Using a combination of chitosan, methyl jasmonate, and cyclodextrin as an effective elicitation strategy for prenylated stilbene compound production in *Arachis hypogaea* L. hairy root culture and their impact on genomic DNA

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Abstract
Peanut (*Arachis hypogaea* L.) hairy roots are a potential tool for stilbene compound production. This study focuses on the most efficient elicitation strategy for inducing stilbene compound production, especially for the prenylated forms of *trans*-arachidin-1 and *trans*-arachidin-3. To the best of our knowledge, this is the first report of simultaneous treatment involving chitosan (CHT), methyl jasmonate (MeJA), and cyclodextrin (CD); CHT + MeJA + CD in peanut hairy roots to induce substantial amounts of *trans*-arachidin-1 and *trans*-arachidin-3 at 72 h of the elicitation period. The results demonstrate the highest amounts of *trans*-arachidin-1 and *trans*-arachidin-3, with 684.30 ± 183.85 and 543.94 ± 171.17 mg/g dry weight of hairy roots, respectively. The highest antioxidant capacity determined by ABTS and FRAP assays was 188.95 ± 0.43 µmol Trolox/g dry weight of hairy roots and 24.61 ± 0.50 mg ascorbic acid/g dry weight of hairy roots, respectively, while the highest phenolic content was also detected in this crude extract, with 8.67 ± 0.11 mg gallic acid/g dry weight of hairy roots. The antioxidant and pro-oxidant activity of the CHT + MeJA + CD crude extract in the protection and damage of DNA is of great interest and can have major positive impacts on health promotion and disease prevention.

Key message
Chitosan, methyl jasmonate, and cyclodextrin induce high stilbene compounds. The antioxidant in crude extract could scavenge free radicals and protect DNA, while pro-oxidant activity causes severe damage to genomic DNA.

Keywords *Trans*-arachidin-1 · *Trans*-arachidin-3 · DNA protection · Genotoxicity · Fenton reaction

Abbreviations
AAE  Ascorbic acid equivalent
ABTS  2,2′-Azino-di-(3-ethylbenzthiazoline sulfonic acid)
Cd  Cadmium
CD  Cyclodextrin
CHT  Chitosan
EDTA  Ethylenediamine tetraacetic acid
EtOH  Ethanol
FeCl3  Ferric chloride
FRAP  Ferric reducing antioxidant power
GAE  Gallic acid equivalent
H2O2  Hydrogen peroxide
HPLC  High-performance liquid chromatography
MeJA  Methyl jasmonate
NaCl  Sodium chloride
NO  Nitric oxide
OH*  Hydroxyl radical
O2**  Superoxide anion radical
PQ  Paraquat
PR  Pathogenesis-related protein
ROS  Reactive oxygen species
SDS  Sodium dodecyl sulfate

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to induce the combination of MeJA and CD can be a good elicitor in peanut hairy root culture. Yang et al. (2015) reported that been applied to stimulate bioactive compound production (Ball et al. 2015). These elicitors therefore stimulate the plant defence mechanism, involving pathogenesis-related protein, antimicrobial peptides, or secondary metabolite production. Secondary metabolite biosynthesis results in the production of bioactive compounds such as anthocyanin, flavonoids, polyphenol, and terpenes. Numerous polyphenolic compounds play a major role in protecting plant cells against external stimuli and pathogen attack (Lattanzio et al. 2006). Stilbenes are polyphenolic compounds and contain aromatic rings in their structure, functioning as phytoalexins to protect plants against pathogens, insects, and oxidative stress (Valletta et al. 2021).

Stilbene compounds such as trans-resveratrol have been found in berries, grapes, and peanuts (Arachis hypogaea L.). In addition, prenylated forms of stilbene derivatives such as trans-arachidin-1 and trans-arachidin-3 are synthesised from trans-resveratrol and can only be found in peanuts (Yang et al. 2016). These compounds have diverse potent biological activities and medical properties. The anti-inflammatory activity of trans-arachidin-1 was demonstrated in RAW 264.7 macrophage cells which exhibited an inhibitory effect on the lipopolysaccharide (LPS)-induced production of prostaglandin E2-mediated inflammation (Djoko et al. 2007). Additionally, trans-arachidin-1 had the highest efficacy for inducing programmed cell death in human leukaemia (HL-60) cells when compared with trans-arachidin-3 and trans-resveratrol, respectively (Huang et al. 2010). The efficacy of trans-arachidin-1 and trans-arachidin-3 for decreasing Rotavirus infectivity titrations indicated the inhibition of viral replication and could be attributed to the antioxidant and anti-inflammatory activity of these stilbene compounds (Ball et al. 2015).

Several studies have reported that peanut hairy root culture can effectively produce stilbene compounds (Abbott et al. 2010; Condori et al. 2010). To date, elicitors have been applied to stimulate bioactive compound production in peanut hairy root culture. Yang et al. (2015) reported that the combination of MeJA and CD can be a good elicitor to induce trans-resveratrol, trans-arachidin-1, and trans-arachidin-3 with high antioxidant activity in peanut hairy root culture. Our previous studies investigated the different sequential elicitation approaches of MeJA plus CD treatment before or after the application of paraquat (PQ) or cadmium (Cd), and the results demonstrated that the sequential elicitation strategy could evoke high levels of stilbene compounds with high biological activity in peanut hairy root culture (Pilaisangsuree et al. 2020; Somboon et al. 2019). However, PQ, which is widely used as a plant herbicide, and the heavy metal Cd are considered toxic to plants and humans. Therefore, the investigation of other safe substances rather than PQ and Cd could be beneficial for stilbene compound production.

Numerous studies have reported that chitosan (CHT) has the potential to induce secondary metabolites with bioactivity production in plants (Jiao et al. 2018). CHT is a natural biopolymer that exists in shrimp or insect shells (Malerba and Cerana 2015) and is low-cost, safe, and non-toxic. Spraying chitosan at the veraison stage of grape bunches increased the trans-resveratrol content 1.6 times and piceid 3.8 times over the untreated bunches. The proteomic approach also demonstrated the upregulation of proteins in relation to the oxidative stress response, such as superoxide dismutase enzymes and stilbene biosynthesis enzymes in the CHT-treated grape skins (Lucini et al. 2018). The combination of chitosan and ultraviolet C irradiation in Vitis vinifera cell suspension cultures demonstrated a significant increase in trans-resveratrol, total phenolic compound, and total flavonoid content. In addition, the synergistic effect of combined elicitation enhanced the activity and gene expression level of chitinase and β-1,3-glucanase enzymes which function as pathogenesis-related proteins in plant defence response (Xu et al. 2016).

DNA damage is associated with ageing and several diseases, such as cancer and neurodegenerative disease. Various studies have reported polyphenolic compounds to be promising antioxidant substances for preventing the cellular oxidative DNA damage caused by free radicals (Kaur et al. 2019). The DNA nicking assay with the Fenton reaction was used to evaluate the protective DNA activity of crude extract. It has been reported that a novel luteolin derivative isolated from Terminalia chebula exhibited high antioxidant activity, thus demonstrating DNA protection from hydrogen peroxide-induced DNA damage (Soumya et al. 2019). However, a high dose of polyphenol pycnogenol extract was observed to be associated with DNA damage, possibly due to pro-oxidant activity and its effect on DNA breakage (Kim and Park 2004).

To date, no studies have investigated the simultaneous and sequential elicitation of CHT, MeJA, and CD for prenylated stilbene derivatives such as trans-arachidin-1 and trans-arachidin-3 production in peanut hairy roots. This is the first study to investigate proper elicitation using various combination strategies involving CHT, MeJA, and CD for stilbene compound induction. The biological activity of
crude extract as a culture medium was evaluated by antioxidant activity and the impact of antioxidant potential against oxidative damage to DNA further investigated.

**Materials and methods**

**Growth conditions of peanut hairy root culture**

The established peanut hairy root line of cultivar Kalasin2 (K2-K599) described by Pilaisangsuree et al. (2018) was subcultured by cutting root tips into 1–2 cm long and transferred to agar-solidified B5: Gamborg’s medium (Gamborg et al. 1968) supplemented with 3% sucrose at 25 ± 2 °C in continuous darkness every two weeks.

**Elicitor treatment strategy**

One gram of K2-K599 root was cultured in a 500-ml Erlenmeyer flask containing 200 ml of ½ MS: Murashige and Skoog (Murashige and Skoog 1962) liquid medium with 1.5% sucrose and shaken at 150 rpm at 25 ± 2 °C in the dark for seven days. The elicitor treatment was carried out at the exponential root growth phase as described in our previous report (Eungsuwan et al. 2021). After seven cultivation days, the spent media was renewed with 200 ml of fresh medium, and the elicitation experiments performed in six groups: 1) control group with 1% acetic acid pH 5.7; 2) treatment with 150 mg/l CHT alone (CHT) as previously described by Chayjarung et al. (2020); 3) treatment with 100 µM MeJA and 6.87 mM CD (MeJA + CD) as previously described by Yang et al. (2015); 4) treatment with 150 mg/l CHT for 24 h followed by 100 µM MeJA and 6.87 mM CD (CHT(MeJA + CD)); 5) treatment with a combination of 100 µM MeJA and 6.87 mM CD for 24 h followed by 150 mg/l CHT (MeJA + CD)_CHT; 6) treatment with a combination of 150 mg/l CHT, 100 µM MeJA, and 6.87 mM CD (CHT + MeJA + CD). All treatments were performed using three biological replicates and harvested after elicitation at 24, 48, and 72 h. All elicitations were carried out at 25 ± 2 °C and incubated on a rotary shaker (150 rpm) under continuous darkness.

**Extraction of stilbene compounds from culture medium**

The elicited culture medium was extracted by partitioning 200 ml of culture medium with equal volume of ethyl acetate at a ratio of 1:1 in a separatory funnel and repeated extraction for three times. The organic phase of the culture medium was evaporated to dryness under vacuum at 40 °C using a rotary evaporator (Büchi). The crude extract was weighed and dissolved in EtOH for further analysis.

**Analysis of stilbene compounds from crude extract by HPLC**

The crude extract was dissolved in EtOH to obtain a 2 mg/ml concentration, filtered through a 0.45-µm nylon membrane filter, and analysed by reversed-phase HPLC according to Limmongkon et al. (2018). Chromatography was performed in a C18 column with a constant flow rate of 1.0 ml/min. The mobile phase consisted of 2% formic acid in water and acetonitrile (70:30). The chromatograms were detected using a UV detector at 306 nm for trans-resveratrol and 340 nm for trans-arachidin-1 and trans-arachidin-3.

**ABTS antioxidant assay**

The ABTS (2,2′- azinobis (3-ethylbenzoline sulfonic acid)) assay followed the method of Rajurkar and Hande (2011). The procedure measures the relative ability of antioxidants to scavenge ABTS radical cations (ABTS +). A solution of 198 µl ABTS + was mixed with 2 µl of crude culture medium extract in a 96-well microplate and incubated at room temperature for 6 min in the dark. The absorbance was measured at 734 nm. Trolox was used as the standard substance, and the result expressed as the TEAC value (Trolox equivalent antioxidant capacity, µmol Trolox/g dry weight of hairy roots).

**FRAP reducing antioxidant assay**

The FRAP (ferric reducing antioxidant power) assay was performed according to Rajurkar and Hande (2011). This assay determined the ability of antioxidants to reduce ferric ions in the form of 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) to ferrous ions in the form of tripyridyl triazine. The reduction began by mixing 198 µl of fresh FRAP working solution (300 mM acetate buffer pH 3.6, 10 mM TPTZ in 40 mM HCl, and 20 mM FeCl3 0.2H2O) and 2 µl of culture medium crude extract. The absorbance was measured at 593 nm after 5 min at room temperature in the dark. The result was reported as the AAE value (ascorbic acid equivalent, mg ascorbic acid/g dry weight of hairy roots) using ascorbic acid as the standard.

**Total phenolic content assay**

The total phenolic content (TPC) assay was used to quantify the total phenolic compound substances according to the Folin-Ciocalteu method (Zheng and Wang 2001). Gallic acid was used as the standard. Ten microlitres of Folin reagent was mixed with 2 µl of culture medium crude extract (2 mg/ml), followed by 10 µl of 20% w/v sodium carbonate and then incubated at room temperature in the dark for 30 min. The absorbance was measured at 765 nm, and the
total phenolic compound content reported as gallic acid equivalent (GAE) mg/g dry weight of hairy roots.

**Genotoxicity evaluation of crude extract**

To evaluate the genotoxicity of crude extract on genomic DNA, the control and elicited crude extracts with different concentrations (10, 20, 50, 100, and 200 µg/ml) were added to 150 ng of blood genomic DNA in a reaction mixture with a final volume of 20 µl. All reactions were incubated at 37 °C for 20 min, and the genomic DNA visualised by 0.8% agarose gel electrophoresis.

**DNA nicking assay**

Genomic DNA from human whole blood sample was prepared using modified salting-out method as described by Chacon-Cortes et al. (2012). Briefly, white blood cells were suspended with lysis buffer (100 mM Tris–Cl; pH 7.6, 40 mM EDTA; pH 8.0, 50 mM NaCl, 0.2% SDS) and subsequently incubated with 0.1 mg/ml proteinase K at 50 °C for 60 min. The 6 M NaCl was used for deproteinization step and the genomic DNA was precipitated using ice-cold absolute ethanol. The DNA nicking assay was performed using genomic DNA with a modification of the Devi et al. (2012) method. The non-toxic crude extract concentrations were chosen from the genotoxicity results and further verified with a DNA nicking assay. The crude extract samples were mixed with 150 ng of genomic DNA and Fenton reaction. Different concentrations of the Fenton reaction were investigated in this study: 1 × Fenton reagent (120 µM FeCl₃, 45 mM H₂O₂, and 75 µM ascorbic acid) and 2 × Fenton reagent (240 µM FeCl₃, 90 mM H₂O₂, and 150 µM ascorbic acid). The reactions were incubated at 37 °C for 20 min with a total reaction volume of 20 µl. The qualification of genomic DNA was determined by 0.8% agarose gel electrophoresis.

**Statistical analysis**

All experiments were conducted in triplicate and expressed as the mean ± SD. Statistical analyses were calculated using two-way ANOVA with SPSS software version 23.0. Statistically significant differences were determined at the p < 0.05 level.

**Results**

**Effect of different elicitors on peanut hairy root tissue and culture medium**

The hairy roots were treated with different elicitors: CHT, MeJA + CD, CHT_(MeJA + CD), (MeJA + CD)_CHT, and CHT + MeJA + CD for 24, 48, and 72 h. After 72 h of elicitation, the characteristic of peanut hairy root tissue treated with CHT alone or a combination of CHT and MeJA + CD exhibited a yellowish colour that was distinct from the control and MeJA + CD-treated groups (Fig. 1). The culture medium elicited with (MeJA + CD)_CHT and CHT + MeJA + CD showed a more intense yellowish colour than the medium treated with CHT alone, MeJA + CD, and CHT_(MeJA + CD) at all time points. The culture medium of the control group without any elicitor treatment exhibited a non-yellowish colour (Fig. 2).

![Fig. 1 Phenotypes of K2-K599 hairy root tissues elicited by different elicitors at 72 h](image-url)
Total antioxidant activity

The antioxidant activities of the crude extract culture medium were determined by ABTS and FRAP assays. The ABTS method evaluated the ability of crude extract to scavenge ABTS\(^{+}\) radical cations and is presented as the TEAC value (Online Resource 1). All crude extract-treated samples except the CHT alone treatment exhibited increasing antioxidant activity upon elicitation. However, the CHT alone treatment showed slightly higher values than the control group. After 24 and 48 h, the (MeJA + CD)_CHT treatment showed the highest antioxidant activity compared with the other elicitor treatments and the control group. At 72 h, the CHT + MeJA + CD treatment exhibited the highest TEAC value of 188.95 ± 0.43 μmol Trolox/g dry weight of hairy roots, which was significantly different from the other treatments except (MeJA + CD)_CHT (Fig. 3a).

The FRAP assay was used to determine the ability of crude extract to reduce ferric (Fe\(^{3+}\)) to ferrous (Fe\(^{2+}\)) iron through the action of an electron-donating antioxidant. The FRAP antioxidant activities of the MeJA + CD, CHT, (MeJA + CD), (MeJA + CD)_CHT, and CHT + MeJA + CD treatments increased with elicitation time, which was in accordance with the results of the ABTS antioxidant activity assay (Fig. 3b) (Online Resource 1). The CHT + MeJA + CD treatment at 72 h exhibited the highest FRAP antioxidant activity of 24.61 ± 0.50 mg ascorbic acid/g dry weight of hairy roots, which was 91.35-fold higher than that of the control group.

Total phenolic contents

The total phenolic content of the crude extract culture medium was determined by the Folin-Ciocalteu method. After 24 and 48 h of elicitation, the (MeJA + CD)_CHT treatment presented the highest phenolic content of 2.67 ± 0.09 and 3.73 ± 0.10 mg gallic acid/g dry weight of hairy roots, respectively. A sharp increase in phenolic content was observed in the CHT + MeJA + CD treated sample at 72 h of elicitation. The highest phenolic content was 8.67 mg gallic acid/g dry weight of hairy roots, which was 137-fold higher than that of the control group (Fig. 3c) (Online Resource 1). The total phenolic content was in accordance with antioxidant capacity, the highest phenolic content being detected at 72 h with the CHT + MeJA + CD treatment.

Stilbene compound determination in crude medium extracts

The amount of stilbene compounds was investigated using HPLC. The HPLC for trans-resveratrol was performed at UV 306 nm, while trans-arachidin-1 and trans-arachidin-3 were detected at UV 340 nm. The HPLC chromatograms detected at UV 340 nm of crude extract treated with the different elicitors at 24, 48 and 72 h were presented (Online Resource 2–4). No stilbene compounds were detectable in the control group. Treatment with MeJA + CD, CHT_(MeJA + CD), (MeJA + CD)_CHT
and CHT + MeJA + CD at 24, 48, and 72 h elicited stilbene compounds, \textit{trans}-resveratrol, \textit{trans}-arachidin-1, and \textit{trans}-arachidin-3, at increasing levels compared with CHT alone (Fig. 4) (Online Resource 1). After 24 h of elicitation, the highest \textit{trans}-resveratrol content was observed in the (MeJA + CD)_CHT treatment, with a value of 2.04 ± 0.23 mg/g dry weight of hairy roots, whereas the highest \textit{trans}-resveratrol contents at 48 and 72 h were detected in the CHT + MeJA + CD treatment, with values of 2.42 ± 0.32 and 2.14 ± 0.44 mg/g dry weight of hairy roots, respectively (Fig. 4a). The highest level of \textit{trans}-arachidin-1 was observed with CHT + MeJA + CD treatment at 72 h, with a value of 684.30 ± 183.85 mg/g dry weight of hairy roots, being 10,940- and 2.5-fold higher than the CHT alone and MeJA + CD treatment groups, respectively (Fig. 4b). \textit{Trans}-arachidin-3 was expressed at the highest levels of 470.02 ± 100.76 and 543.94 ± 171.17 mg/g dry weight of hairy roots after 48 and 72 h elicitation, respectively, being 6,277- and 12,054-fold higher than the treatment with CHT alone and 3.3- and two-fold higher than the treatment with MeJA + CD (Fig. 4c).

### Genotoxicity of crude medium extracts

Various concentrations (10, 20, 50, 100, and 200 µg/ml) of crude medium extract from control, CHT alone, MeJA + CD, CHT (MeJA + CD), MeJA + CD) CHT and CHT + MeJA + CD treated hairy roots at 24, 48, and 72 h were incubated directly with genomic DNA for 20 min. As shown in Fig. 5a, b and c), the sample from the control group had no effect on DNA, with visible DNA bands at all concentrations and in all time point studies. The crude extract from 72 h of the CHT-treated group resulted in a weak intensity of the DNA band with higher concentrations of 100 and 200 µg/ml, suggesting a correlation between genotoxic activity and a higher concentration of crude extract (Fig. 5f, lanes 5–6). Treatment of MeJA + CD and CHT MeJA + CD crude extract with 100 and 200 µg/ml resulted in light and diffuse DNA bands starting from 48 and 72 h of elicitation, indicating the genotoxic activity of high concentrations of crude extract on DNA (Fig. 5h, i, j, k and l, lanes 5–6). Both samples from the (MeJA + CD)_CHT and CHT + MeJA + CD treated groups expressed very strong genotoxic activity.
to DNA, resulting in low-intensity bands being observed after treatment periods of 24, 48, and 72 h (Fig. 5m–r, lanes 5–6). The bright and strong diffused bands observed upon treatment with 100 and 200 µg/ml concentrations of CHT + MeJA + CD hairy roots at 72 h could be due to the high amounts of stilbene compounds in the crude extracts being absorbed in the UV spectrum (Fig. 5r, lanes 5–6).

**DNA nicking assay with Fenton reaction**

Two different concentrations (10 and 20 µg/ml) of crude extract from various treatment groups demonstrating non-toxicity to DNA were chosen for the DNA nicking assay with the Fenton reaction. The Fenton reaction was performed with two different concentrations (1 × and 2 ×) of Fenton reagent. The purpose of the Fenton reaction is to induce OH radicals for oxidative damage to the DNA strand. Figure 6a–r, lane 1 shows intact genomic DNA with a high molecular weight. The incubation of 1 × Fenton reagent with genomic DNA demonstrated a smeared band, indicating DNA damage triggered by OH radicals generated from the Fenton reaction (Fig. 6a–r, lane 2). By incubating all crude extract samples with concentrations of 10 and 20 µg/ml together with DNA and the Fenton reaction for 20 min, the high-molecular-weight genomic DNA clearly appeared, suggesting the protective property of crude extract to avoid damage to DNA from OH radicals (Fig. 6a–r, lanes 3 and 4). Interestingly, the 20 µg/ml CHT + MeJA + CD crude extract in the 1 × Fenton reaction displayed a smeared band of DNA, which could be due to the pro-oxidant activity of the high amounts of stilbene compound present in the sample (Fig. 6r, lane 4).

A higher concentration of 2 × Fenton reagent was applied to the reaction mixture to generate more OH radicals, subsequently causing severe damage to genomic DNA, as can be seen from the smeared band in Fig. 6a–r, lane 5. All crude extracts (10 and 20 µg/ml) were added to reagent tubes containing genomic DNA and 2 × Fenton reagent and incubated for 20 min. The appearance of genomic DNA was different in the 2 × Fenton reaction compared with that in the 1 × Fenton reaction. Genomic DNA was observed as incomplete high-molecular-weight DNA with a smeared tail, which could be due to the inadequacy of antioxidant substances in the crude extract to cope with the large number of free radicals.
generated in the 2 × Fenton reaction system (Fig. 6a–r, lanes 6–7). High-molecular-weight DNA bands with smeared, fragmented tails were observed in the control, CHT, and MeJA + CD samples during all elicitation periods (Fig. 6a–i, lanes 6–7). Interestingly, the large-sized DNA band with a smeared tail was only visible in the CHT (MeJA + CD) treated sample at 24 and 48 h (Fig. 6j–k, lanes 6–7), while an entirely smeared tail was observed after 72 h of elicitation (Fig. 6l, lanes 6–7). According to the high amounts of stilbene compound with high antioxidant activity in the (MeJA + CD)_CHT and CHT + MeJA + CD treated samples, a very faint large DNA band with a long fragmented tail was detected at 24 h of elicitation time (Fig. 6m, p, lanes 6–7), and the sole smeared tail was observed with the sample at 48 and 72 h of elicitation (Fig. 6n, o, q, and r, lanes 6–7), indicating high potent pro-oxidant activity of crude extract from (MeJA + CD)_CHT and CHT + MeJA + CD treatment, synergistically causing severe damage to DNA.

To verify the switching between antioxidant and pro-oxidant properties of crude extract, a lower concentration of all treated crude extracts (5 µg/ml) at 72 h of elicitation time was examined with 1 × Fenton reagent and genomic DNA. The control and CHT crude extracts were unable to recover the high-molecular-weight genomic DNA, which was obviously observed with the smeared band (Fig. 7, lanes 3–4), indicating insufficient antioxidant activity to scavenge OH radicals in the Fenton reaction. The MeJA + CD, CHT (MeJA + CD) and MeJA + CD (MeJA + CD) samples presented incomplete DNA protection ability, as indicated by the more intense high-molecular-weight DNA bands with some smearing tails (Fig. 7, lanes 5–7) compared with the control and CHT samples (Fig. 7, lanes 3–4). Surprisingly, the 5 µg/ml CHT + MeJA + CD crude extract exerted DNA protection activity and showed a strong high-molecular-weight genomic DNA band with only a faint smearing tail (Fig. 7, lane 8). This suggested that a low concentration of CHT + MeJA + CD crude extract can act as an antioxidant instead of having pro-oxidant activity compared with a high-concentration extract, as demonstrated in Fig. 6r, lanes 3–4.

**Effect of crude extract and Fenton reaction on genomic DNA**

The Fenton reagent was sequentially added to the reaction to prove the effect of the crude extract and Fenton reagent on genomic DNA. The genomic DNA was completely degraded after incubation with 1 × Fenton reagent at 37 °C for 20 min (Fig. 8, lane 2) compared with the intact genomic DNA.
The addition of 20 µg/ml CHT + MeJA + CD crude extract at 48 h of elicitation together with DNA and Fenton reagent, incubated for 20 min, obviously demonstrated DNA protective activity, with a visible high-intensity genomic DNA band (Fig. 8, lane 3). Surprisingly, the opposite result was observed in the sequential experiment demonstrated in lanes 4 and 5. Since the result showed a degraded DNA band (Fig. 8, lane 4) in which genomic DNA was first incubated with CHT + MeJA + CD crude extract for 10 min, Fenton reagent was then added and further incubated for 10 min. This possible pro-oxidant activity of the CHT + MeJA + CD crude extract is related to DNA genotoxicity. In contrast, the first incubation of Fenton reagent with CHT + MeJA + CD crude extract for 10 min, followed by the addition of genomic DNA, incubated for another 10 min, led to the recovery of high-molecular-weight genomic DNA, indicating that the antioxidant activity of the crude extract could scavenge free radicals from the Fenton reaction and thereby protect DNA from excessive OH radicals (Fig. 8, lane 5).

**Discussion**

Plants are often exposed to different elicitors: abiotic and/or biotic. These elicitors cause oxidative stress, generating reactive oxygen species (ROS) in plant cells and stimulating various plant defence responses, such as secondary metabolite and pathogenesis-related protein production (Rejeb et al. 2014). Our previous study investigated different combinations of abiotic elicitors, such as PQ, Cd, MeJA, and CD, in peanut hairy root culture, and the results demonstrated that all these elicitors, especially the combination and sequential elicitor treatment, could induce diverse amounts of stilbene.
trans-Resveratrol was not much different; however, the prenylated stilbenes of trans-arachidin-1 and trans-arachidin-3 showed greater diversity in amounts between each combination of elicitors (Pilaisangsuree et al. 2020; Somboon et al. 2019; Wongshaya et al. 2020).

As a natural, safe, non-toxic, and inexpensive biopolymer found in the exoskeletons of crustaceans, insects, and fungal cell walls, CHT can be applied as a stimulating substance for plant development and used as an elicitor of the plant defence response. It was reported that treatment with 0.1% CHT increased the production of curcumin and stimulated rhizome development (Anusuya and Sathiyabama 2016). The elicitation of *Isatis tinctoria* L. hairy root cultures by 150 mg/l CHT for 36 h was reported to enhance total flavonoids by 7.08-fold, including quer cetin, isorhamnetin, isoliquiritigenin, and rutin, compared with the control (Jiao et al. 2018). CHT (10 mg/l) was reported to stimulate low amounts of trans-resveratrol and trans-pterostilbene production in peanut hairy roots (Medina-Bolivar et al. 2007). Malerba and Cerana (2015) demonstrated that CHT induced high-level production of *H₂O₂*, *O₂*, and NO in *Acer pseudoplatanus* L. culture cells compared with the control. Additionally, the combination of MeJA and CD elicitation in *Nicotiana tabacum* cell culture could induce ROS, including *H₂O₂* and NO, in cell culture (Almagro et al. 2012). These elicitors can stimulate ROS production, thus inducing oxidative stress in the plant cell. The induction of antioxidant substances to cope with oxidative stress in cells could be involved in the plant defence response.

Due to lower biological activity observed from hairy root tissue compared to culture medium extract (unpublished data), the present study demonstrated the activity of crude extract prepared from the culture medium. The study indicates the highest antioxidant activity measured by the ABTS and FRAP methods in peanut hairy root culture medium elicited with the combination of CHT, MeJA, and CD (CHT + MeJA + CD) in a simultaneous strategy. The sequential elicitation strategy of pre-treatment with MeJA + CD followed by CHT, (MeJA + CD)_CHT, revealed a close range of antioxidant activity to CHT + MeJA + CD, while pre-treatment with CHT prior to MeJA + CD (CHT_ (MeJA + CD)) exhibited lower antioxidant activity. These results accord with the total phenolic compound assay. A strong positive linear correlation was found between the ABTS assay and the FRAP method, with *r* = 0.9866 indicating that the antioxidant activity of the crude extract could be related to electron-donating properties. These results suggest that simultaneous elicitation with CHT + MeJA + CD and the sequential treatment strategy of (MeJA + CD)_CHT could present a synergistic effect to induce a substantial amount of antioxidant substances in plant cells. In addition, the order of elicitor treatment may also play a significant role in antioxidant compound production, as seen by the antioxidant activity obtained from different sequential elicitations of (MeJA + CD)_CHT and CHT_(MeJA + CD). Jaisi and Panichayupakaranant (2020) also demonstrated that simultaneous treatment with chitosan and L-alanine enhanced plumbagin production to 14.62 mg/g dry weight, while the sequential addition of CD followed by CHT induced plumbagin production to 14.44 mg/g dry weight in *Plumbago indica* root culture.
A yellowish colour was observed on the peanut hairy root tissue treated with CHT alone or in combination with CHT and MeJA + CD, suggesting the production of prenylated stilbene compounds have been produced in these treated cultures (Sobolev et al. 2006). A slight yellow colour was observed in hairy root tissue treated with CHT alone or the co-treatment of MeJA and CD (MeJA + CD), while a strong yellow colour was mostly present in all combinations between CHT and MeJA + CD, CHT + (MeJA + CD), (MeJA + CD)_CHT, and CHT + MeJA + CD, in accordance with the yellow colour observed in the medium of hairy root culture. This also implies that prenylated stilbene compounds such as trans-arachidin-1 and trans-arachidin-3 were secreted into the culture medium. The darkest yellow colour of hairy root tissue and culture medium was inspected in the treatment of (MeJA + CD)_CHT and CHT + MeJA + CD, and also associated with the highest induction of trans-arachidin-1 and trans-arachidin-3 detected in the culture medium. It has been reported that plant exudates such as bioactive secondary metabolites can be secreted from plant roots and function as defense response against a broad array of root surrounding microbes or insects. The ability of plant cells to secrete exudates into the culture medium indicated the adaptation of plants to the stress environment. The cellular injury from fungal attack caused the increasing level of trans-resveratrol in the culture medium of Vitis vinifera cell suspension culture and it has been suggested that the secretion of trans-resveratrol was probably related to active transport mechanism such as ABC transporter (Santamaria et al. 2011). Our previous research on elicitation with the plant herbicide PQ demonstrated that elicitation of PQ prior to the combination of MeJA + CD, PQ + (MeJA + CD), resulted in the highest amounts of trans-arachidin-1 and trans-arachidin-3, with 178 ± 0.05 and 444 ± 0.06 mg/g dry weight of hairy roots, respectively (Somboon et al. 2019). In the study of elicitation with heavy metal Cd, the treatment of MeJA + CD prior to Cd, (MeJA + CD)_Cd, represented the highest amounts of trans-arachidin-1 and trans-arachidin-3, with only 9.67 ± 6.67 and 78.84 ± 32.60 mg/g dry weight of hairy roots, respectively (Pilaisangsuree et al. 2020). The present study concerning the treatment involving a combination of a non-toxic CHT elicitor (CHT + MeJA + CD) illustrates the most promising results, with the highest amounts of trans-arachidin-1 (684.30 ± 183.85 mg/g dry weight of hairy roots) and trans-arachidin-3 (543.94 ± 171.17 mg/g dry weight of hairy roots) in a 72-h elicitation period. This suggests that the simultaneous treatment of CHT + MeJA + CD is the most favourable elicitor for peanut hairy root culture to induce high amounts of trans-arachidin-1 and trans-arachidin-3.

Our elicitation experiment demonstrates that increasing amounts of stilbene compounds such as trans-arachidin-1 and trans-arachidin-3 have high antioxidant activity and therefore could be applied as DNA protective substances. All crude extracts were examined for genotoxic properties by direct incubation with genomic DNA. The genotoxic activity of crude extracts was observed from samples with strong antioxidant activity, especially those treated with high concentrations of (MeJA + CD)_CHT and CHT + MeJA + CD. It has been reported that under certain conditions, the conjugated double bond in the molecular structure coordinates to redox ions and may lead to free radical formation, which behaves as a pro-oxidant substance and induces oxidative DNA damage (Sotler et al. 2019). Our present study also demonstrates the strong antioxidant activity and high reducing power of FRAP in the crude extracts of (MeJA + CD)_CHT and CHT + MeJA + CD treated samples. The antioxidant activity of the crude extract could scavenge free radicals generated by the Fenton reaction, thus preventing oxidative DNA damage. However, the potent antioxidant activity of stilbene compounds in higher concentrations of (MeJA + CD)_CHT and CHT + MeJA + CD crude extracts exhibited pro-oxidant activity, thereby causing additive effects on DNA damage in the Fenton reaction. An increasing concentration of myricetin reportedly enhances DNA damage due to the formation of stable Cu(II) complexes via the Fenton reaction, causing a synergistic effect on DNA damage through free radical formation (Chobot and Hadacek 2011). By incubating genomic DNA with CHT + MeJA + CD crude extract before adding Fenton reagent, the appearance of DNA degradation demonstrates that crude extracts probably act as pro-oxidants. The crude extract could supposedly reduce molecular oxygen directly into superoxide anion radicals and interact with DNA strands (Hayyan et al. 2016), thus causing oxidative damage and genomic DNA fragmentation. The subsequent addition of Fenton reagent to the reaction increased the free radical amount in the system, enhancing DNA fragmentation via oxygen-derived species. Conversely, the incubation of Fenton reagent with CHT + MeJA + CD crude extract before adding genomic DNA led to the recovery of high-molecular-weight genomic DNA, indicating that the crude extract prevents DNA damage by quenching the OH radicals generated by the Fenton reaction (Borra et al. 2014), and therefore could inhibit oxidative DNA damage.

Conclusion

Our study demonstrates for the first time, the simultaneous and sequential elicitation of CHT, MeJA, and CD in peanut hairy root culture. The synergistic effect of simultaneous CHT + MeJA + CD elicitation at 72 h could give rise to the efficient production of trans-arachidin-1 and trans-arachidin-3 with high antioxidant activity when compared to the treatment with either CHT alone or MeJA + CD.
The sequential treatment of MeJA + CD prior to CHT in (MeJA + CD)_CHT or post-treatment of MeJA + CD in the CHT_(MeJA + CD) approach could stimulate distinct amounts of trans-arachidin-1, trans-arachidin-3, and antioxidant substances. The genotoxic activity and DNA protective ability of crude extract containing highly prenylated stilbene compounds provide important information for its application as an effective substance for human health and disease prevention. Further investigation should be conducted to fully reveal the molecular mechanism of prenylated stilbene compound production in response to external stimuli.

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