

Elicitation improves the production of bioactive compounds and antioxidant activity in cell suspension culture of *Withania coagulans* (stocks) Dunal

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Abstract

Abiotic and biotic elicitors may induce higher synthesis of bioactive compounds in medicinal plants. In the present study, the impact of abiotic (salicylic acid (SA)) and biotic (cellulase from *Aspergillus niger* (CE)) elicitors with 200 mg/L concentration at 24, 48 and 72 h treatment durations was evaluated on total phenolic and flavonoid contents (TPC and TFC) and withaferin A and withanolide A compounds (analyzed by HPLC) as well as free radicals scavenging activity in cell suspension culture derived from leaf and stem explants of *Withania coagulans* (stocks) Dunal for the their production in commercial level. The results of this investigation revealed that all elicitors treated cell cultures obviously promoted TPC, TFC, withaferin A and withanolide A accumulation as well as antioxidant activity. Also, these characteristics increased as treatment duration of SA and CE elicitors enhanced in both leaf and stem cell extracts and reached a maximum at 72 h treatment duration. In most treatments, TPC and TFC of leaf cell extracts did not has significant difference with TPC and TFC of stem cell extracts. In contrast, the leaf cell extracts had higher withaferin A and withanolide A amounts when compared to stem cell extracts under all treatments. The highest values of TPC, TFC, withaferin A, withanolide A, and antioxidant activity observed in SA elicitor treated leaf cell extracts at 72 h treatment duration (16.31 mg GAE/g DW and 1.61-fold, 13.21 mg QE/g DW and 2.46-fold, 14.46 µg/g DW and 3.08-fold, 6.67 µg/g DW and 1.99-fold, and 83.73% and 1.78-fold higher than control culture, respectively). Therefore, elicitation can be applied as a promising strategy for large-scale production of *W. coagulans* valuable secondary metabolites in cell suspension culture at commercial level.

1. Introduction

Withania coagulans (stocks) Dunal, belonging to *Solanaceae* family, is one of the significant medicinal plants, an evergreen and small shrub and use for remedy sensible debility, rheumatism, flatulent colic, ulcer, dyspepsia and dropsy (Atta-ur-Rahman et al., 2003; Jain et al., 2012). It has various pharmaceutical properties like hepato-protective, antidiabetic, antidepressant, antimicrobial, cardiovascular, anti-cancer, free radical scavenging and immunosuppressive (Haq et al., 2013; Teixeira da Silva et al., 2015).

These medicinal properties have been related to a large number of biological active compounds including phenolic compounds, alkaloids, saponins, tannins, steroids and withanolides presence in this plant (Teixeira da Silva et al., 2015). The main phytochemical compounds of *Withania* genus are withanolides, which are a group of C-28 steroidal lactones (Jha et al., 2005). The first known member of withanolides is withaferin A which was extracted from *W. somnifera* (Lavie et al., 1965).

Among known withanolides, Withaferin A and withanolide A are main withanolides in different tissues of the plant (Singh et al., 2018). Withanolide A play a significant role in the remedy of neurodegenerative illnesses such as cognitive function impairment, Parkinson, Alzheimer and convulsions (Tohda et al., 2005).

W. coagulans naturally propagates from seeds, but self-incompatibility and unisexual nature of flowers limit the seed setting (Jain et al., 2009; Rathore et al., 2016). Overexploitation from wild resources for medicinal industries, no cultivation practices for this plant, use by animals as a feed and reproductive failure have marked this plant as threatened in its habitat and critically endangered (Jain et al., 2009; Teixeira da Silva et al., 2015; Rathore et al. 2016). Hence, alternative approaches for the production of this plant and its bioactive compounds must be applied instead harvest of raw material from wild plants. Biotechnological methods, particularly cell, tissue and organ culture can use for the synthesis of secondary metabolites which indirectly conserve vital plants by reducing the use of wild plants (Ramachandra Rao and Ravishankar, 2002; Teixeira da Silva et al., 2015).

A high synthesis source of bioactive compounds is cell suspension culture. The continuous, predictable and reliable production of these compounds and independent of seasonal constraints are the advantages of this approach (Jha et al., 2005; Karuppusamy, 2009). Numerous trials have been performed for the synthesis of bioactive compounds in cell, tissue and organ culture in various plants (Nagella and Murphy, 2010; Sivanandhan et al., 2012; Naik and Al-Khayri, 2018; Koul and Mallubhotla, 2020; Kanthaliya et al, 2023). Nevertheless, the commercial utilization of the secondary metabolites in cell suspension culture is limited due to low quality and yield and biosynthetic instability (Bourgaud et al., 2001; Yue et al., 2016). Therefore, the strategies must be used for the improvement of bioactive compounds synthesis in cell, tissue and organ cultures. The elicitation treatment including abiotic and biotic elicitors can be induced higher production of bioactive compounds in cell, tissue and organ cultures (Zhao et al., 2005; Kim et al., 2007; Hussein et al., 2012).

Abiotic elicitors like methyl jasmonate and salicylic acid are signaling molecules in the plants, which mediate plants resistance against pathogen and disease (Wasternack, 2007; Sivanandhan et al., 2013). Several researchers have been reported that abiotic elicitors led to the stimulation of secondary metabolites synthesis in plants (Sivanandhan et al., 2013; Manivannan et al., 2016; Yu et al., 2019; Wen et al., 2019; Al-Khayri and Naik, 2020; Koul and Mallubhotla, 2020). Among the biotic elicitors, fungi in numerous studies have been used to boost the bioactive compounds synthesis in the cell, tissue and organ cultures (Baldi et al., 2008; Ahlawat et al., 2017; Linh et al., 2021; Sak et al., 2021).

In *W. coagulans* (stocks) Dunal medicinal plant, little informations are available about its cell suspension culture and elicitation treatment for the enhancement of secondary metabolites. Therefore, this trial was established for the study of the influence of abiotic (salicylic acid) and biotic (cellulase enzyme from *Aspergillus niger*) elicitors on bioactive compounds and antioxidant potential in the cell suspension culture of *W. coagulans*.

2. Material and methods

2.1. Chemicals

withaferin A and withanolide A standards and all the chemicals applied for the preparation of medium, elicitor treatments and secondary metabolites analysis were provided from Sigma Aldrich (St. Lois, MO, USA).

2.2. Plant material and the production of in vitro sterile seedlings

Seeds of *Withania coagulans* (stocks) Dunal were collected from Mehrestan region of Saravan in Iran. For the sterilization of seeds, they were washed with sterile distilled water and treated with 10% sulfuric acid for 2 min. Then the seeds were transferred into 10% sodium hypochlorite for 10 min and finally, they were washed 3 times with sterile distilled water. The sterile seeds were cultured in vitro on half MS medium (Murashige and Skoog, 1962) and the cultures were incubated with a 14-h light/10-h dark cycle at 25 °C for 3 weeks.

2.3. Callus induction

For callus induction, the leaf and stem explants from 21-days-old in vitro seedlings were cultured separately on MS medium containing 2.0 mg/L 2,4-dichlorophenoxyacetic (2,4-D), 0.5 mg/L Kinetin (KIN) and 3% sucrose and were incubated at 25 °C and dark for 4 weeks.

2.4. Establishment of cell suspension cultures and assessment of growth kinetics

Produced calli (3 g/L) were transferred into 250 mL erlenmeyer flasks containing 50 mL MS liquid medium supplemented with 2.0 mg /L 2,4-D, 0.5 mg /L KIN and 3% sucrose for cell suspension culture. The cultures were kept under dark and 25 °C on a rotary shaker at 120 rpm. For the assessment of growth kinetics, the cells were harvested at 2 days intervals until 30 days with three replicates and weighted after filtering by Whatman No. 1 filter paper.

2.5. Elicitor application in cell suspension cultures

Cellulase (CE) from *Aspergillus niger* and salicylic acid (SA) elicitors were used in this study. On the 27th day, CE and SA elicitors with 200 mg L⁻¹ concentration were added to cell suspension cultures and placed on a rotary shaker at 120 rpm. The control samples were the cell suspension cultures without elicitor. The cells were harvested at 24, 48 and 72 h intervals after the treatment of elicitors. The experiment was carried out based on completely randomized design with three replicates. Harvested cells were freeze-dried and used for phytochemicals analyses.

2.6. Biochemical assays

2.6.1. Total phenolic content (TPC)

For the preparation of cell extracts, 1 g of the dried and powdered cell samples were added to 10 mL of 80% methanol in 15 mL tubes and placed on a rotary shaker at 110 rpm for 5 h at room temperature.

Then, the extracts were sonicated (Bondelin-Sonorex) for 45 min, centrifuged for 10 min at 8000 rpm and finally, the supernatant of samples were filtered using a 0.45 µm acrodisc syringe filter with nylon membrane. The obtained extracts employed for the biochemical assays.

For the measurement of TPC, the method of Ardestani and Yasdanparast (2007) with minor modifications was used. For this aim, 0.5 mL of the prepared extracts, 2 mL of 7.5% sodium carbonate solution and 2.5 mL of the 10% Foline-Ciocalteu's reagent were added to a 10 mL test tube, and then solutions were left in a hot water bath at 45 C for 15 min. The absorbance of examples were measured by a spectrophotometer at 765 nm. The TPC was calculated based on Gallic acid equivalents (GAE) per dry weight (mg/g).

2.6.2. Total flavonoid content (TFC)

The measurement of TFC was done based on the method of Zhang et al. (2015) with partial changes. 125 µL of the extracts were mixed with 75 µL of 5% NaNO₂ and after incubation at room temperature for 6 min, 150 µL of 10% AlCl₃ was added to them. After 5 min, 750 µL of 1 M NaOH and 1400 µL of distilled water were mixed with the obtained solutions and remained for 15 min at room temperature. By a spectrophotometer, the absorbance of each sample was measured at 510 nm. The TFC was reported based on Quercetin equivalents (QE) per dry weight (mg/g).

2.6.3. Free radicals scavenging potential

DPPH (2,2-diphenyl-1-picrylhydrazyl) was applied for measurement of antioxidant activity of cell extracts based on the method of Girennavar et al. (2007) with minor modifications. For this purpose, 0.1 ml of cell extracts were added to 2 ml of 0.1 mM methanolic solution of DPPH and kept in room temperature for 30 min under dark condition. The absorbance of the samples (containing of cell extracts (A_s)) and control (solution without cell extract (A_c)) was evaluated at 515 nm. Based on the following formula, the inhibition percentage of each sample was calculated:

$$\% \text{inhibition} = [(A_c - A_s) / A_c] \times 100$$

2.6.4. The measurement of withaferin A and withanolide A compounds by HPLC

Chromatography analysis was done by using a high-performance liquid chromatography (HPLC) system (Unican-Crystal-200, England) according to described method of Sivanandhan et al. (2012) with minor changes and by a reverse-phase C18 column (250 mm × 4.6 mm, 5 µm). 20 µL of extracts was injected and investigated at 250 nm. The mobile phase was containing of 40% acetonitrile, 1% acetic acid and 59% distilled water which was injected to the column whit the rate of 1 mL/min. The value of withaferine A and withanolide A in cell methanolic extracts was determined based on their retention times and peak area with using of pure standard samples and reported as µg per g of dry weight.

2.7. Statistical data analysis

The data were subjected to analysis of variance (ANOVA) by the Statistical Analysis System (SAS, Ver. 9.1) program and reported as the mean $\pm$ standard. The Least Significance Difference (LSD) test was performed for specification significant differences among amounts of treatments at probability level of 0.05.

3. Results and discussion

In the present study, two elicitors (salicylic acid and cellulase enzyme) with 200 mg/L concentration and three treatment durations (24, 48, and 72 h after elicitors treatment) were evaluated for the production of total phenolic, total flavonoid, withanoloides (withaferin A and withanolide A) and radical scavenging activity in cell suspension culture derived from leaf and stem explants of *W. coagulans*.

3.1. Growth kinetics

The production of secondary metabolites and biomass accumulation occur during exponential growth phase of cell growth cycle (Ouyang et al., 2005; Liu et al., 2007). Hence, in this study, before adding elicitors into cell suspension culture, cell growth kinetics was determined. The biomass accumulation on fresh weight (FW) basis of cells in cell suspension culture of *W. coagulans* are represented in Fig. 1. After cells culture, cells growth significantly increased from 6th day to 26th day (exponential growth phase) and reached a maximum biomass on 26th day of growth cycle (14.20 g/L). After the 26th day, between 27 and 30 days of growth cycle, cells growth remained constant. Therefore, elicitors were added to cell suspension cultures on 27th day of growth cycle. Sivanandhan et al. (2014) investigated kinetics of biomass accumulation and withanolides production in cell suspension culture of *W. somnifera* and reported that the cell growth cycle consisted of two exponential growth (days 7–28) and death (days 29–42) phases and biomass and withanolides content achieved a maximum on 28th day. Ahlawat et al. (2017) also stated that biomass accumulation and withanolides content significantly enhanced between 8 and 22 days (exponential growth phase) and then decreased between 23 and 26 days (death phase) in cell suspension culture of *W. somnifera* and reached maximum values on 22th day.

3.2. Influence of elicitors on total phenolic and flavonoid contents (TPC and TFC)

The elicitation treatment including abiotic and biotic elicitors is one of the most effective methods to improve secondary metabolites accumulation in cell, tissue and organ cultures (Zhao et al., 2005; Kim et al., 2007; Hussein et al., 2012). The type of elicitor and treatment duration influence secondary metabolites values in in vitro culture (Klimek-szczkutowicz, et al., 2022). In present study, the impact of salicylic acid (SA) and cellulase (CE) elicitors on TPC and TFC accumulation in cell suspension culture derived from leaf and stem explants of *W. coagulans* at 24, 48 and 72 h treatment durations after elicitation are represented in Fig. 2. All elicitor treated cell cultures obviously promoted TP and TF accumulation which indicates that elicitation can be as a promising approach for economic production of secondary metabolites in cell suspension culture of *W. coagulans*. Several researchers reported that

elicitation treatment could influence the secondary metabolites synthesis in different plants. Kim et al. (2010) observed that an *Aspergillus niger* cellulase treatment improved nutritional compounds like polyphenols, total catechins, epigallocatechin, epicatechin and gallic acid in the hot water extract of green tea in comparison to the non-treated control. The increase of secondary metabolites under SA elicitor treatment was obtained in cell suspension culture of *Salvia miltorrhiza* (Yu et al., 2018). Linh et al. (2021) stated that the elicitation by the fungi induced the biosynthesis of bioactive compounds (total alkaloids and vinblastine) of *Catharanthus roseus* plant in cell suspension culture compared to the control cultures. Also, the improvement of TPC and TFC was obtained by biotic elicitors (yeast extract, pectin and alginate) in in vitro propagated shoots of *Pueraria tuberosa* (Kanthaliya et al., 2023).

In present investigation, the amounts of TPC and TFC increased as treatment duration of SA and CE elicitors enhanced in both leaf and stem cell extracts and reached a maximum at 72 h treatment duration (Fig. 2). Similar to our results, Simic et al. (2014) reported that the synthesis of bioactive compounds (hypericin and pseudohypericin) was increased by pectin and dextran elicitors at late treatment duration in shoot cultures of *Hypericum perforatum*. Wen et al. (2019) found that the amounts of bioactive compounds (TP, TF and polysaccharide) enhanced with prolonged duration of SA elicitor treatment and reached their maximum values at 48 h exposure time in cell culture of *Orostachys cartilaginosa*.

Our results showed that in all treatments, TPC of leaf cell extracts did not have significant difference with TPC of stem cell extracts, and on average, it enhanced by 11.7, 24.0 and 50.0% under CE elicitor treatment and 22.0, 33.1, and 63.1% under SA elicitor treatment at 24, 48 and 72 h treatment durations, respectively as compared to their control cultures. Also, TFC of leaf cell extracts did not indicate significant difference with TFC of stem cell extracts except CE treatment at 72 h exposure time and SA treatment at 24 h exposure time that TFC of leaf cell extracts was significantly higher when compared to TFC of stem cell extracts. SA elicitor increased TFC of leaf and stem cell extracts, on average by 108.0 and 148.1% at 48 and 72 h treatment durations, respectively, and CE elicitor promoted its by 18.0 and 46.8% at 24 and 48 h exposure times, respectively, in comparison to their control cultures (Fig. 2). Generally, these findings indicate that SA elicitor can more effectively induce the synthesis of secondary metabolites in comparison to CE elicitor. SA as a signaling molecule mediates defensive system of plants against pathogens and stimulate the production of secondary metabolites through the induction of genes expression of synthesis pathway of them (Zhao, et al., 2005).

The highest amounts of TPC and TFC obtained under SA treatment at 72 h exposure time in both leaf and stem cell extracts (TPC: 16.31 and 15.67 mg GAE/g DW and TFC: 13.21 and 12.64 mg QE/g DW, in leaf and stem cell extracts, respectively). These results were similar to previous findings, which showed that SA elicitor were effectively enhanced the synthesis of biological active compounds in in vitro cultures (Sivanandhan et al., 2013; Al-Khayri and Nike, 2020; Koul and Mollubhetla, 2020).

3.3. Impact of elicitors on withaferin A and withanolide A contents

The values of withaferin A and withanolide A in *W. coagulans* cell suspension cultures was determined by HPLC analysis. Withaferin A and withanolide A are main withanolides in different tissues of the *Withania* genus that play a significant role in the treatment of neurodegenerative diseases and possess various pharmaceutical properties like antimicrobial, hepato-protective, antidiabetic, antidepressant, cardiovascular, anti-cancer, free radical scavenging and immunosuppressive (Tohda et al., 2005; Haq et al., 2013; Teixeira da Silva et al., 2015; Singh et al., 2018). Hence, in present study, the effect of cellulase (CE) and salicylic acid (SA) elicitors on the production of these important medicinal compounds (Withaferin A and withanolide A) in cell suspension culture of *W. coagulans* was investigated.

Our results indicated that both elicitors at all treatment durations had significantly positive effects in the improvement of both compounds accumulation in both leaf and stem cell extracts compared to their control cultures (Fig. 3). CE elicitor induced higher production of withaferin A and withanolide A at 24, 48 and 72 h treatment durations by 1.10, 1.65 and 2.25 and 1.08, 1.68 and 1.95 times in leaf cell extracts and 1.23, 1.61 and 1.76 and 1.71, 2.52 and 3.13 times in stem cell extracts, respectively as compared to their control cultures. SA elicitor improved withaferin A and withanolide A contents from 4.69 to 14.46 and from 3.36 to 6.67 µg/g DW in leaf cell extracts and from 2.80 to 6.88 and from 1.12 to 4.78 µg/g DW in stem cell extracts, respectively (Fig. 3). In agreement with our findings, Sivanandhan et al. (2012) reported that abiotic (aluminum chloride) and biotic (chitosan) elicitors enhanced the content of withanolides (withanolide A, withanolide B, withaferin A and withanoside) in *W. somnifera* adventitious root culture. In another study, the accumulation of withanolide A, withanone and withaferin A was observed in *W. somnifera* hairy root culture under methyl jasmonate and SA treatments (Sivanandhan et al., 2013). Ahlawat et al. (2017) found that cell homogenates of fungal elicitors significantly enhanced withanolides (withanolide A, withaferin A and withanone) content in *W. somnifera* cell suspension culture and *piriformospora indica* fungal cell homogenate at 3.0% (v/v) concentration and 7 days exposure time indicated the highest withanoloides production. Therefore, elicitation can improve the synthesis of these important medicinal compounds in in vitro cultures for commercial production of them.

In this study, as the exposure time after SA and CE elicitation increased, the content of both compounds enhanced and reached a maximum value at 72 h exposure time (Fig. 3). According to our results, the enhancement of stilbenes (trans-resveratrol and trans-piceid) with the increase of treatment duration of cellulase, cell wall homogenate from fungus, jasmonate and sodium orthovanadate elicitors in *Vitis vinifera* cell cultures was observed (Sak et al., 2021).

In the present research, the leaf cell extracts had more content of these compounds as compared to stem cell extracts under all treatments (Fig. 3). Similar to our results, the difference in withanoloides content in different portions of the *Withania* genus has been stated by other researchers (Dhar et al. 2006; Senthil et al., 2015).

Our results revealed that the highest increase of withaferin A and withanolide A synthesis was in leaf and stem cell extracts elicited with SA at 72 h exposure time that it was 3.08 and 1.99-fold higher in leaf cell extracts and 2.46 and 4.27-fold higher in stem cell extracts when compared to their control cultures,

respectively. The highest amounts of withaferin A and withanolide A observed in SA elicitor treated leaf cell extracts at 72 h treatment duration (14.46 and 6.67 µg/g DW, respectively). Control culture of stem had the lowest value of these compounds (2.80 and 1.12 µg/g DW, respectively) (Fig. 3). Regard to these findings, SA elicitor with 72 h exposure time in cell cultures derived from leaf explants is the best treatment for more the synthesis of withanoloids in cell suspension culture of *W. coagulans* in commercial level.

3.4. The effect of elicitors on radical scavenging potential

Radical scavenging activity (RSA) on DPPH in cell suspension culture of *W. coagulans* after treatment by SA and CE elicitors is indicated in Fig. 4. Our results showed that RSA was significantly increased by both elicitors at all treatment durations in both leaf and stem cell extracts. According to our results, Simic et al. (2014) found that polysaccharide elicitors significantly induced antioxidant activity in *Hypericum perforatum* shoot cultures. *Scrophularia kakudensis* cell suspension cultures treated with abiotic elicitors (methyl jasmonate, SA and sodium nitroprusside) were indicated the increase of free radical scavenging potential in comparison with non-treated cultures (Manivannan et al., 2016). Also, Kanthaliya et al. (2023) reported higher antioxidant activity under biotic elicitors treatment compared to control culture in in vitro propagated shoots of *Pueraria tuberosa*.

Therefore, elicitation can apply as a suitable tool for the increase of antioxidant properties in important medicinal plants.

In present investigation, the lowest and highest RSA recorded in control culture of stem (38.33%) and SA treated leaf cell extracts at 72 h exposure time (83.73%), respectively that were similar with the results of bioactive compounds content (TPC, TFC, withaferin A and withanolide A). These results indicated that antioxidant property of *W. coagulans* relate to presence these compounds and elicitation was improved antioxidant property through the induction the synthesis bioactive compounds. Our findings were similar to other researchers results such as Manivannan et al. (2016) (cell suspension cultures of *Scrophularia kakudensis*), Al-Khayri and Naik (2020) (cell suspension culture of date palm), and Klimak-Szczkutowicz et al. (2022) (watercress microshoot cultures) that reported the enhancement of radical scavenging potential in in vitro cultures by elicitors was due to the induction of secondary metabolites accumulation.

4. Conclusions

The present study showed that salicylic acid and cellulase elicitors in all treatment durations and both cell cultures derived from leaf and stem explants induced more accumulation of total phenolic and flavonoid, withaferin A and withanolide A as well as antioxidant activity in cell suspension culture of *W. coagulans*. The highest bioactive compounds and antioxidant potential occurred in SA elicitor treated leaf cell extracts at 72 h treatment duration. SA elicitor was effectively improved the accumulation of secondary metabolites compared to CE elicitor. Therefore, elicitation as a promising tool can be used to improve the synthesis of secondary metabolites for the production in commercial level. Also, cell suspension culture can be a suitable strategy to conserve natural resources indirectly.

Declarations

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Data will be made available by the corresponding author. The authors declare that there is no conflict of interest regarding the publication of this paper.

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Figures

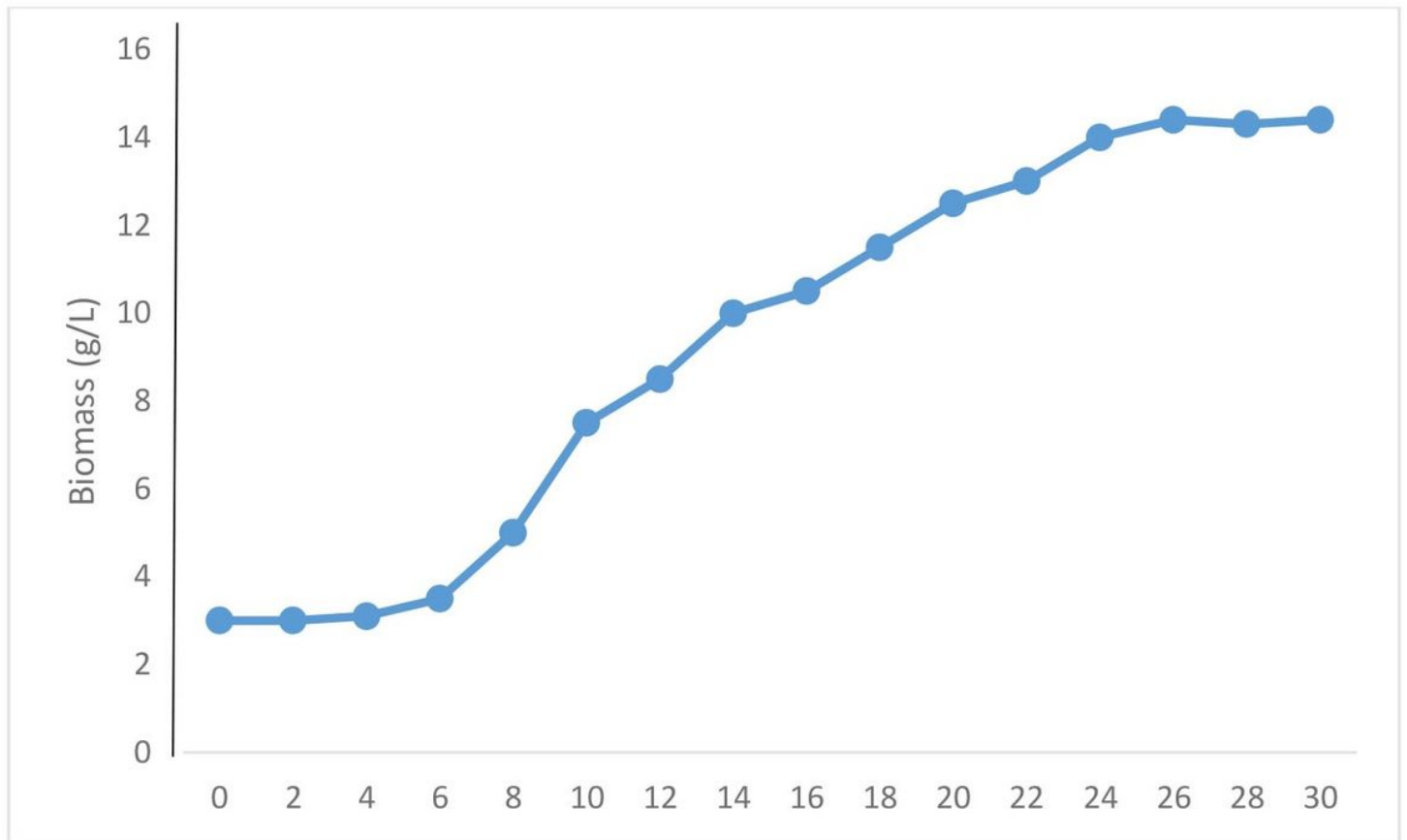
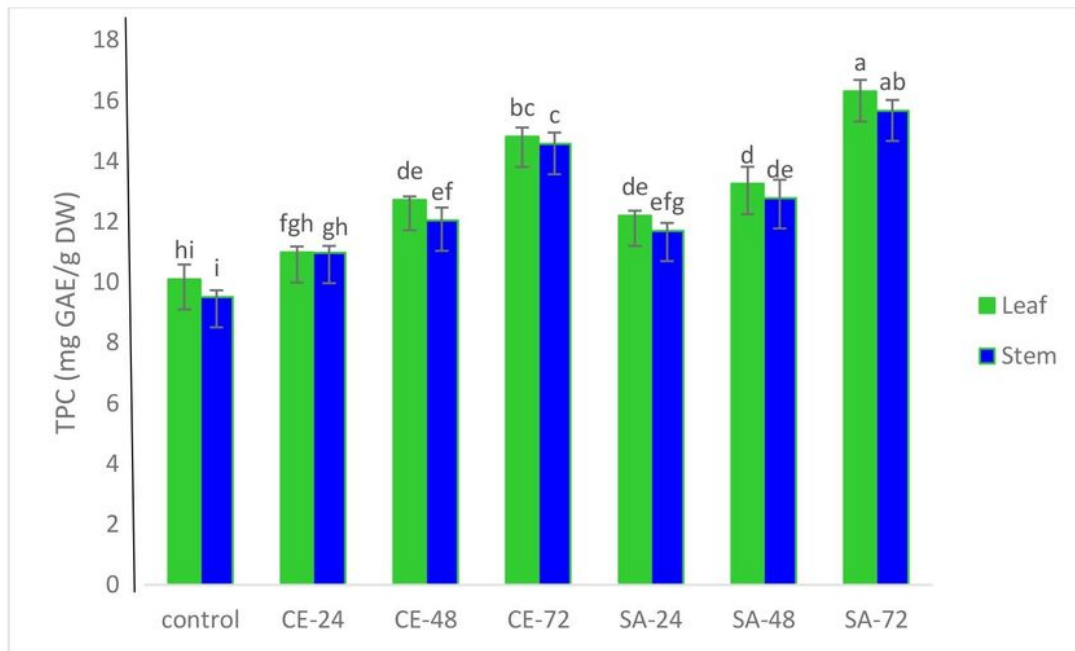
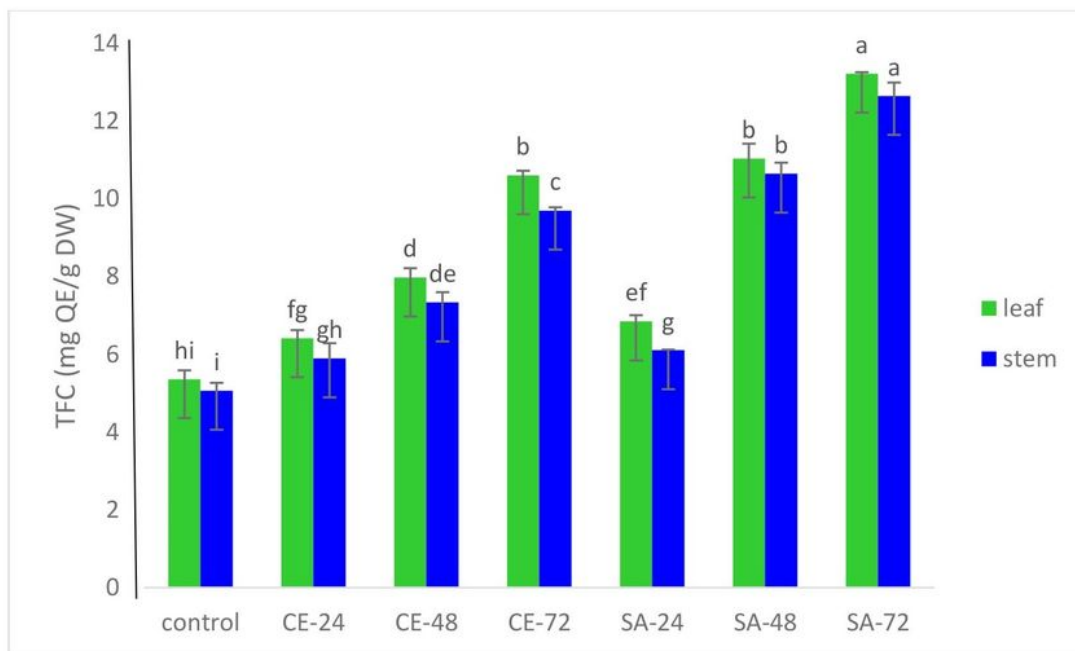


Figure 1

Growth curve in cell suspension culture of *W. coagulans*.



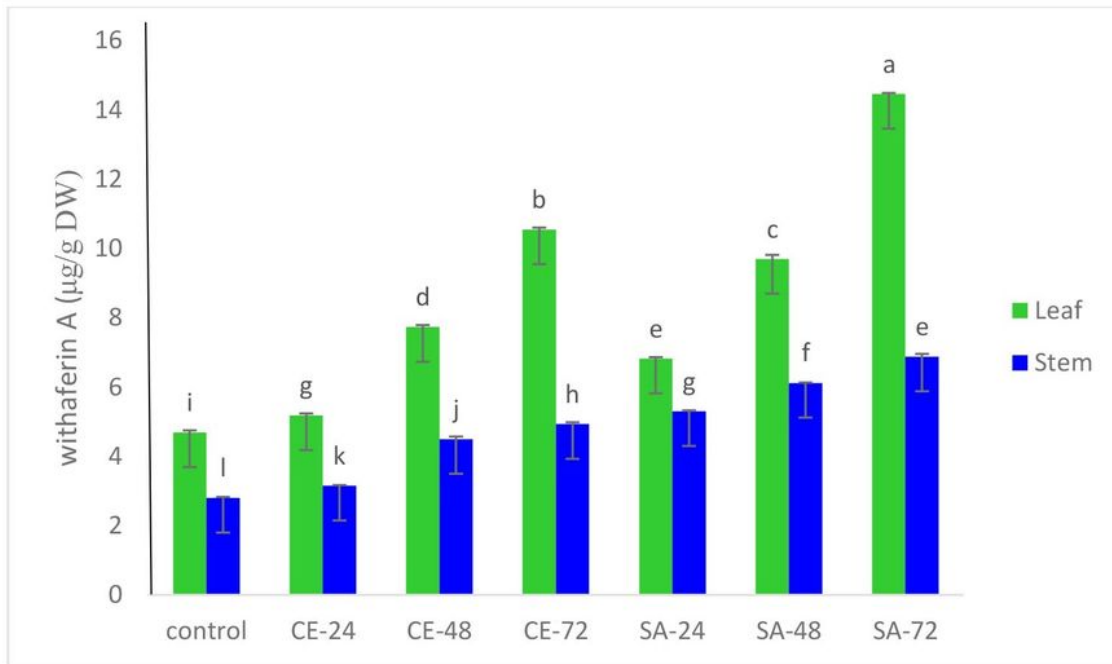
(a)



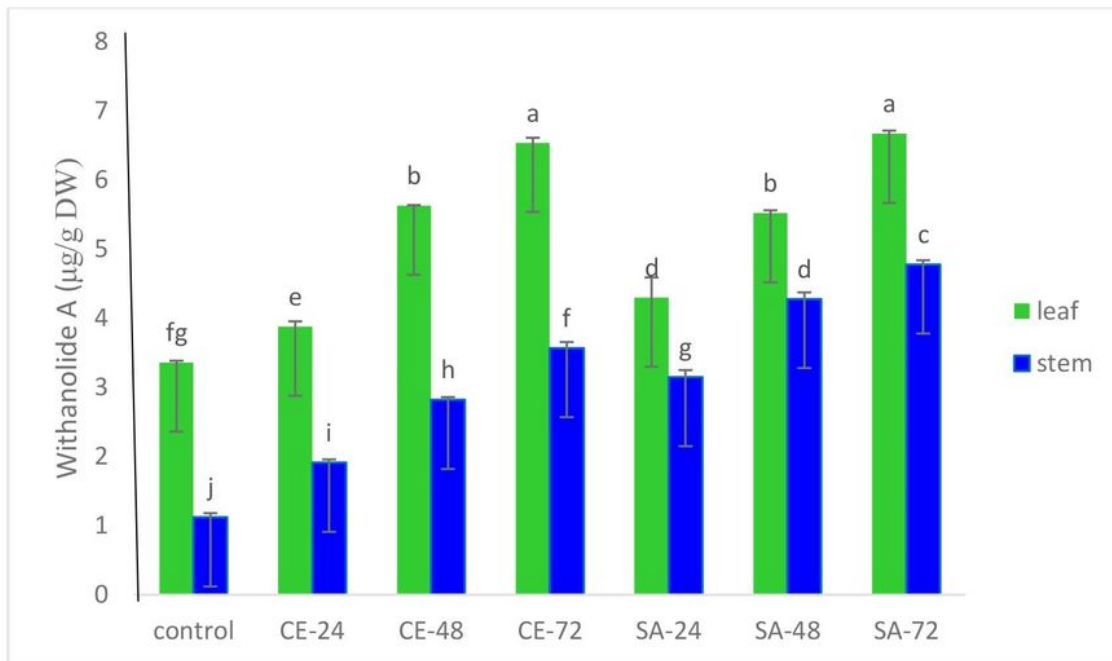
(b)

Figure 2

Total phenolic content (TPC) (a) and total flavonoid content (TFC) (b) in cell suspension cultures derived from leaf and stem explants of *W. coagulans* at 24, 48, and 72 h treatment durations after addition of salicylic acid (SA) and cellulase (CE) elicitors. GAE: gallic acid equivalents, QE: quercetin equivalents, DW: dry weight. Different letters indicate the significant difference at $p \leq 0.05$ level of probability.



(a)



(b)

Figure 3

Withaferin A (a) and Withanolide A (b) contents in cell suspension cultures derived from leaf and stem explants of *W. coagulanc* at 24, 48, and 72 h treatment durations after addition of salicylic acid (SA) and cellulase (CE) elicitors. DW: dry weight. Different letters indicate the significant difference at $p < 0.05$ level of probability.

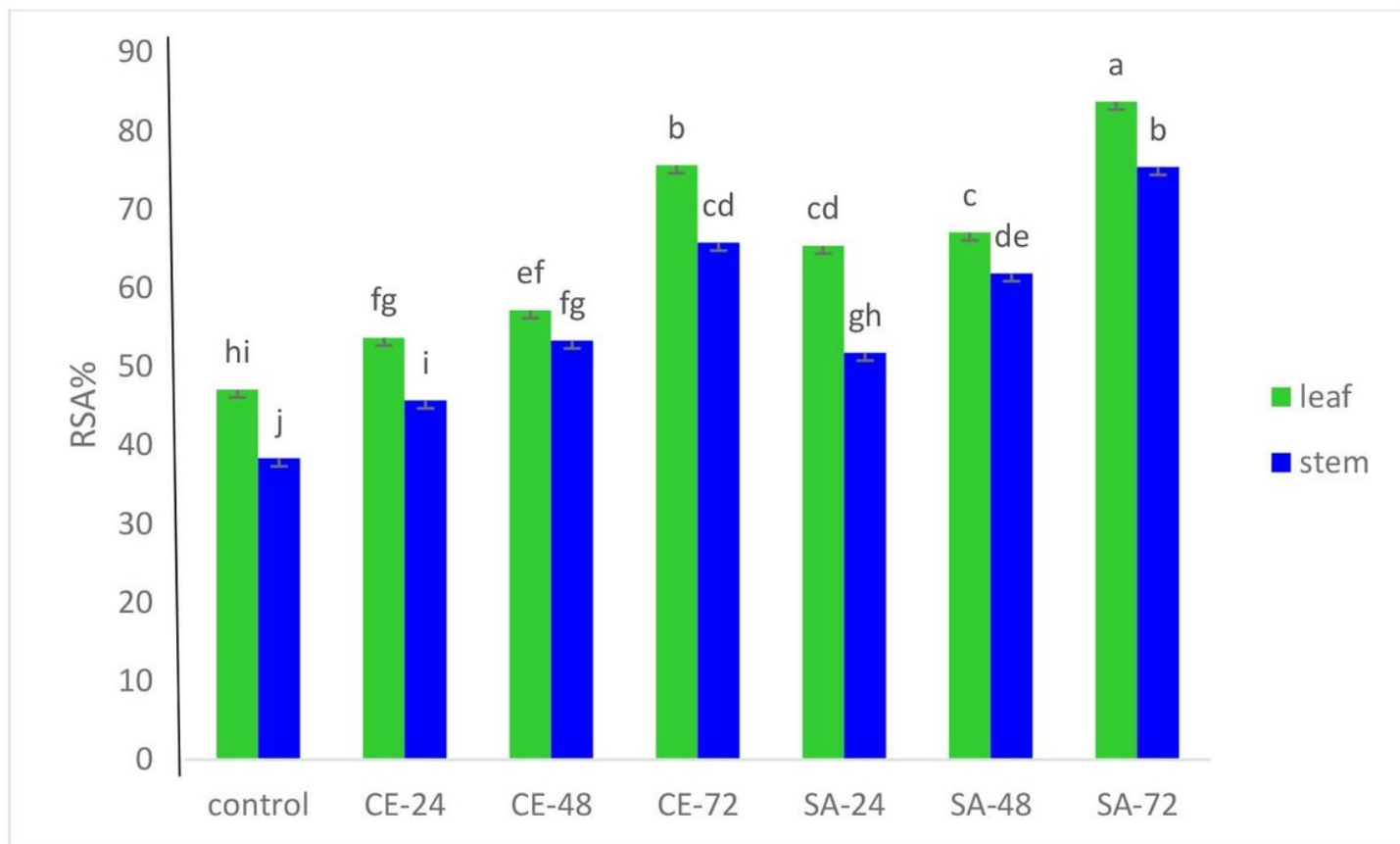


Figure 4

The percentage of radical scavenging activity (RSA) in cell suspension cultures derived from leaf and stem explants of *W. coagulanc* at 24, 48, and 72 h treatment durations after addition of salicylic acid (SA) and cellulase (CE) elicitors. Different letters indicate the significant difference at $p < 0.05$ level of probability.