 50 g/day, after four weeks their blood
60 glucose level has been significantly reduced [16].

61 The production of secondary metabolites in different plant culture techniques using
62 biotechnological interventions is an attractive substitute to the isolation from whole plant
63 materials [16]. Using plant cell cultures coupled with elicitation strategies such as introduction of
64 physical and chemical entities to the cell culture *per se* or growth conditions, as a biological
65 platform for the synthesis of secondary metabolites is a promising approach for the sustainable
66 production in modern medicinal and aromatic industry [17].

67 Due to growing interests on lignans and neolignans, alternate platforms for their production other
68 than conventional plant cultivation are needed. Cell and tissue culture techniques could be used
69 for the enhanced biosynthesis of these metabolites. Chitosan is a polycationic β -1,4 linked D-
70 glucosamine polymer known to act as a bioactive antifungal agent [18] though the elicitation of
71 pathogenesis-related proteins in the host [19] and stimulation of phytoalexin production [20].
72 Chitosan has not been exploited previously for the accumulation of these valuable lignans and
73 neolignans in cell cultures of flax. Therefore, in current study effect of chitosan is evaluated
74 based on its impact on the biosynthesis and dynamics of these anti-cancer lignans and
75 neolignans. A validated reverse-phase high performance liquid chromatography (RP-HPLC
76 DAD) was used for the quantification of lignans and neolignans. As per our knowledge, this is
77 the first report on enhanced biosynthesis of lignans (secoisolariciresinol diglucoside (SDG) and
78 lariciresinol diglucoside (LDG)) and neolignans (dehydrodiconiferyl alcohol diglucoside (DCG)
79 and guaiacylglycerol- β -coniferyl ether diglucoside (GGCG)) using chitosan as a stimulator of
80 lignans and neolignans production in flax cell cultures. Current research has a potential to be
81 scaled up to bioreactor levels for the feasible biosynthesis of these commercially important
82 metabolites.

83 **2. Materials and Methods**

84 ***2.1 Plant materials***

85 Flax seeds (brown variety) were collected from malakand division hills (natural habitat) Khyber
86 Pakhtunkhwa, Pakistan. The flax cultivar Barbara was supplied by Coopérative Terre de Lin (St
87 Pierre le Viger, France). The generation of the RNAi plants and the homozygous transgenic lines
88 were obtained as previously described [21].

89 For germination, seeds were surface sterilized using 0.1% mercuric chloride and 70% ethanol for
90 30 and 60 seconds respectively, subsequently washed 3 times with dH₂O (autoclaved water) and
91 dried using sterilized filter paper sheet. The sterilized seeds were inoculated on MS (Murashige
92 and Skoog basal Media) following the protocol described earlier [22]. Growth room temperature
93 was maintained at 25±2°C and 16/8 h (light/dark) photoperiod having 40 µmol/m²/s light
94 intensity for all cultures was adjusted.

95 ***2.2 Establishment of callus cultures***

96 For establishing callus, stem explant was excised from 4 week-old plantlets (*in vitro*). Explant
97 was inoculated using the optimized protocol [23]. Stem explants (~1.0 cm long) were inoculated
98 on MS media modified with NAA (α -naphthalene acetic acid) 1.0 mg/L, sucrose 30g/L, agar
99 8g/L and pH was adjusted to 5.6 using 2 M NaOH (sodium hydroxide) prior to autoclaving
100 (121°C, 20 min) and kept at above said conditions for growth.. The explant derived calli were
101 subcultured after 4 weeks to ensure 100% callogenesis. The experiment was executed in
102 triplicates. Chemicals used in experimentation were all purchased from Sigma Corporation
103 (USA).

104 **2.3 Cell suspension culture**

105 For cell suspension culture, the 3-weeks old calli (subcultured) were inoculated in Erlenmeyer
106 flasks (250 ml) containing liquid MS media with sucrose (30g/L) and 1.0 mg/L NAA. Flasks
107 were kept on gyratory shaker ($25 \pm 2^\circ\text{C}$) at constant agitation (120 rpm) in 16/8 h (light/dark)
108 photoperiod with light intensity of $40 \mu\text{m}^2/\text{s}$ for 15 days. For inoculum preparation 250 ml
109 Erlenmeyer flasks containing 100 ml Media (each flask) were used.

110 **2.4 Elicitor preparation and treatment**

111 Chitosan ($\text{C}_6\text{H}_{11}\text{NO}_4$)_n (deacetylating grade: 70-85%) was used as an elicitor. Chitosan was
112 dissolved in 0.1% acetic acid at 50°C with continuous stirring for 5 hours. For elicitation liquid
113 MS media was prepared having sucrose 30g/L and NAA 1.0 mg/L. To that media different
114 concentrations (0.1, 0.5, 1.0, 10, 20, 50, 100, 200 and 500 mg/L) of chitosan were added. Prior to
115 autoclaving (121°C , 20 min), pH was set to 5.6. Experiment was performed in Erlenmeyer flasks
116 (100 ml) containing 40 ml of media. To each flask 10 ml of inoculum (15 days old fine cells) was
117 added. Triplicate flasks were used for each concentration. Cultures were kept on gyratory shaker
118 ($25 \pm 2^\circ\text{C}$) at constant agitation (120 rpm) in 16/8 h (light/dark) photoperiod. MS media
119 containing sucrose 30g/L and NAA 1.0 mg/L without any elicitor was used as control.
120 Observation of the experiment and tracking data of the growth dynamics and secondary
121 metabolites accumulation was executed with the gap of 5 days interval for 50 days.

122 **2.5 Biomass determination**

123 To determine the fresh weight (FW), respective cell cultures were harvested through filtration
124 using $0.45 \mu\text{m}$ stainless steel sieve (Sigma), to remove any attached media, cell cultures were
125 gently washed using dH_2O . To remove the excess water, cells were gently rubbed on filter paper

126 sheets and cells were weighed for FW. To obtain dry weight (DW) cell were dried in oven (45°C,
127 48 hours).

128 **2.6 RNA extraction**

129 Total RNA was extracted from 100 mg of liquid nitrogen ground frozen tissues using the Plant
130 GeneJet RNA Purification Kit (Life Technologies) according to supplier instructions.
131 Quantification of RNA was each performed using a fluorometer, with the Quant-iT RNA Assay
132 Kit (Invitrogen) adapted for the Qubit fluorometer, according to the manufacturer's protocol.

133 **2.7 RT-qPCR analysis**

134 Reverse transcription was performed using a First-strand cDNA synthesis kit (Thermo).
135 Quantitative PCR was performed in 96-well plates with a PikoReal real time PCR system
136 (ThermoFisher) using the DyNAmoColorFlash SYBR Green qPCR Kit (ThermoFisher). Each
137 reaction was made in 10 µL (1 µM of each primer pairs, 0.5 µL diluted cDNAs and 2 × SYBR
138 Green mix). All PCR reactions were carried out with the following protocol: 7 min at 95 °C, 40
139 cycles of 10 s at 95 °C, 10 s at 55 °C and 30 s at 72 °C. The specificity of the amplified product
140 was confirmed for each primer pair, via a melting curve. Data analysis was carried out with
141 Pikoreal software. Three biological replicates and two technical repetitions were performed for
142 each sample. Relative transcript levels were obtained using specific primers (Table S1), designed
143 with Primer3 software , and normalized via the comparative $\Delta\Delta Cq$ method using two validated
144 housekeeping reference genes selected by Huis et al. ²⁴: *LuCYC* encoding for cyclophilin and
145 *LuETIF5A* encoding for Eukaryotic Translation Initiation Factor 5A. Results were presented on a
146 heat map format using the MeV software computed with a hierarchical clustering analysis (HCA)
147 representation employing the Euclidean distance as a clustering method with a complete linkage
148 clustering as parameters.

149 **2.8 PAL activity**

150 Soluble proteins were extracted from 1.5 g of fresh frozen tissue by homogenization in 3 ml of
151 0.1 M sodium borate buffer (SBB) pH 8.8 containing 10 mM β -mercaptoethanol and kept on ice
152 for 30 min. After centrifugation (10 min, 16,000 g) at 4°C the supernatant was collected and used
153 in the assay. Protein concentrations were quantified using a Qubit fluorimeter and the Quant-iT
154 Protein Assay Kit (Invitrogen) according to the manufacturer's protocol.

155 PAL specific activity was assayed spectrophotometrically by monitoring the production of *trans*-
156 cinnamate at 290 nm as previously described [25]. The reaction mixtures contained 50 μ g proteins
157 and 50 mM l-phenylalanine in 5 ml of SBB (pH 8.8) and were incubated at 40°C.

158 **2.9 CCR and CAD activities**

159 Soluble proteins were extracted by grinding 1.5 g of fresh frozen tissue in a pre-chilled mortar
160 containing a cold extraction buffer (0.1 M Tris-HCl pH 7.5, 5% w:v ethylene glycol, 2% w:v
161 polyvinyl polypyrrolidone and 0.1 M β -mercaptoethanol). The crude extract was centrifuged (10
162 min, 16,000 g) at 4°C and the supernatant was used in the assays. Protein concentrations were
163 quantified using a Qubit fluorimeter and the Quant-iT Protein Assay Kit (Invitrogen) according
164 to the manufacturer's protocol.

165 CCR specific activity was determined spectrophotometrically as described previously [26] using
166 feruloyl-CoA as substrate. Feruloyl-CoA was synthesized according to the optimized protocol
167 reported previously [27].

168 CAD specific activities were determined spectrophotometrically as described earlier [28]

169 **2.10 Plant extracts preparation**

170 Extracts of cell cultures were prepared following the protocol described previously [29] with
171 slight modifications. Dried cell cultures were grounded finely and mixed with methanol in 1:5
172 (100 mg in 500 μ l). All mixtures were sonicated for 30 minutes and followed by vortexing for 5
173 minutes, this process is repeated twice. These mixtures were then kept on gyratory shaker ($25 \pm$
174 2°C) at constant agitation (80 rpm) for 24 hours. The sonication and vortexing were repeated and
175 finally the mixtures were centrifuged at 10,000 rpm for 10 minutes and supernatant was collected
176 and subsequently syringe filtered and stored at 4°C to be analyzed. For the extraction of lignans
177 and neolignans from cell cultures of flax the protocol described by [4] was followed. Extraction
178 of lyophilized cells was carried out using 80% v/v (20 ml) methanol (aqueous) using ultra
179 sonication (USC1200TH) having 30 kHz frequency for 1 hour at $25 \pm 2^{\circ}\text{C}$. The mixture was
180 subjected to centrifugation. The supernatant was extracted and evaporated (40°C) and followed
181 by suspending (4 h at 40°C) in 0.1 M (1 ml) 4.8 pH buffer (citrate-phosphate). In order to release
182 the aglycones the buffer was equipped with β -glucosidase from almonds (5 units/ml; Sigma
183 Adrich).

184 **2.11 Determination of total phenolic production**

185 Total phenolic content (TPC) was evaluated according to the previously reported protocol [22]
186 using Folin-Ciocalteu (FC) reagent. Twenty microliter of sample extract and 90 μ l of FC reagent
187 were mixed and diluted 10x using dH₂O followed by incubation for 5 minutes at $25 \pm 2^{\circ}\text{C}$.
188 Sodium carbonate (6%, w/v) was added to the mixture. Absorbance was recorded by Absorbance
189 Microplate Reader (ELx808 BioTek, USA) at 725 nm. In order to plot the calibration curve
190 ($R^2=0.967$), 0-40 μ g/ml of gallic acid used as standard. TPC was expressed as equivalents of
191 gallic acid (GAE)/g of dry weight). Equation (I) was used to calculate the total phenolic
192 production (TPP).

193 Total phenolic production $\frac{mg}{l} = DW \left(\frac{g}{l}\right) \times TPC \left(\frac{mg}{g}\right)$ _____ (I)

194 TPP was expressed in mg gallic acid/l.

195 ***2.12 Quantification of lignans and neolignans by RP-HPLC***

196 The extract was centrifuged for another time and prior to injection the resultant supernatant was
197 filtered (0.45 μ m). The quantification of Lignans and neolignans aglycones was carried out using
198 RP-HPLC by Varian liquid chromatographic system equipped with a Varian Prostar 230 pump, a
199 Metachem Degasit, a Varian Prostar 410 autosampler and a Varian Prostar 335 Photodiode Array
200 Detector (PAD) and controlled by Galaxie version 1.9.3.2 software. For separation the method
201 described by [21] was followed using Purospher RP-18 (Merck) column (250 \times 4.0 mm i.d.; 5
202 μ m). Calibration curves were used to perform the quantification of Lignans and neolignans. In
203 order to compare the results with published literature easily, results were expressed as mg of
204 glycosides like SDG, LDG, DCG and GGCG equivalent per gram of dry weight. Molecular
205 weight of compounds was used for conversion. Equation (II) was used to calculate the
206 productivity of lignans and neolignans:

207 Lignans and neolignans productivity $\frac{mg}{l} = DW \left(\frac{g}{l}\right) \times \text{accumulation} \left(\frac{mg}{g}\right)$ _____ (II)

208 ***2.13 Method validation***

209 The quantification was performed with standard calibration curves obtained using five standard
210 dilutions ranging from 50 to 1000 μ g/ml. Each standard solution was injected in triplicate.
211 Arithmetic means of each triplicate were calculated. The linear regression equations were carried
212 out by plotting the peak areas against the injected amounts of standard compounds. The linearity
213 was demonstrated by coefficient of determination (R^2). The limits of detection (LOD) and the
214 limits of quantification (LOQ) were determined based on the signal-to-noise ratio (S:N) of

215 approximately 3:1 and 10:1, respectively. Accuracy was evaluated by measuring recovery rates.
216 Dried flax cell cultures were homogenized and separated into two parts of equal mass, one of
217 which was spiked with a known volume of stock solutions. The spiked and non-spiked parts were
218 analyzed by HPLC in triplicate following the procedures described. The recovery rates were
219 calculated according to the following formula:

220
$$\text{Recovery rate} = (\text{amount in spiked part} - \text{amount in non-spiked part}) / (\text{spiked amount}) \times 100.$$

221 The method precision and stability were evaluated by determining the intraday and interday
222 variations respectively, which were calculated from data obtained by the repeated injections of
223 standard solutions. The intraday variation was obtained by five replicates in a day and the
224 interday variation was determined by three injections over three continuous days. Retention times
225 and peak areas were assessed. The precision was further checked by measuring the repeatability
226 using five continuous injections of the same extracted sample. The precision was expressed as
227 the relative standard deviation (RSD, %).

228 ***2.14 Determination of antioxidant activity***

229 For evaluation of antioxidant activity 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical
230 scavenging assay (FRSA) was performed according to the reported protocol [30] with minor
231 alterations. Twenty microliter of sample extract mixed with 180 μl of DPPH (3.2 mg/100 ml
232 methanol) and the resultant mixture was incubated at $25 \pm 2^\circ\text{C}$ for 60 minutes and followed by
233 the addition of 160 μl of dH_2O . Absorbance was recorded by Absorbance Microplate Reader
234 (ELx808 BioTek, USA) at 517 nm. In order to plot the calibration curve ($R^2=0.989$) methanolic
235 extract of DPPH solution (0.5 ml) was used as standard. The following equation (III) was used to
236 calculate the free radical scavenging activity as percentage of discoloration of DPPH.

237 Free radical scavenging activity (%) = $100 \times \left(1 - \frac{Ac}{As}\right)$ _____ (III)

238 where AC stands for absorbance of the solution when sample extract was mixed at a specific
239 concentration, and AS denotes the absorbance of standard (DPPH solution).

240 Ferric reducing antioxidant power (FRAP) was measured as described by [31] with some
241 modifications. Briefly, 10 μ l of the extracted sample was mixed with 190 μ l of FRAP (10mM
242 TPTZ; 20mM FeCl₃ 6H₂O and 300mM acetate buffer pH3.6; ratio 1:1:10 (v/v/v)). Incubation
243 lasted 15min at room temperature. Absorbance of the reaction mixture was measured at 630nm
244 with a BioTek ELX800 Absorbance Microplate Reader. Assays were made in triplicate and
245 antioxidant capacity was expressed as Trolox C equivalent antioxidant capacity (TAEC).

246 ***2.15 Statistical analysis***

247 All the experiments were carried out in a synchronized manner and repeated twice. Each
248 treatment was consisted of triplicates. All the mean values were analyzed using Pareto analysis of
249 variance (ANOVA). The significance at P<0.05 was determined by Duncan's multiple range test
250 (DMRT, Windows version 7.5.1, SPSS Inc., Chicago) Graphs were generated using Origin pro
251 (8.5).

252 **3. Results and Discussion**

253 ***3.1 Influence of different concentrations of chitosan on biomass accumulation and (neo)*** 254 ***lignans biosynthesis***

255 The effects of different concentrations of chitosan (0.1-500 mg/L) were evaluated on cell cultures
256 of *L. usitatissimum* for biomass accumulation (FW and DW). Data recorded for chitosan-treated
257 (each concentration) and control cell cultures after 30 days. All treatments were also analyzed for

258 its total phenolic content (TPC). Maximum biomass accumulation (both fresh weight (FW) and
259 dry weight (DW)) occurred at 10 mg/l of chitosan (hereafter called CHI-10) as compared to
260 control. Morphological changes induced by different concentrations of chitosan (0.1-500 mg/L)
261 treatments of flax cell suspensions are presented in Fig. 2A. The cell culture treated with 10 mg/l
262 chitosan also presented the highest accumulation of TPC (Table 1) evidencing an activation of
263 phenylpropanoid metabolism.

264 Following this first evaluation, to assess the impact of chitosan on phenylpropanoid pathway,
265 transcript accumulation of monolignols as well as lignans and neolignans (i.e., monolignol-
266 derived products) biosynthetic genes by RT-qPCR (Fig. 3) and enzyme activity of key enzymes
267 involved in monolignols biosynthesis (Fig. 4) in flax cell suspensions treated with 0 (control,
268 CTL), 1 (CHI-1), 10 (CHI-10) and 100 (CHI-100) mg/l chitosan were evaluated.

269 The expression of i) monolignols biosynthetic genes *LuPAL* encoding for a phenylalanine
270 ammonia lyase, *LuCCR* encoding for a cinnamoyl-CoA reductase and *LuCAD* encoding for a
271 cinnamyl alcohol dehydrogenase, previously characterized in flax cell suspension challenging
272 with elicitors [32], ii) lignans biosynthetic genes, encoding for biochemically characterized
273 enzymes, *LuDIR5* encoding for a (-)-pinoresinol forming dirigent protein [33, 34], *LuPLR1*
274 encoding for a (-)-pinoresinol/(-)-lariciresinol reductase [35, 36] and *LuUGT74S1* encoding for a
275 (+)-secoisolariciresinol uridine glycosyltransferase [37], and iii) neolignans biosynthetic genes
276 *LuPCBER* encoding for a phenylcoumaran benzylic ether reductase [35] (Fig. 3A), were
277 monitored after 8h, 24h and 48h after chitosan addition and their relative gene expression levels
278 (relative to control cell suspension) are presented in Fig. 3B. Here, chitosan treatment triggered
279 the expression of monolignols, lignans and neolignans biosynthetic genes. This gene expression
280 stimulation appeared to be dependent on the chitosan concentration applied with a highest
281 stimulatory effect observed with 10 mg/l chitosan treatment (CHI-10, Fig. 3B). The gene

282 expression kinetic also appeared specific to the metabolic steps thus highlighting the possibility
283 of a coordinated regulation of these metabolic pathways.

284 PAL, CCR and CAD specific activities were then monitored in control and chitosan-treated cell
285 suspensions during a 48h period following chitosan addition (Fig. 4). In a good agreement with
286 the gene expression analyses, a rapid, strong and dose-dependent stimulation of PAL activity was
287 observed in all treated cell cultures, reaching a maximum activation 24h after chitosan addition
288 (Fig. 4A). In the same way, a very similar rapid, transient and dose-dependent activation of both
289 CCR and CAD activities were observed for chitosan-treated cells (Fig. 4B, C). From these
290 enzyme activities data, it appeared that saturation occurred beyond 10 mg/l chitosan addition.

291 Activation of genes, at both expression and enzyme levels, during the early steps of the
292 phenylpropanoid pathway in response to biotic or abiotic stresses has already been reported in
293 other plant systems. The PAL enzyme catalyzes the entry point of L-phenylalanine into the
294 phenylpropanoid pathway. This enzyme is known to play a crucial role in plant defense
295 mechanisms and is presumably responsible for the increased carbon flux into this pathway
296 leading to an increased biosynthesis of defense/stress-related compounds deriving from this
297 phenylpropanoid pathway [38]. Chitosan induces the enzymatic defensive mechanisms in plants
298 by producing chitinases, pectinases and glucanases enzymes and stimulates plant's immunity
299 which results in enhanced accumulation of biomass while some reports suggest that chitosan is
300 also responsible for enhancing the availability and uptake of water as well as essential nutrients
301 by regulating the osmotic pressure of cells [39, 40]. In agreement with our results Mathew &
302 Sankar (2012) [41] also reported up to 3.5 times enhancement in biomass accumulation in
303 chitosan treated cell cultures of three species of *Ocimum* i.e. *O. basilicum*, *O. sanctum* and *O.*
304 *gratissimum*. Similar results were also reported by [42] for *O. basilicum* after application of

305 chitosan. From its polycationic β -1,4 linked D-glucosamine polymer chemical structure, chitosan
306 could be consider either as a biotic elicitor or fertilizer as a source of nitrogen and sugars [18].

307 Here, gene expression analyses and enzyme kinetics, confirmed the trend observed for TPC
308 measured in the corresponding cell suspensions (Table 1). From all these results, 10 mg/L
309 concentration of chitosan was selected for further investigation.

310 ***3.2 Effect of chitosan on biosynthesis and productivity of lignans and neolignans***

311 For determination of trends in growth kinetics and biomass accumulation data was monitored
312 periodically at an interval of 5 days for a total of 50 days (Fig. S1). The growth curves of cell
313 cultures of flax grown in 10 mg/L of chitosan exhibited nearly same degree of lag phase (10
314 days), log phase (exponential phase) starting from day 10 to day 30 comprised of 20 days and
315 followed by 10 days of stationary phase from day 30 to day 40. From day 40 onwards decline
316 phase was observed (Fig. 2B, Fig. S1).

317 Chitosan exhibited a positive influence on accumulation of total phenolic content (Fig. S2). Upon
318 analysis it was noted that highest TPC accumulation of 19.36 mg/g DW and a productivity of
319 315.89 mg/l were recorded at 30th day of inoculation. The probable explanation behind this
320 enhancement is that chitosan upon contact with plant cells induces antioxidant defense
321 mechanism, as a result plant synthesise phenolic compounds to scavenge harmful reactive
322 oxygen species (ROS) [43].Chitosan also has a possible key role in signaling pathways of
323 biological synthesis of phenolics [44]. The accumulated TPC in Flax cell cultures grown in 10
324 mg/L of chitosan is 2.1 times (19.36 mg/g DW) higher than control (9.1 mg/g DW) (Fig 5).
325 Similar findings were reported previously [45] who obtained a 2 fold increase in phenolic content
326 in chitosan elicited adventitious root cultures of *Morinda citrifolia*.

327 Lignans and neolignans are polyphenols formed via phenylpropanoid metabolic pathway from a
328 common precursor (the monolignol coniferyl alcohol). These polyphenolic compounds are
329 pharmacologically important [46].

330 To get further insight in their accumulation profile and kinetics, the main lignans (SDG and
331 LDG) and neolignans (DCG and GGCG) accumulated in flax cell cultures were quantified using
332 RP-HPLC method (Figure S3). For this purpose the HPLC-DAD method used to quantify these
333 lignans and neolignans, in the different plant materials analyzed in the present study, was
334 validated in term of precision, accuracy, stability and repeatability. Results of this validation are
335 presented in Table S2. Representation of the peak area and standard concentrations revealed high
336 linear correlations in the range of 50-1000 $\mu\text{g/mL}$. The linear regression of the 5-point calibration
337 graph showed a R^2 -value ranging from 0.9989 for LDG to 0.9998 for DCG, whereas the slope of
338 the standards covering the analytical range varied at most 1% relative standard deviation (RSD)
339 over a four weeks period. In term of limits of detection (LOD, $S/N = 3$) and limits of
340 quantification (LOQ, $S/N = 10$), values obtained were 3.4 ng and 10.6 ng for LDG, 2.7 ng and
341 9.2 ng for SDG, 3.1 ng and 10.0 ng for DCG, and 4.2 ng and 13.4 ng for GGCG, respectively.
342 The determination of the instrumental precision was realized by five injections of the same
343 sample. The chromatographic method used proved its precision (intraday precision) with RSD
344 values ranging from 0.45 (SDG) to 1.25 (LDG). The same sample was injected six times (0, 6,
345 12, 24, 48 and 72 h after its preparation) in order to evaluate the method stability (interday
346 precision). The small observed values for the RSD, from 0.84 (SDG) to 1.65 (GGCG) confirmed
347 the good stability of the extracted sample. Application of the whole extraction procedure three
348 times to the same batch of material allowed the verification of the high repeatability with RSD
349 values ranging from 0.99 (SDG) to 2.16 (LDG). The separation method accuracy measurement
350 was assessed with standard addition at three concentration levels (50%, 100% and 150%) and a
351 good recovery of the compounds ranging from 97.5% (DCG) to 102.5 (GGCG) was observed.

352 In current study, lignans (SDG and LDG) and neolignans (DCG and GGCG) were accumulated
353 in their glycosylated forms since aglycones were not detected before β -glucosidase hydrolysis
354 (data not shown). The maximum accumulation of lignans and neolignans were observed at day
355 30 and 35, respectively (Figure 5). All these current results are in agreement with previously
356 reported results [4, 19, 20]

357 Upon evaluation of growth dynamics it was noted that the maximum accumulation of SDG
358 occurred at 35th day (stationary phase) of inoculation (Fig 5A). Results revealed that maximum
359 accumulation of lariciresinol diglucoside (LDG) was observed at 40th day of inoculation in cell
360 cultures of *L. usitatissimum* (Fig 5B). The accumulation of LDG in late stationary phase is
361 showing its involvement in alleviating nutrients depletion-induced stress.

362 Furthermore, the maximum accumulation of dehydrodiconiferyl alcohol glucoside (DCG) was
363 observed at 30th day of inoculation in flax cell cultures (Fig 5C). Surprisingly, chitosan showed
364 inhibitory effects on the accumulation of guaiacylglycerol- β -coniferyl alcohol ether glucoside
365 (GGCG) (Fig. 5D). This observation will deserve future study in order to understand the partition
366 regulation of the monolignol into these different monolignol-derived products upon chitosan
367 treatment.

368 LDG and DCG showed growth-associated behavior, maximum accumulation observed with
369 enhanced biomass. However, highest accumulation of SDG in late stationery phase showed that
370 its biosynthesis was growth non-associated and its involvement if ameliorating stresses induced
371 by nutrient depletion. Similar enhancing effects of chitosan on accumulation of lignans and
372 neolignans have been reported [43].

373 From these data, discrepancies can be pointed in our results from the expression levels (Fig. 3),
374 enzyme activities (Fig. 4) and lignans and neolignans production (Fig. 5). First, the expression

375 levels shown in Fig. 3 suggested a very rapid response of gene expression to chitosan induction
376 after 8-24h of chitosan treatment. On the contrary, the lignans and neolignans production reached
377 the highest levels after 35 days, whereas the expression of related genes appeared to slightly
378 decrease after 48 h of treatment with the 10 mg/L chitosan treatment. This first discrepancy
379 between the accumulations of lignans and neolignans concentration and their biosynthetic gene
380 expression could result from the regulation of key genes involved in monolignol biosynthesis, as
381 it has been reported for the effects of ABA and GA on lignin accumulation by Kim et al. [50] and
382 Zhao and Dixon [51] as well as for the accumulations of lignans and neolignans [52]. For
383 example, CAD and/or COMT genes could be the target of such regulation [50,53] thus modifying
384 the availability of the precursor monomers for lignin or lignans and neolignans biosynthesis. For
385 instance, CAD is of particular interest, since here the CCR and CAD enzyme activities, could
386 also appear not consistent with their corresponding gene expression levels. Indeed, the CCR and
387 CAD enzyme activities reached their maxima 8h post-treatment and then decreased, whereas
388 their expression remained at very high levels. Nevertheless, we have to keep in mind that CCR
389 and CAD both belong to small multigene families, therefore the enzyme activities measured can
390 result from the action of distinct enzymes encoded by different genes involved in different
391 biosynthetic pathways. Here, we have determined gene expression profile of CCR and CAD
392 genes previously associated with the production of lignans and neolignans in flax cell
393 suspensions [35]. However, other CCR and CAD genes could be associated with the production
394 of lignin as reported by Le Roy et al. [54] and can contribute to the overall enzyme activities
395 measured. The induction of gene expression associated with a decrease in enzyme activity could
396 not only be due to a differential transcriptional regulation within a multigene family, but also
397 result from posttranslational regulation events also known to occur in the phenylpropanoid
398 pathway. This have been, for example, described by Allwood et al. [55] evidencing the
399 implication of phosphorylation event(s) and the involvement of a protein kinase in the

400 posttranslational regulatory mechanism of the PAL enzyme. Moreover, to date no data about the
401 turnover of proteins involved in production of lignans and neolignans are available.

402 Last, these apparent discrepancies between gene expression levels, enzyme activities and lignans
403 and neolignans production might be due to the fact that the first results provide an indication of
404 the gene expression of the enzyme activity at a precise time whereas metabolite accumulation
405 summarize the effect during the whole duration of the experiment.

406 Finally, we cannot exclude a possible metabolization of chitosan into a more or less active
407 elicitor compound(s) by flax cells following its addition to the culture medium.

408 ***3.3 Impact of chitosan-treatment on antioxidant potential of flax cell culture compared to flax*** 409 ***seeds***

410 DPPH assay was conducted to examine the antioxidant potential of the flax cell cultures. It was
411 noted that the cell cultures exhibited highest antioxidant potential (93.6%) at its 30th day of
412 inoculation as compared to control cell cultures (Fig. 6). ROS are normally formed during light
413 dependent processes in plants and can lead to oxidative stress which can be extremely harmful
414 for photosynthetic cells and can damage cellular compounds like membrane lipids, proteins or
415 nucleic acid [47]. Chitosan and its derivatives are involved in accumulation of hydrogen peroxide
416 (H₂O₂) in plants which has a key role in oxidative burst and stimulation of scavenging system of
417 ROS in plants [48, 49]. Chitosan is considered to be responsible for alleviation of membrane
418 lipid peroxidation which results in lowering the phytotoxicity in plant cells by reducing the high
419 chemical oxygen demand. All such antioxidative activities contribute additively to the free
420 radical scavenging capacity of a medicinal plant, therefore, free radical scavenging activity was
421 performed in order to analyze and compare the antioxidant potential of chitosan elicited and
422 control cell cultures of Flax. Results showed that 10 mg/L chitosan efficiently enhanced the free

423 radical scavenging (93.6 %) compared to control (69 %). Similar results were observed for
424 chitosan treated cell cultures of *Ocimum basilicum* [41].

425 Last but not least, Table 2 presents the comparison of the accumulation of lignans and neolignans
426 and resulting antioxidant activity of the extracts prepared from control and chitosan-treated flax
427 cell suspensions vs wild seeds (from Barbara cultivars known to be highly productive in term of
428 lignans accumulation) [34]; and *LuPLRI* gene silenced transgenic flax [21]. Seeds of wild type
429 flax mainly accumulated SDG whereas seeds of transgenic RNA silencing *PLRI* transgenic flax
430 mainly accumulated neolignans. On the contrary flax cell suspensions accumulated both lignans
431 and neolignans, and chitosan treatment was able to enhanced lignans and neolignans
432 accumulation in this *in vitro* system. Moreover, flax seeds are cultivated only once a year
433 whereas flax cell suspension constituted a continuous and efficient production system. Last
434 important point, our *in vitro* production system is non transgenic and therefore could be less
435 controversial in term of public acceptance, safety and usefulness. Altogether these results clearly
436 indicated that flax cell suspension and in particular chitosan treatment are effective production
437 systems of antioxidant lignans and neolignans.

438 **4. Conclusion**

439 In current study, we evaluated the impact of a number of concentrations from 0.1 to 500 mg/L of
440 chitosan in order to elicit the biosynthesis of pharmaceutically important polyphenols i.e. lignans
441 (SDG and LDG) and neolignans (DCG and GGCG) in *L. usitatissimum* cell cultures. Among all,
442 10 mg/l chitosan proved to be highly effective in the stimulation of gene expressions, enzyme
443 activities and metabolite accumulation of anticancer lignans and neolignans than control cells.
444 Beside, chitosan (10 mg/L) also remarkably enhanced the accumulation of biomass (FW and
445 DW) and antioxidant potential of the flax cells. The productivity of lignans and neolignans as
446 well as antioxidant potential of these chitosan-treated cells were higher than those of flax

447 evidencing the interest of this non transgenic *in vitro* system. Based on the current findings it is
448 suggested that chitosan treatment of cell cultures of *L. usitatissimum* using chitosan (10 mg/L)
449 for the enhanced productivity of anticancer polyphenols is a feasible and promising approach.

450 **Authors Contributions**

451 WA performed experiments of cell culture, elicitation with chitosan and analyses of TPC, TFC
452 and antioxidant activity. AZ and MN assisted WA with all of these experiments. BHA conceived
453 idea, supervised research and critically evaluated MS. CH contributed to HPLC analysis,
454 performed gene expression analysis and enzymatic measurements and contributed to the
455 conception, writing and critical reading of the manuscript. LG and SD performed HPLC. SR and
456 JD contributed to enzymatic measurements and HPLC quantification. NGG discussed research
457 and critically reviewed the manuscript.

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464 **Conflict of interest**

465 Authors declare no conflict of interest

466 **References**

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Figures Legends

Figure 1. Schematic representation of biosynthetic pathways of lignans and neolignans, quantified in cell cultures of flax using RP-HPLC-DAD adopted from Anjum et al.,⁴ DIR: dirigent protein; PLR: pinoresinol lariciresinol reductase; UGT/UDP-GT: UDP-Glucosyl Transferase.

Figure 2. A. Morphological characteristic of flax cell cultures grown in 0.1-500 mg/L of chitosan at day 30 of culture. B. Morphological characteristic of the different growth phases of flax cell cultures grown in 10 mg/L of chitosan. B1: lag phase. B2: exponential phase. (Log) B3: stationary phase. B4: death phase.

Figure 3. A. Monolignol and monolignol-derived biosynthetic pathway occurring in flax cell suspension (from Hano et al., 2006a). PAL: phenylalanine ammonia-lyase; CCR: cinnamoyl-CoA reductase; CAD: cinnamyl alcohol dehydrogenase; DIR5: dirigent protein 5; PLR1: pinoresinol lariciresinol reductase 1; UGT74S1: secoisolariciresinol uridine glucosyl transferase; PCBER: phenylcoumaran benzylic ether reductase; L-Phe: L-phenylalanine; SDG: secoisolariciresinol diglucoside; LDG: lariciresinol diglucoside; DCG: dehydrodiconiferyl glucoside; GGCG: guaiacylglycerol- β -coniferyl ether diglucoside. B. Normalized relative gene expression profiles in flax cell suspension as determined by RT-qPCR (normalized with cyclophilin (*CYCI*) and Eukaryotic Translation Initiation Factor 5A (*ETIF5A*)) following exogenous addition of chitosan at 1 (CHI-1), 10 (CHI-10) and 100 (CHIU-100) mg/l. Results are presented as a clustering classification realized by a hierarchical clustering analysis (HCA) based on the complete linkage Pearson uncentered correlation method performed with MeV.

Figure 4. Time course of specific PAL (A), CCR (B) and CAD (C) activities of the soluble protein fraction of control and chitosan-treated (at 1 (CHI-1), 10 (CHI-10) and 100 (CHIU-100) mg/l) cell suspension cultures. CTL is control cells. Bars represent SE of 3 independent experiments.

Figure 5. Biosynthesis and production dynamics of lignans SDG (A) and LDG (B) as well as neolignans DCG (C) and GGCG (D) in chitosan-treated cell cultures of *Linum usitatissimum* (CHI-10) versus control (CTL) flax cell cultures. Values are mean of three triplicates \pm SE

Figure 6. Antioxidant potential (radical scavenging activity) of cell cultures of *Linum usitatissimum* grown in chitosan (CHI-10) versus control (CTL) cell cultures.

Supplementary Figure Legends

Figure S1. Biomass accumulation in cell cultures of *Linum usitatissimum* grown in chitosan. CTL: control, CHI-10: chitosan 10 mg/L, FW: fresh weight, DW: dry weight.

Figure S2. Total Phenolic content and production of cell cultures of *Linum usitatissimum* grown in chitosan (CHI-10) versus control (CTL) cell cultures.

Figure S3: Typical HPLC chromatogram of a Flax cell culture extracts. IS: internal standard (enterolactone)

Table 1: Growth (fresh weights (FW) and dry weights (DW)) and total phenolic concentrations (TPC) in control and chitosan-treated (at different concentration levels) flax cell cultures

Treatment	FW	DW	TPC
(mg/l)	(g/l)	(g/l)	(mg/g DW)
Control	60.35 ± 3.02	5.50 ± 0.07	9.1 ± 0.1
0.1	187.08 ± 5.16	12.55 ± 0.15	10.37 ± 0.13
0.5	130.58 ± 4.39	9.71 ± 0.09	13.9 ± 0.26
1	176.08 ± 4.89	13.10 ± 0.27	14.32 ± 0.29
10	390.66 ± 8.11	16.31 ± 0.33	19.36 ± 0.39
20	193.25 ± 5.05	10.24 ± 0.28	15.34 ± 0.33
100	121.66 ± 3.23	11.32 ± 0.11	13.76 ± 0.27
200	56.33 ± 2.71	5.50 ± 0.08	10.9 ± 0.14
500	35.83 ± 1.19	12.55 ± 0.17	8.1 ± 0.09

Table 2. Comparison of lignans and neolignans accumulations and antioxidant activities in flax cell culture (control and chitosan-treated) vs seeds (wild type seeds and RNAi-*LuPLR1* transgenic seeds).

	<i>in vitro</i> cell cultures		seeds	
	Control	Chitosan-treated	Wild type (cv Barbara)	RNAi- <i>LuPLR1</i> transgenic seeds
SDG^a (mg/g DW)	4.73 ± 0.22 ^B	23.15 ± 2.04 ^A	20.62 ± 4.71 ^A	0.62 ± 0.15 ^C
LDG^a (mg/g DW)	8.91 ± 0.57 ^B	18.26 ± 1.07 ^A	ND ^D	Traces ^C
DCG^a (mg/g DW)	17.52 ± 0.84 ^B	56.25 ± 6.35 ^A	ND ^C	14.22 ± 3.67 ^B
GGCG^a (mg/g DW)	5.52 ± 0.28 ^A	1.42 ± 0.67 ^B	ND ^D	Traces ^C
DPPH (%RSA) ^b	68.36 ± 5.12 ^{BC}	93.37 ± 8.93 ^A	78.25 ± 11.42 ^B	52.14 ± 10.26 ^C
FRAP (TEAC) ^c	287.63 ± 5.38 ^C	485.28 ± 12.14 ^A	319.34 ± 6.57 ^B	156.45 ± 4.12 ^D

ND: not detected below the LOD; Traces: detected below LOQ (see Table 2); ^a maximum accumulation for control and chitosan-treated flax cell suspension; ^b expressed as percentage of radical scavenging activity measured at day 30 for control and chitosan-treated flax cell suspensions; ^c expressed in mM of Trolox C equivalent antioxidant capacity (TEAC) measured at day 30 for control and chitosan-treated flax cell suspensions; Data are expressed as the mean of n = 4 independent experiments ± standard deviation of the mean and different superscript majuscule letters indicate significant differences between conditions ($P < 0.05$).

able S1. Primers for expression analysis of flax selected-DIR

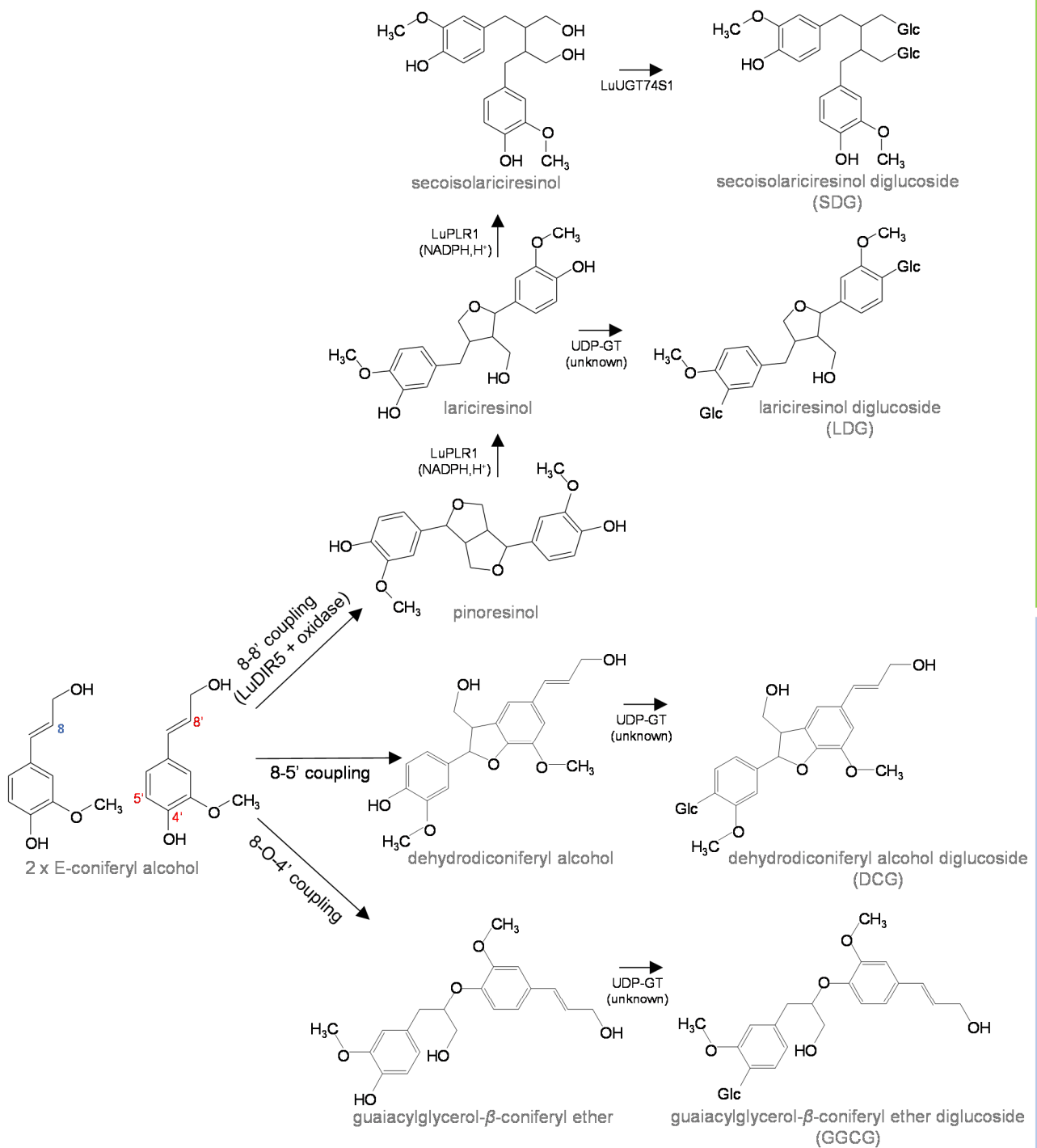
Gene	Phytozome ID	Designation	Sequence 5' → 3'	Length (bp)
<i>LuDIR5</i>	Lus10028749	Forward	TCATTCTTCATCTTGCCTTCCT	141
		Reverse	CATGGCTTATTGGGATTTCTTC	
<i>LuPLR1</i>	Lus10012145	Forward	TATGGAGATGGCAACGTCAA	181
		Reverse	GAAGTTGGTTGCCTGAGAGC	
<i>LuUGT74S1</i>	Lus10017825	Forward	GGA CTCGTTGTGCATGGTG	200
		Reverse	TCAGCTCTCACTCCGGTTTT	
<i>LuPCBER</i>	Lus10026348	Forward	TCAATGACCTTGTGGCTCG	179
		Reverse	CGGCTCGATCTCAAAGTAGG	
<i>LuPAL</i>	Lus10026518	Forward	ATGGACTGCCATCGAATCTC	175
		Reverse	AATCCAACGAGTTCACGTC	
<i>LuCAD</i>	Lus10019811	Forward	TGAAGCACTGGATCATCTCG	161
		Reverse	TCAACTCCCATCGACCTTC	
<i>LuCCR</i>	Lus10041651	Forward	ATTCCCCGAGTATCCTGTCC	191
		Reverse	TGTGCTGCTGTTGTTTAGGG	
<i>LuCYC</i>	Lus10012167	Forward	TGATTGCGGTCAGCTGTAAG	147
		Reverse	AGGTGAAACGCTAGGCAGAA	
<i>LuETIF5A</i>	Lus10036801	Forward	TGCCACATGTGAACCGTACT	159
		Reverse	CTTTACCCTCAGCAAATCCG	

Table S2: Validation parameters of the HPLC-DAD method for the quantification of the main lignans and neolignans from flax cell suspension extract

Compound	Retention time (min)	Regression equation	R ²	Linear Range (µg/ml)	LOD (ng)	LOQ (ng)	Intraday Precision n ^b	Interday Precision ^b	Repeatability	Recovery
LDG	31.87	y=0.208x-1.979	0.998	50-1000	3.4	10.6	1.25	1.44	2.15	98.7
SDG	27.12	y=0.221x-0.063	0.999	50-1000	2.7	9.2	0.45	0.84	0.99	100.4
DCG	36.45	y=0.230x-0.708	0.999	50-1000	3.1	10.0	0.86	1.48	1.81	97.5
GGCG	33.11	y=0.239x-1.375	0.999	50-1000	4.2	13.4	0.79	1.65	2.03	102.5

^a quantified under their aglycone form; ^b expressed as % RSD (relative standard deviation); ^c expressed as % recovery.

Figure 1:

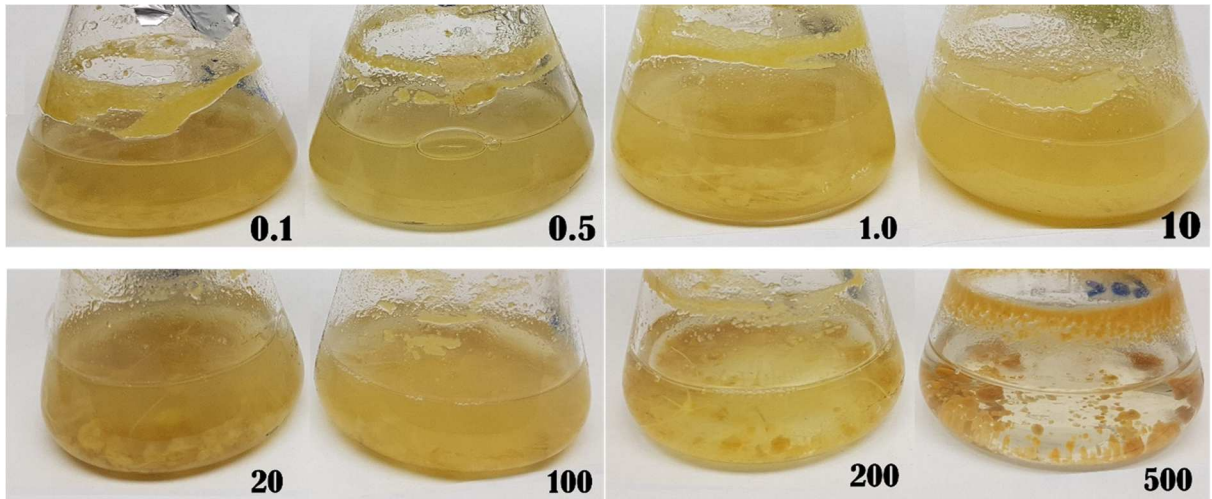


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Figure 2:

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B.

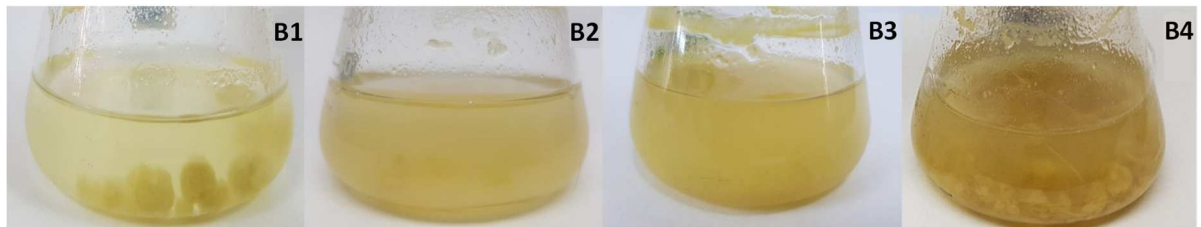
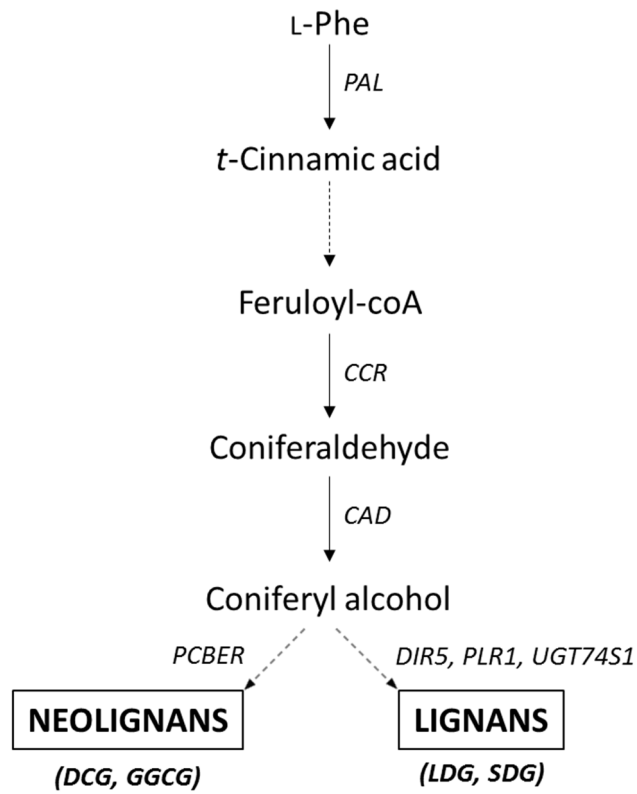


Figure 3:

A.



B.

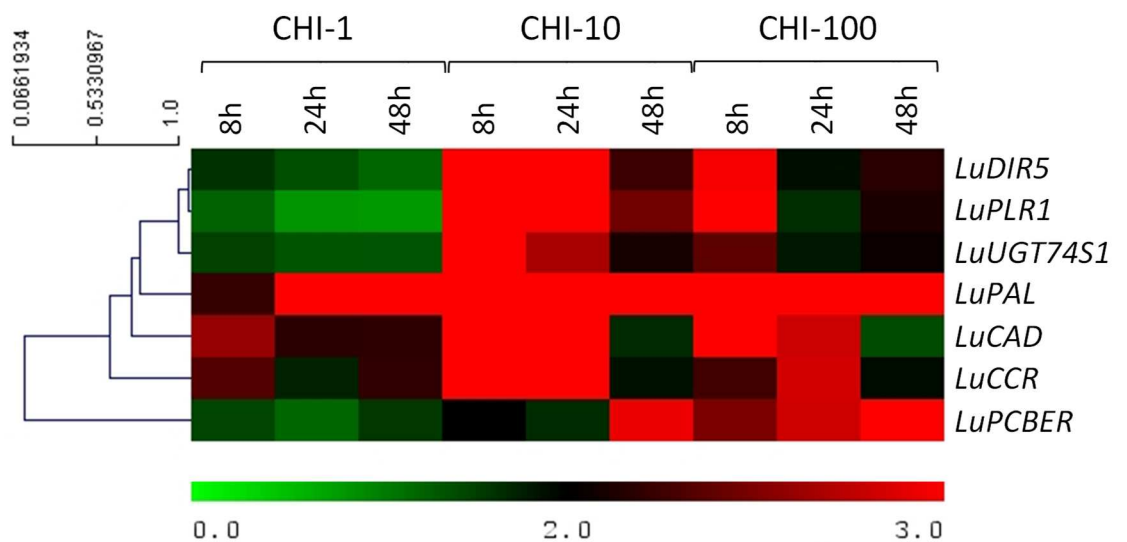


Figure 4:

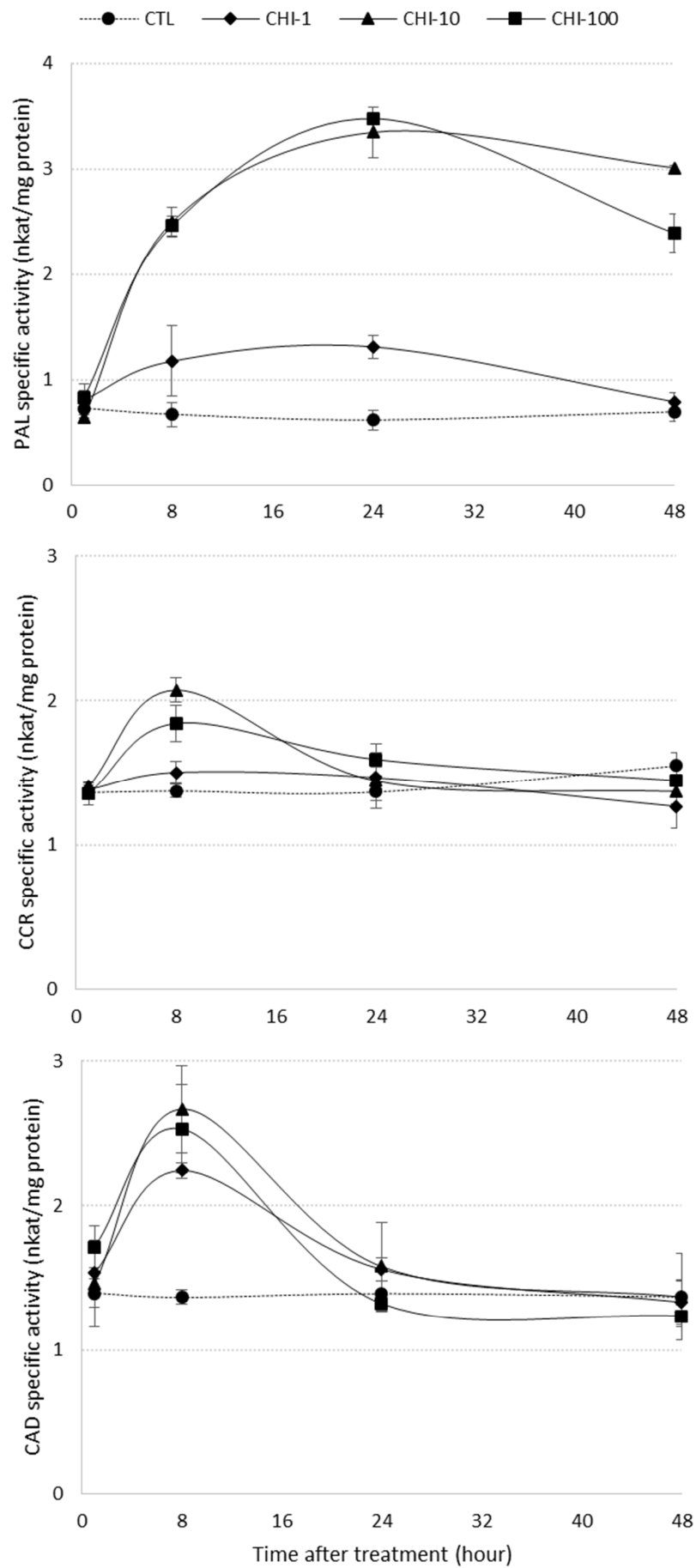


Figure 5:

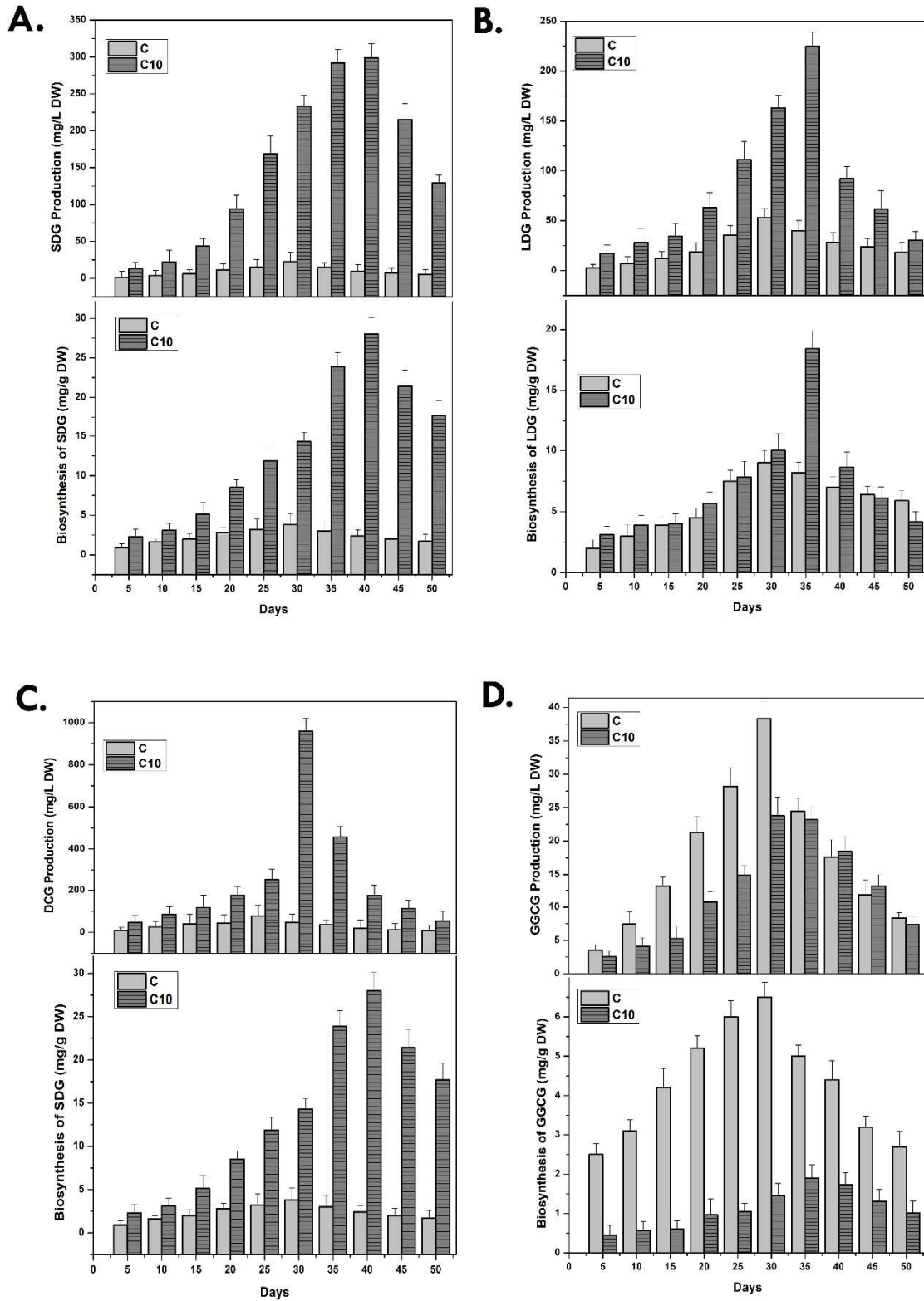
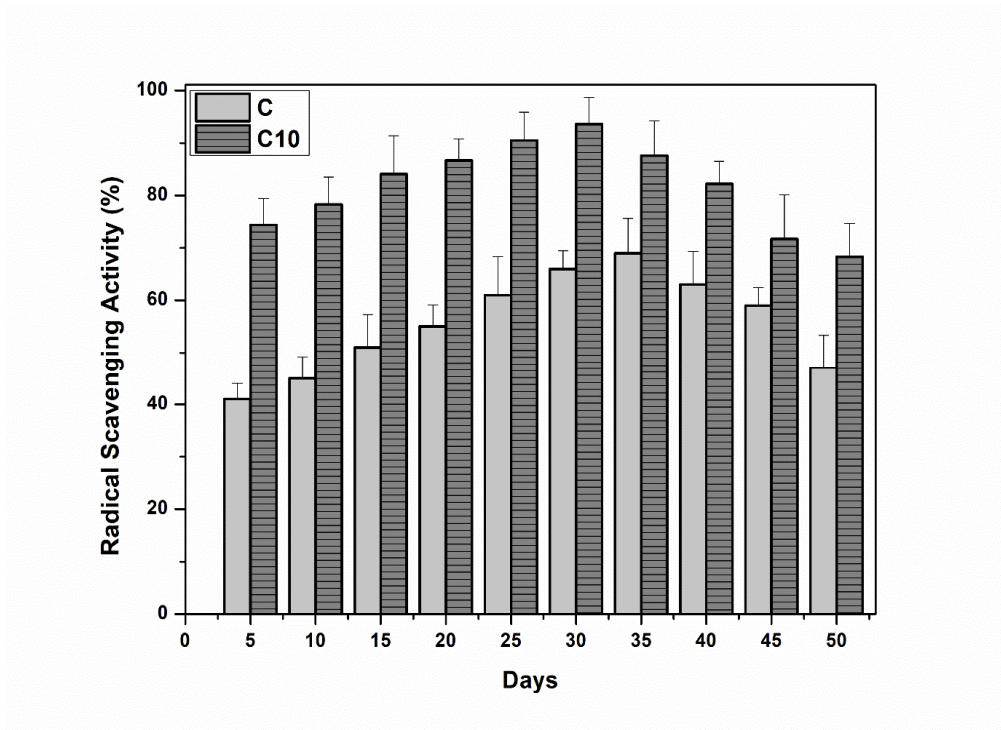
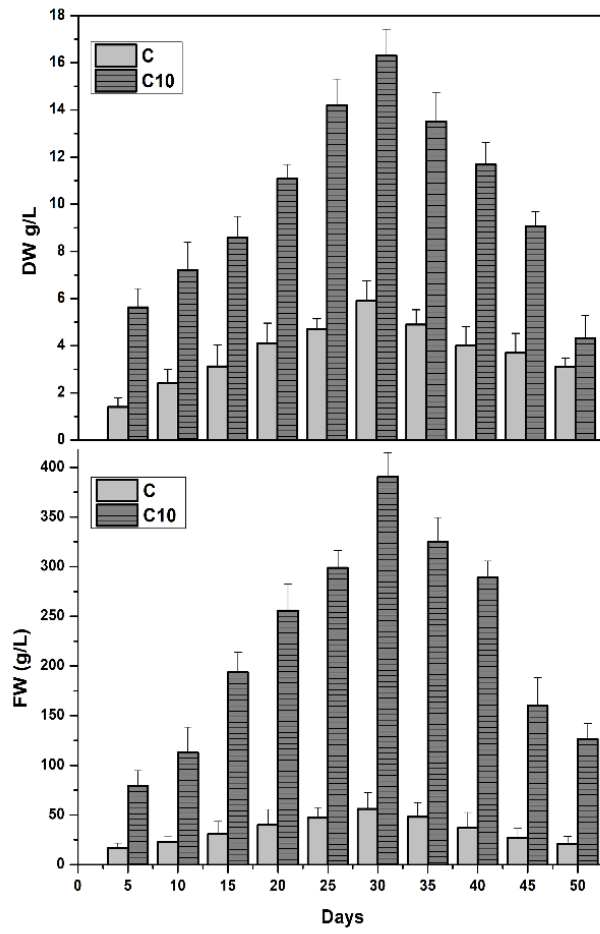


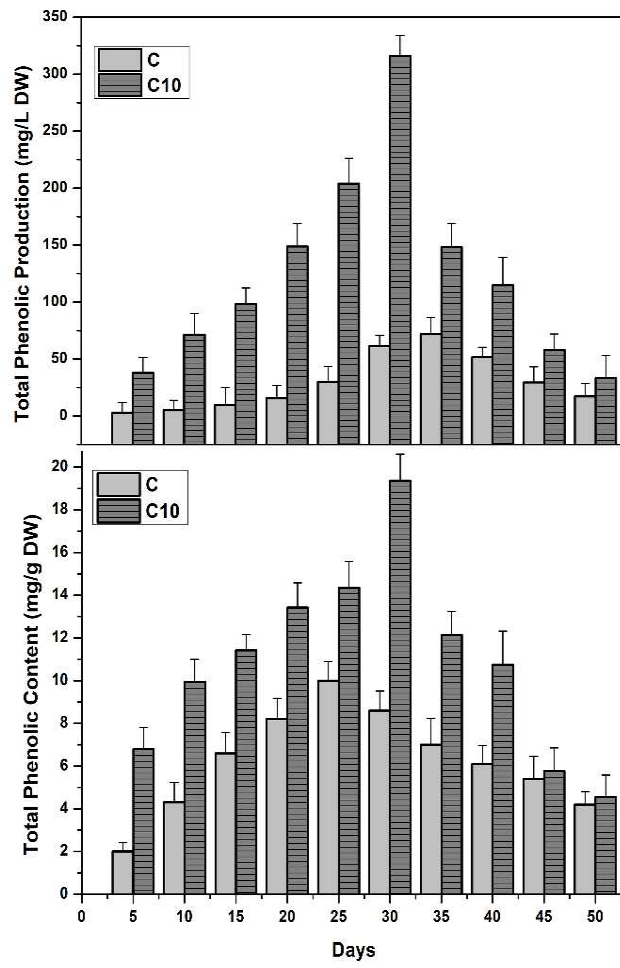
Figure 6:



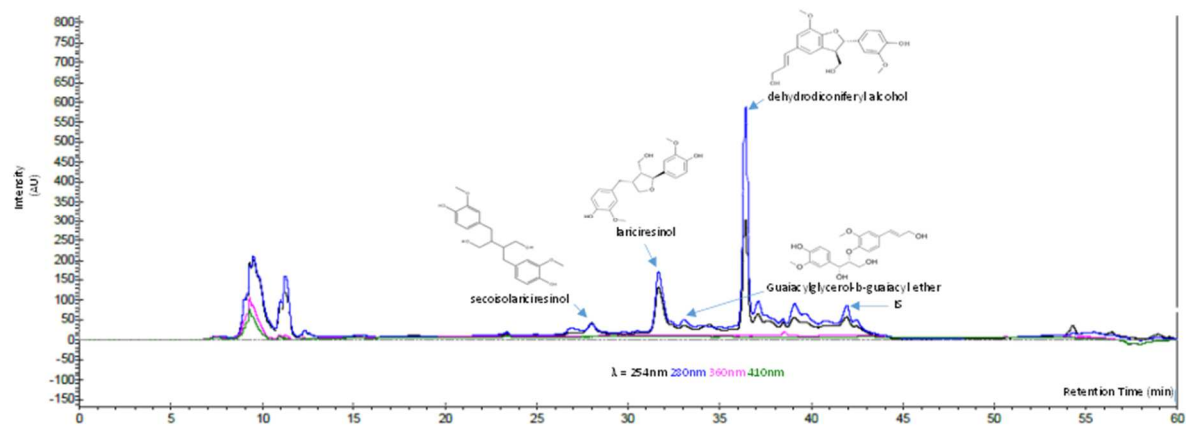
Supplementary Figure S1:

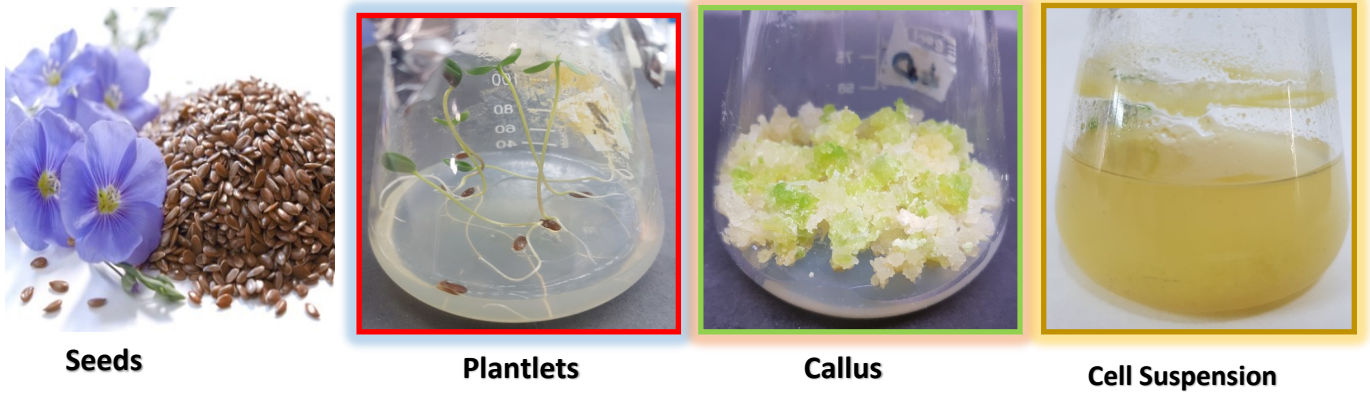


Supplementary Figure S2:



Supplementary Figure S3:





Chitosan Treatment



RT-qPCR Analysis

PAL Activity

CCR and CAD Activity

Antioxidant Potential

Analytical Scheme

RP-HPLC Analysis

Total Phenolic Production

