

OPTIMIZING PODOPHYLLOTOXIN YIELD IN *PODOPHYLLUM HEXANDRUM* ROYLE IN-VITRO CULTURES: THE ROLE OF VARIOUS ELICITORS

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Abstract

The objective of this study was to assess the impact of various elicitors on the production of podophyllotoxin (PTOX), a potent anticancer agent, in in-vitro cultures of *Podophyllum hexandrum*, a plant known for its medicinal properties. The study focused on enhancing the production of PTOX in callus suspension cultures through the application of Chitosan, Sodium Chloride (NaCl), Salicylic Acid ($C_7H_6O_3$), and Sodium Alginate (NaAlg). Among these, Chitosan proved to be the most effective in stimulating PTOX production, followed by NaCl, NaAlg, and Salicylic Acid. The optimal concentrations for elicitation were determined as 50 mg L⁻¹ for both Chitosan and NaCl, 15 mg L⁻¹ for Salicylic Acid, and 20 mg L⁻¹ for NaAlg. The peak yield of PTOX, measured at 619.33 µg/g dry weight, was achieved using Chitosan at a concentration of 150 mg L⁻¹. However, it was observed that higher concentrations of Chitosan adversely affected the growth of the cultures. Additionally, the accumulation of PTOX was found to be dependent on the culture's growth phase, with the highest levels recorded during the exponential phase. Remarkably, cell suspension cultures treated with NaAlg demonstrated a fivefold increase in PTOX production compared to the untreated control, highlighting the potential of this approach in enhancing PTOX yield. This study establishes that employing elicitors can significantly augment the production of PTOX in *P. hexandrum* cultures. Chitosan, in particular, emerges as a promising elicitor for the biosynthesis of PTOX in callus suspension cultures of *P. hexandrum*.

Keywords: PTOX, cell suspension culture, elicitors, chitosan, NaCl, Sodium Alginate, Salicylic Acid

INTRODUCTION

P. hexandrum Royle, a perennial rhizomatous herb, grows in the Himalayan subalpine forests (Sharma *et al.*, 2000; Nadeem *et al.*, 2007; Chalise *et al.*, 2021). Its rhizome produces a resin called podophyllin, which contains several lignans that have antitumor effects. The most potent cytotoxic lignan is PTOX, which makes up 0.36-1.08% of the rhizome's dry weight (Uden *et al.*, 1989; Nadeem *et al.*, 2007; Aftab *et al.*, 2018). PTOX is in high demand, but *P. hexandrum* is rare because it has a long juvenile phase, low fruit set, and environmental adversities such as climate change. The plant is "critically endangered" due to overexploitation and lack of systematic cultivation (Foster, 1993; Choudhary *et al.*, 1998; CITES (2000); Hamayun *et al.*, 2006). Despite its great medical value, PTOX synthesis methods are inefficient and costly (Berkowitz *et al.*, 2000; Farkya *et al.*, 2004). Therefore, extracting PTOX from plants remains the only feasible option (Canel *et al.*, 2000). Many efforts have been made to enhance PTOX production from various plant sources (Konuklugil *et al.*, 1999; Smolny *et al.*, 1998; Mishra *et al.*, 2019; Shah *et al.*, 2021). However, isolating pharmaceutical compounds from wild plant biomass poses several challenges. Plant populations can be destroyed by overexploitation or natural disasters, affecting drug availability and quality. Additionally, different genotypes and environmental conditions can influence the drug profile of wild plants, resulting in purity issues. To overcome these problems, conventional and in vitro propagation techniques for *Podophyllum* sp. have been developed (Arumugam *et al.*, 1990; Majumder *et al.*, 2008; Satake *et al.*, 2015).

Since total chemical synthesis of PTOX is complicated and expensive, biotechnological approaches, particularly plant cell and tissue cultures, appear to be attractive alternatives for the production of this pharmaceutically important lignan. Induction of callus culture from *P. peltatum* and detection of PTOX from such cultures was reported in previous studies by (Kadkade, 1981; Uden *et al.*, 1989; Guerriero *et al.*, 2018; Muhammad *et al.*, 2021). Initiated PTOX-producing callus cultures from in vitro plantlets of the Indian *Podophyllum*; dark-grown. PTOX could be detected from all the callus lines that survived after 1 year of initiation, induced from different juvenile and mature explants. Callus cultures producing PTOX have also been initiated from needles of *C. drummondii*, *L. album*, *L. nodiflorum*, leaves of *Juniperus chinensis*, *P. peltatum* and *P. hexandrum* (Uden *et al.*, 1990; Konuklugil *et al.*, 1999; Smolny *et al.*, 1998; Muranaka *et al.*, 1998; Chattopadhyay *et al.*, 2002; Majumder *et al.*, 2008; Anbazhagan *et al.*, 2008).

To improve PTOX accumulation in in vitro cultures, several studies have optimized culture conditions, nutrient levels, and elicitor addition. Culture conditions directly affected biomass yield and PTOX accumulation. Callus cultures of *P. hexandrum* accumulated less PTOX in light than in dark (Archana & Lakshmi, 2000). Likewise, dark conditions enhanced cell growth and PTOX accumulation in suspension cultures of *P. hexandrum* (Uden *et al.*, 1989; Chattopadhyay *et al.*, 2002). Elicitation is a manipulative technique that increases secondary metabolite production (Gundlach *et al.*, 1992; Yukimune *et al.*,

1996; Furmanowa *et al.*, 1997; Berim *et al.*, 2005; Bhattacharyya *et al.*, 2012; Malik *et al.*, 2014; Largia *et al.*, 2022).

Plants produce secondary metabolites as a defense or wound response, and elicitors mimic these responses. Elicitors are signal molecules that activate novel genes encoding enzymes in different biosynthetic pathways. Many studies have reported the positive effects of elicitors on bioactive secondary metabolite accumulation (Uden *et al.*, 1998; Zabetakis *et al.*, 1999; Zhao *et al.*, 2005; Van *et al.*, 2005; Yousefzadi *et al.*, 2010; Satake *et al.*, 2015). For example, chito-oligosaccharides increased PTOX accumulation in callus cultures of *J. chinensis* (Muranaka *et al.*, 1998). Methyl jasmonate enhanced PTOX accumulation in suspension cultures of *Linum album* line X4SF (Furden *et al.*, 2005). Salicylic acid and Methyl Jasmonate significantly increased PTOX accumulation in cell suspension cultures of *P. hexandrum* (Majumder *et al.*, 2008). Based on the above literature, the present study aims to elicit PTOX in the cell suspension culture of *P. hexandrum* callus with various elicitors.

MATERIALS AND METHODS

Sterile conical flasks (50 to 500 ml), an orbital shaker, sterilized forceps, sterile filter paper, sterile beakers (50, 100, and 250 ml), sterilized scalpel blades and handles, test tubes, double-distilled water, a spirit lamp, cotton, teepol, a designated culture room, laminar airflow equipment, a pH meter, and a weighing balance. These items were essential to establish cell suspension cultures originating from calli obtained from cultured leaf explants.

Initiation and maintenance of suspension culture derived from callus tissue

For the initiation of suspension culture, the most compact calli that were derived from the leaf explants were selected. Different concentrations of auxins and cytokinins were added to the liquid MS medium that was used for initiating and maintaining the friable callus. The medium was autoclaved to sterilize it. The friable callus from one of the flasks was taken out with sterile forceps and sliced into 4-5 pieces of around 1 g each with a sterile scalpel blade. The pieces were then inoculated into the flasks that had 20 ml of liquid medium. The flasks were kept in the dark on an orbital platform shaker at $22 \pm 2^\circ\text{C}$ for cell separation. The cell suspension cultures were transferred to fresh medium every week.

Enhancing cell proliferation in an orbital shaker

Cell proliferation was optimized in an orbital shaker. The cells were grown in 500 ml flasks, each with 150 ml of MS medium supplemented with various concentrations of plant growth regulators and Ascorbic acid as an antioxidant agent (**Table.1**). The pH of the medium was set to 5.8 before autoclaving it. The cultures were maintained on an orbital shaker at a rotational speed of 120 rpm at 22°C in the dark. The suspension cultures were subcultured every 3-4 weeks.

Table 1: Composition of liquid MS medium for enhancing cell proliferation from callus induced from *P. hexandrum* leaf explants

Medium code (MS basal)	Cytokinins (mg L ⁻¹)	Auxins (mg L ⁻¹)	Antioxidants (mg L ⁻¹)
M1	BA (0.5-1.5 mg L ⁻¹)	2,4-D (0.5-3 mg L ⁻¹)	Ascorbic acid (50-100 mg L ⁻¹)
M2	KN (0.2-1 mg L ⁻¹)	2,4-D (2-4 mg L ⁻¹)	=
M3	BAP (0.5-2.5 mg L ⁻¹)	NAA (0.5-1mg L ⁻¹)	=
M4	(TDZ) (0.5-2 mg L ⁻¹)	NAA (0.5-1 mg L ⁻¹)	=

Treatment of Cell Suspension Cultures with Elicitors for the Enhanced Production of PTOX

The study investigated the effects of different elicitors, including sodium chloride, sodium alginate, salicylic acid, and chitosan, on the enhanced PTOX production in cell suspension cultures of *P. hexandrum*. Three different concentrations of each elicitor were tested to determine the optimal concentration for enhancing PTOX yield (**Table 4**). The concentrations were systematically varied to determine the optimal concentration that would promote higher PTOX yield without inducing adverse effects. The calli were subcultured every 15 days and transferred to fresh media. After 45 days of growth, the calli were treated with the elicitors for 24 to 48 hours. The elicitors were added to the culture media to stimulate the production of PTOX in the callus cells. The cultures were incubated at 25°C and 100 rpm in the dark. The treated callus cultures were monitored and analyzed to assess the effectiveness of the elicitors in promoting PTOX production through HPLC method.

Table 2: Different Elicitors for the enhancing PTOX yield in callus culture (MS media)

Treatments	Elicitors	Concentration (mg/L)
1	Sodium Chloride	25
2		50
3		100
1	NaAlg	20
2		25
3		50
1	Salicylic acid	5
2		10
3		15
1	Chitosan	100
2		150
3		200

Extraction and HPLC analysis of PTOX

Sample extraction and Preparation

Pieces of soft callus mass were dried at 45°C for 48 hours, weighed, crushed, and ground using a mortar and pestle. Ground samples (~0.5g dry weight) were soaked in ethanol for 24 hours, then filtered and evaporated to obtain the solid residue. The residue was

sonicated for approximately 15-30 minutes to enhance extraction of PTOX. After sonication, the extract was centrifuged to separate the supernatant, which contained PTOX, from the solid residue.

Preparation of (Standard) Calibration Solutions

Calibration solutions of known concentrations of PTOX were prepared in analytical grade methanol, which was also used as the extraction solvent for the callus sample. A stock solution of 1 mg/mL PTOX was prepared by dissolving PTOX standard in methanol. A series of calibration solutions with different concentrations (10 µg/mL to 100 µg/mL) were prepared by diluting appropriate volumes of the stock solution with methanol.

Calibration Curve

The calibration solutions were injected into the High Performance Liquid Chromatography (HPLC) system, and the chromatograms were recorded. The HPLC system consisted of a reverse-phase C18 column (250 x 4.6 mm, Bonda pack) and a UV-Vis detector (Shimadzu: Model: SPD-20A/LC-10A). A mobile phase of water: acetonitrile: methanol (40:30:25) was used as the eluent with a flow rate of 1 mL/min. An injection volume of 20 µL and a detection wavelength of 254 nm were used for all samples. The retention time of PTOX was about 8 min. A calibration curve was plotted with the peak area of PTOX versus its concentration in the calibration solutions. The peak area was chosen as the parameter for the calibration curve because it was more sensitive and accurate than the peak height.

HPLC Analysis

The solid residue obtained from the sample extraction was dissolved in methanol to achieve a final concentration of 1 mg/mL. Subsequently, an aliquot of 20 µL (microliters) of this solution was injected into the HPLC system. The chromatogram was monitored for the presence of the PTOX peak at a retention time of approximately 8 min. Quantification of PTOX in the callus sample was performed by comparing its peak area to the calibration curve, constructed by plotting the peak area of PTOX versus its concentration in the calibration solutions. The calibration curve, generated with five points ranging from 0.1 to 1.0 µg/mL, served as the basis for calculating the concentration of PTOX in the callus sample. The results were reported as PTOX content in the callus tissue in µg/g. To evaluate precision and accuracy, replicates of standard solutions and callus samples were analyzed. The mean and standard deviation of the PTOX content in the callus tissue were determined to be 0.56 ± 0.03 µg/g based on three replicates.

RESULTS AND DISCUSSION

In this experimental study, cell suspension cultures on MS media were exposed to different elicitors such as NaAlg, sodium chloride, salicylic acid, and chitosan at various concentrations for enhanced PTOX production. The results reveal that elicitors were the most effective in inducing the highest yield of PTOX compared to the control (**Table.3**).

These elicitors were introduced to the calli during the exponential growth phase, specifically in the third week for the biosynthesis of PTOX.

Table 3: PTOX content in callus culture of *P. hexandrum* with various elicitors

Elicitors	Treatments (mg/L)	PTOX content (µg/g DW)
Control	---	200.74
Chitosan	100	604.00
Chitosan	150	619.33
Chitosan	200	597.70
NaCl	25	472.66
NaCl	50	583.66
NaCl	100	457.33
NaAlg	20	410.70
NaAlg	25	416.66
NaAlg	50	423.00
Salicylic acid	05	383.00
Salicylic acid	10	392.00
Salicylic acid	15	401.66

Effect of Chitosan on PTOX Production in Cell Suspension Cultures of Rhizome-Derived Callus of *P. hexandrum*

The results showed that chitosan significantly enhanced the PTOX content in the callus cultures, with the highest increase (6.4-fold) (619.3 µg/g DW) observed at 150 mg L⁻¹ concentration (**Table.3** and **Figure.1**). However, higher concentrations of chitosan (200 mg L⁻¹) had a negative effect on PTOX production, indicating a dose-dependent response. The optimal concentration of chitosan was found to be 50 mg L⁻¹, which induced a 6.4-fold increase in PTOX content compared to the control. The PTOX accumulation was also influenced by the growth phase of the culture, with the highest level recorded at the exponential phase and the lowest level at the stationary phase. Chitosan treatments exhibited a range of PTOX yields, with the highest mean observed at 150 mg L⁻¹ (619.33 µg/g DW). This suggests that an intermediate concentration of chitosan is more effective in enhancing PTOX production compared to lower or higher concentrations. These findings suggest that chitosan can be used as an effective elicitor to stimulate PTOX biosynthesis in *P. hexandrum* callus cultures, and that the optimal concentration and timing of chitosan application should be carefully determined to achieve the maximum yield.

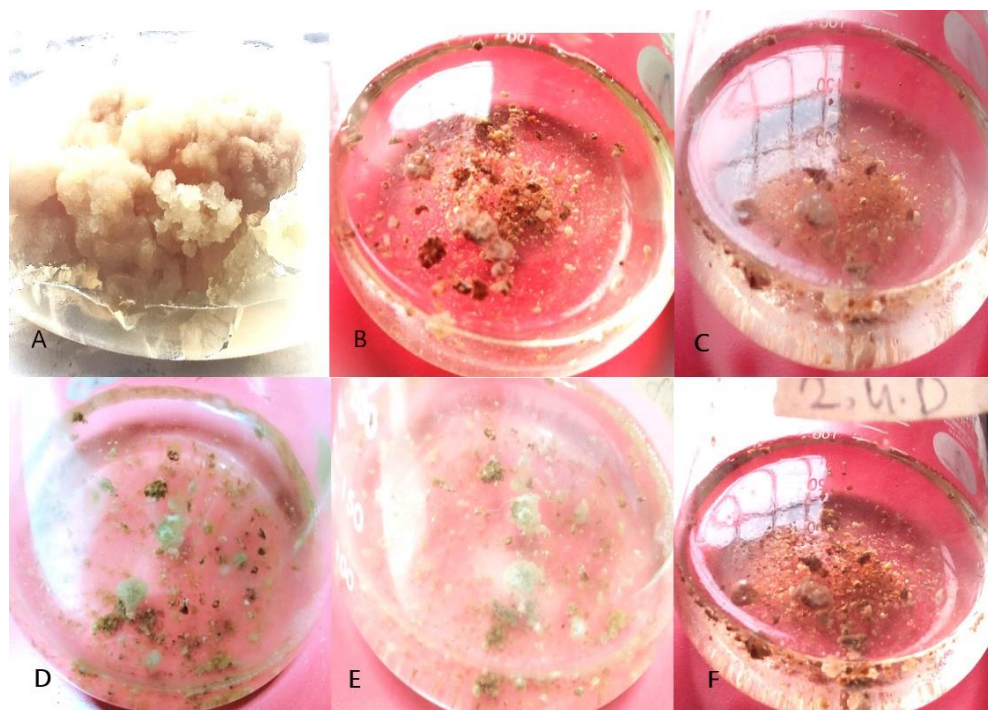


Figure 1

- A:** Callus culture induced from explants on MS medium supplemented with BA 1mg L⁻¹ and 2, 4-D 1.5mg L⁻¹
- B:** Callus suspension culture elicited at Chitosan (150mgL⁻¹)
- C:** Callus suspension culture of *P. hexandrum* elicited at Sodium Chloride (50mgL⁻¹)
- D:** Callus suspension culture elicited at Sodium Alginate (50mgL⁻¹)
- E:** callus suspension culture elicited at Sodium Alginate (50mgL⁻¹)
- F:** Callus suspension culture elicited at Salicylic acid (10mgL⁻¹)

The data obtained from this study are consistent with previous studies that reported the positive effect of chitosan on PTOX production in different plant species (Ferri & Tassoni, 2011). For example, Giri et al. (2016) reported that chitosan at 100 mg L⁻¹ increased the PTOX content by 3.1-fold in *P. peltatum* callus cultures. Nandy et al. (2021) demonstrated that chitosan at 0.5% increased the PTOX content by 3.6-fold in *P. hexandrum* cell suspension cultures. Ahmad et al. (2007) found that chitosan at 100 mg/L increased the PTOX content by 2.9-fold in *L. album* cell suspension cultures. These studies indicate that chitosan can act as a universal elicitor for PTOX production in different plant systems. A study by Singh et al reported that chitosan at 100 mg/L increased the PTOX content by 3.7-fold compared to the control, while salicylic acid at 100 µM decreased it by 50%.

A study by Sharma et al (2016) investigated the effects of chitosan and methyl jasmonate on the production of PTOX in the callus of *P. hexandrum*. They found that chitosan at 100

mg/L increased the PTOX content by 2.9-fold compared to the control. The mechanism by which chitosan enhances PTOX production is not fully understood, but it may involve the activation of defense-related genes and enzymes that are involved in the biosynthesis of PTOX. Chitosan can trigger the defense responses and secondary metabolite production in plants by mimicking the pathogen attack or wounding stress (Singh *et al.*, 2011; Bhattacharyya *et al.* 2012). According to a study by Rizwan *et al.* (2021), chitosan induced the expression of genes encoding phenylalanine ammonia-lyase (PAL), which is key enzyme in the lignan biosynthetic pathway that leads to PTOX formation (Govindaraju *et al.*, 2018). These results suggest that chitosan may enhance PTOX production by upregulating the lignan biosynthetic pathway in plant cells.

Effect of Sodium Chloride on PTOX Production in Cell Suspension Cultures of rhizome -Derived Callus of *P. hexandrum*

Sodium Chloride was the second most effective elicitor for PTOX production (583.66 µg/g DW), with the optimal concentration of 50 mg L⁻¹ (**Table.3**). NaCl can cause osmotic stress in plant cells, which may stimulate the accumulation of PTOX and other secondary metabolites. However, higher concentrations of NaCl (>100 mg L⁻¹) had a negative effect on both growth and PTOX production, indicating a threshold level of salt tolerance. The sodium chloride applied to cell suspension culture also triggered PTOX at three different concentrations (25mg L⁻¹, 50mg L⁻¹, 100mg L⁻¹) respectively. The results from the data (**Table.3 and Figure.1**) revealed that sodium chloride at 100 mg L⁻¹ enhanced PTOX content by 5.3 folds, higher than the control calli. The contents of the PTOX in the suspension cells after NaCl treatment varied with different concentrations time when exposed for the duration of 24hrs. The contents of the PTOX in the suspension cells were significantly increased with increased in the concentrations of NaCl from 25mg L⁻¹ to 100mg L⁻¹. NaCl treatments also showed variability in PTOX yield, with the highest mean at 50 mg L⁻¹ (583.66 µg/g DW). Interestingly, a concentration of 100 mg L⁻¹ NaCl resulted in a lower PTOX yield, indicating a non-linear response to NaCl concentration. NaCl could be suggested as applicable elicitor because of its ability to induce PAL in *P. hexandrum* cultures which serves as a precursor required for the biosynthesis of lignan (Kadkade, 1982; Yan *et al.*, 2019).

Effect of Salicylic Acid on PTOX Production in Cell Suspension Cultures of rhizome-Derived Callus of *P. hexandrum*

The cell suspension culture of *P. hexandrum* leaf-derived calli was tremendously initiated (Figure 3a), followed by strategic elicitation with a gradient of Salicylic Acid concentrations ranging from 5 to 15 mg/l inducing PTOX yield (**Table.3 and Figure.1**). Maximum PTOX (401.66) production was obtained at 15mg L⁻¹ Salicylic Acid. Through careful HPLC analysis, a striking and noteworthy 4-fold increase in PTOX accumulation was observed, specifically within the 24-hour timeframe of the cell suspension culture experiment, in stark comparison to the parallel control cell suspension culture (Fig. 3). Salicylic acid treatments exhibited a similar trend, with the highest mean at 15 mg L⁻¹ (401.66 µg/g DW). The results suggest that moderate concentrations of salicylic acid may be more

effective in eliciting PTOX production in *P. hexandrum* callus cultures This observation, while deeply captivating, also highlights the potency of Salicylic Acid in orchestrating a pronounced metabolic response in *P. hexandrum* cells. The influence of salicylic acid transcended mere metabolite accumulation, extending its influence to the broader growth and viability spectrum of *P. hexandrum* cells within the suspension culture system.

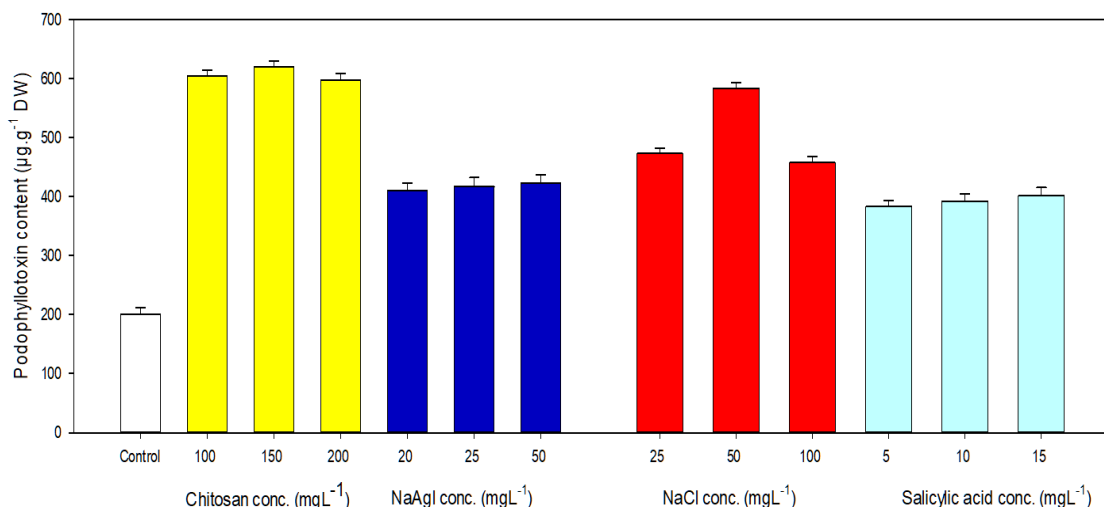


Figure 1: Effect of Chitosan, NaAl, NaCl and Salicylic Acid on PTOX Production in *P. hexandrum* Rhizome-Derived Callus Cultures

Salicylic Acid (SA) can increase secondary metabolites like PTOX in plant cell cultures. SA is a plant defense signal that activates secondary metabolite synthesis. Khawar et al. (2012) showed that SA boosted taxol, another secondary metabolite, in *Taxus chinensis* cells. This suggests that SA can also enhance PTOX in *P. hexandrum* cells.

Zanetti et al (2000) used supercritical fluid technology to make SA–alginate microparticles and tested them on *Linum album* hairy roots. They increased PTOX by 1.8-fold with 0.5% (w/w) SA–alginate. SA stimulates enzymes and genes for secondary metabolites in plants. Li et al. (2013) showed that SA induced artemisinin genes in *Artemisia annua*. This suggests that SA can also activate PTOX genes in *P. hexandrum* cells. Yousufzadi et al (2010) investigated the effects of SA that improved PTOX in *L. album* cells by 3-fold with 10 µM for 3 days. SA also upregulated PAL, CCR and CAD genes, which start PTOX synthesis. SA can control PTOX production by activating phenylpropanoid genes. Similarly, Wasternack and Hause (2013) studied the effects of SA on PTOX production in suspension cultures of *J. virginiana* and *L. flavum*. They compared the effects of Salicylic Acid (SA) with those of Cinnamic Acid (CA) and Jasmonic Acid (JA), which are also potential elicitors. They found that CA had the best effect on PTOX production, followed by SA and JA. In a nutshell, the use of Salicylic Acid as an elicitor to enhance the production of PTOX in *P. hexandrum* cell suspension cultures hold significant promise.

Effect of Sodium Alginate (NaAlg) on PTOX Production in Cell Suspension Cultures of rhizome-Derived Callus of *P. hexandrum*

The results presented in **Table.3** and **Figure.1** demonstrate a substantial production of PTOX in the 24-hour cell suspension culture treated with sodium alginate at different concentrations compared to the control (2.7 $\mu\text{g/g DW}$) cell suspension culture. NaAlg treatments displayed an increasing trend in PTOX yield with higher concentrations, with the highest mean at 50 mg L^{-1} (423.00 $\mu\text{g/g DW}$). This suggests a positive correlation between NaAlg concentration and PTOX production. The optimal PTOX yield (423 $\mu\text{g/g DW}$) was shown with the concentration of NaAlg 20 mg/L in suspension culture. This observation strongly suggests that the application of sodium alginate as an elicitor has effectively induced the biosynthesis of PTOX in *P. hexandrum* cell cultures. This increase in PTOX production could be attributed to the activation of specific biosynthetic pathways or upregulation of key enzymes involved in PTOX synthesis due to the stress response triggered by sodium alginate. The concentration of sodium alginate used in the study (20–50 mg L^{-1}) also plays a crucial role in elicitation efficiency. It's possible that the optimal concentration lies within this range, as exceeding it might lead to toxicity or adverse effects on cell growth and viability. Further optimization studies could help identify the precise concentration that maximizes PTOX production without negatively impacting the culture's overall health.

The use of plant cell suspension cultures is gaining attention for producing valuable secondary metabolites like PTOX, known for its pharmaceutical applications, especially in cancer treatment. Zhao et al. (2020) emphasize the potential of cell culture methods for sustainable and controlled PTOX production. NaAlg, derived from brown seaweeds, stands out for its biocompatibility, biodegradability, and gel-forming ability. SA hydrogels, formed by cross-linking with divalent cations like calcium, find applications in drug delivery, tissue engineering, and cell encapsulation. In plant cell cultures, NaAlg hydrogels show promise for enhancing secondary metabolite production, contributing to plant adaptation to environmental stresses (Shah, 1975; Cottrell & Kovacs, 1980; Connick et al., 1984; Andres, 1987; Golkar et al., 2019). In the study described, researchers employed sodium alginate as an elicitor in *P. hexandrum* rhizome-Derived calli cell suspension culture to enhance PTOX production. Elicitors are substances that stimulate the production of secondary metabolites in plant cells, and sodium alginate, a biopolymer derived from seaweed, has been reported to have elicitation effects on various plant cell cultures (Srujana and Bhagat., 2022; Khan et al., 2017; Mishra et al., 2019). In a study by Iqbal et al. 2000, they evaluated the effect of NaAlg and CaCl_2 on the development of synthetic seeds of *P. peltatum*. They found that NaAlg at 3% and CaCl_2 at 100 mM were the best concentrations for synthetic seed formation, and that these synthetic seeds had higher PTOX content than the control seeds. Sodium alginate is known for its ability to mimic the mechanical stress experienced by plants in their natural environment. This stress can lead to the activation of defense mechanisms, including the production of secondary metabolites. The elicitation effect of sodium alginate might have initiated a similar stress response in the cell cultures, resulting in enhanced PTOX production.

Additionally, sodium alginate might have facilitated better nutrient uptake and utilization, leading to increased biosynthesis of PTOX (Khan *et al.*, 201)

In conclusion, the use of Chitosan as an elicitor in *P. hexandrum* cell suspension culture has proven to be effective in enhancing PTOX production. The elicitors Chitosan, NaCl, NaAlg, and Salicylic acid influence PTOX yield in *P. hexandrum* callus cultures, but optimal concentrations vary. The 5.0-fold increase observed in the 24-hour cell suspension culture demonstrates the potential of this approach for obtaining higher yields of PTOX, a compound with significant pharmaceutical importance. This study contributes to the growing body of research on improving secondary metabolite production through plant cell culture techniques and highlights the potential of sodium alginate as a valuable elicitor in such systems.

CONCLUSION

P. hexandrum, recognized for its high PTOX content, is a crucial species in cancer treatment due to its potent anti-cancer lignin derivatives. This compound is instrumental in producing semi-synthetic derivatives such as etoposide and teniposide, which are effective against lung cancer, leukemia's, and various solid tumors. As a result, the demand for Podophyllum and its derivatives continues to rise. However, the natural slow-growing nature of *P. hexandrum* presents significant challenges in meeting the increasing demand for its valuable secondary metabolite, PTOX, necessitating biotechnological methods to boost its production. This study focused on augmenting PTOX production in cell suspension cultures of *P. hexandrum* through the application of different elicitors. The results identified chitosan as the most efficient elicitor, with NaCl, NaAlg, and salicylic acid following in effectiveness. The optimal concentrations for these elicitors were established as 50 mg/L for chitosan and NaCl, 15 mg/L for salicylic acid, and 20 mg/L for NaAlg. Notably, chitosan, at a concentration of 150 mg/L, significantly enhanced PTOX production, reaching a peak content of 619.33 µg/g dry weight. It's important to note, however, that higher chitosan concentrations adversely affected the growth of the cultures. Furthermore, the study revealed that PTOX accumulation was influenced by the growth phase of the culture, with the highest levels observed during the exponential phase. Impressively, the cell suspension culture treated with NaAlg exhibited a substantial 5.0-fold increase in PTOX production compared to the control. These findings underscore the effectiveness of using elicitors, especially chitosan, in enhancing PTOX production in *P. hexandrum* cultures. This approach could significantly advance biotechnological strategies for synthesizing PTOX."

Conflict of Interest:

The authors declare no conflict of interest.

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