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ELICITOR-MEDIATED AUGMENTATION OF PODOPHYLLOTOXIN BIOSYNTHESIS IN *PODOPHYLLUM HEXANDRUM* ROYLE: UNVEILING A REMARKABLE ANTICANCER POTENTIAL

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Abstract

Podophyllotoxin (PTOX), also known as podophyllin, is one of the most remarkable secondary metabolites of *Podophyllum hexandrum* (*P. hexandrum*). PTOX showcases a broad spectrum of therapeutic properties. Notably, PTOX and its derivatives demonstrate anticancer, antimicrobial, antiviral, antifungal, anti-inflammatory, antineoplastic, insecticidal, antiparasitic, and radioprotective effects. These compounds are utilized in the treatment of leukemia and rheumatoid arthritis. The objective of this investigation was to assess the impact of various elicitors on podophyllotoxin (PTOX) production, a valuable anticancer agent, in in-vitro cultures of *P. hexandrum*, a medicinal plant. The addition of chitosan, sodium chloride (NaCl), salicylic acid, and sodium alginate (NaAlg) enhanced podophyllotoxin production in callus suspension cultures. Results indicated that chitosan exhibited the highest efficacy among the elicitors, followed by NaCl, NaAlg, and salicylic acid. Optimal elicitor concentrations were determined to be 50 mg L-1 for chitosan, 50 mg L-1 for NaCl, 15 mg L-1 for salicylic acid, and 20 mg L-1 for NaAlg. Although the highest PTOX yield (619.33 µg/g DW) was achieved with chitosan at 150 mg L-1, higher concentrations adversely affected growth. PTOX accumulation was also influenced by the growth phase of the culture, with the exponential phase exhibiting the highest levels. Treatment with NaAlg resulted in a fivefold increase in PTOX compared to the control, highlighting the potential of this method for augmenting PTOX production. This study underscores the efficacy of elicitors in enhancing PTOX production in *P. hexandrum* cultures. Chitosan emerged as the most potent compound for stimulating podophyllotoxin production in callus suspension cultures of *P. hexandrum*, suggesting its promise as an elicitor for PTOX biosynthesis.

Key words: podophyllotoxin, cell suspension culture, elicitors, chitosan, NaCl, sodium alginate, and salicylic acid

1. Introduction

Medicinal plants play a crucial role in providing health, economic, aesthetic, and ecological benefits to the impoverished indigenous communities and the environments of tribal-dominated areas where access to medical facilities is limited (Mondal et al., 2023). plants serve as valuable sources of bioactive compounds, highlighting the significant potential for future utilization in the advancement of phytotherapy (Kitic et al., 2022). Plants rich in lignans have been utilized for centuries, dating back to at least 1000 years ago, as folk remedies in traditional Chinese, Japanese, and other Eastern medicinal practices (Cunha et al., 2012; Sharifi-Rad et al.,2 022). Lignans exhibit a plethora of biological activities that render them therapeutically valuable, including immunomodulatory effects, impacts on the cardiovascular system, anti-leishmania properties, hypolipemic effects, anti-inflammatory actions, as well as anti-fungal and anti-rheumatic properties (De Silva et a., 2019; Osmakov et al., 2022). These compounds can be isolated from various parts of plants, including leaves, stems, roots, rhizomes, seeds, and fruits, among others (Doughari et al., 2012; Sagar et al., 2018; Chepe et al., 2020). PTOX, a secondary metabolite, is primarily extracted from the roots and rhizomes of species belonging to the genus Podophyllum (family Berberidaceae). Within these species, a resin called podophyllin is produced, containing PTOX as its most abundant lignan (Chattopadhyay et al.,2002; Ahmad et al., 2007 Shah et la., 2021; Ivanova et al., 2021; Muhammad et al., 2021).

One of the most renowned aryltetralin lignans, distinguished for its potential in combating cancer and viral infection, is podophyllotoxin (PTOX; $C_{22}H_{22}O_8$) (Teponno et al., 2016; Cui et al., 2020). Podophyllotoxin being the most potent cytotoxic lignan is, 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). Podophyllotoxin is in high demand, but *P. hexandrum* is rare because it has a long juvenile phase, low fruit set, and environmental adversities such 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, podophyllotoxin synthesis methods are inefficient and costly (Berkowitz et al., 2000; Farkya et al., 2004). Therefore, extracting podophyllotoxin from plants remains the only feasible option (Canel et al., 2000). Many efforts have been made to enhance podophyllotoxin production from various plant sources (Konuklugil et al., 1999; Smollny 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 address these challenges and ensure a steady and sufficient supply of podophyllotoxin (PTOX), researchers have developed in vitro propagation techniques for Podophyllum sp. (Arumugam et al., 1990; Majumder et al., 2008; Satake et al., 2015).

The complexity and expense of total chemical synthesis make biotechnological approaches, particularly plant cell and tissue cultures, appealing alternatives for producing podophyllotoxin, a crucial pharmaceutical lignan (Ozyigit et al., 2023; Bouzroud et al., 2023). Previous studies, including those by Kadkade (1981), Uden et al. (1989), Guerriero et al. (2018), and Muhammad et al. (2021), reported the induction of callus culture from *P. peltatum* and the detection of podophyllotoxin from such cultures. They initiated podophyllotoxin-producing callus cultures from in vitro plantlets of the *P. hexandrum*, cultivated under dark conditions. Podophyllotoxin was detectable in all surviving callus lines one year after initiation, induced from various juvenile and mature explants. Additionally, callus cultures producing podophyllotoxin have 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; Smollny et al., 1998; Muranaka et al., 1998; Chattopadhyay et al., 2002; Majumder et al., 2008; Anbazhagan et al., 2008).

To enhance podophyllotoxin accumulation in in vitro cultures, numerous studies have focused on optimizing culture conditions, nutrient levels, and the addition of elicitors. These factors directly impact biomass yield and the accumulation of podophyllotoxin. For instance, it has been observed that callus cultures of *P. hexandrum* accumulate less podophyllotoxin under light conditions compared to dark conditions (Archana & Lakshmi, 2000). Similarly, dark conditions have been found to promote cell growth and podophyllotoxin accumulation in suspension cultures of *P. hexandrum* (Uden et al., 1989; Chattopadhyay et al., 2002). Elicitation, a manipulative technique aimed at enhancing secondary metabolite production, has been employed in several studies (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 part of their defense or wound response mechanisms, and elicitors are substances that mimic these responses. These signal molecules activate genes encoding enzymes in various biosynthetic pathways, leading to the production of novel compounds. Numerous studies have highlighted the positive impact of elicitors on the accumulation of bioactive secondary metabolites (Uden et al., 1998; Zabetakis et al., 1999; Zhao et al., 2005; Van et al., 2005; Yousufzadi et al., 2010; Satake et al., 2015). For instance, chito-oligosaccharides were found to increase podophyllotoxin accumulation in callus cultures of J. chinensis (Muranaka et al., 1998), while methyl jasmonate enhanced podophyllotoxin levels in suspension cultures of Linum album line X4SF (Furden et al., 2005). Additionally, salicylic acid and methyl jasmonate were shown to significantly boost podophyllotoxin accumulation in cell suspension cultures of *P. hexandrum* (Majumder et al., 2008).
Based on the findings from previous literature, the present study aims to elicit podophyllotoxin production in the cell suspension culture of *P. hexandrum* callus using various elicitors, as outlined below.

2. Materials and Methods

2.1 Essential Equipment for Establishing Cell Suspension Cultures from Leaf Explants

Sterile conical flasks (with capacities ranging from 50 to 500 ml), an orbital shaker, sterilized forceps, filter paper, sterile beakers (available in 50, 100, and 250 ml sizes), sterilized scalpel blades and handles, test tubes, double-distilled water, a spirit lamp, cotton, teepol, a dedicated culture room, laminar airflow equipment, a pH meter, and a precision weighing balance were all necessary components for establishing cell suspension cultures originating from calli obtained from cultured leaf explants.

2.2 Callus initiation in explants of *P. hexandrum*

Leaf explants were utilized for callus initiation, cultured on Murashige and Skoog (MS) media supplemented with various hormone combinations as outlined in Table 1). To prevent callus necrosis and browning, ascorbic acid (at concentrations of 50 and 100, mg L-1) was incorporated into the MS media. The cultured explants were incubated in a growth room at a temperature of 26 ± 2 ℃ under dark conditions for a duration of 45 days. Subsequently, after callus initiation, the cultures were maintained at the same temperature and relative humidity (54-65%) but subjected to a 16-hour photoperiod using cool fluorescent tubes. The percentage of callus induction was recorded for each combination. To mitigate browning and necrosis, explants were subcultured onto fresh media every two weeks, following a partially modified protocol based on the methodologies described by Sharma et al. (2022) and Nadeem et al. (2010).

2.3 Initiation and Maintenance of Suspension Culture calli

To initiate suspension culture, we selected the most compact calli derived from rhizome explants. These calli were obtained from wound-induced tissue responses. Next, we prepared liquid MS (Murashige and Skoog) medium with varying concentrations of auxins and cytokinins. After autoclaving the medium for sterilization, we took friable callus from one of the flasks using sterile

forceps. The callus was then sliced into 4-5 pieces, each weighing approximately 1 g, using a sterile scalpel blade. These callus pieces were inoculated into flasks containing 20 ml of the liquid medium. The flasks were kept in the dark on an orbital platform shaker at a constant temperature of 22±2°C to facilitate cell separation. Finally, we transferred the cell suspension cultures to fresh medium every week to maintain their growth and viability.

2.4 In vitro cell growth proliferation using an orbital shaker

Cell proliferation was maximized using an orbital shaker. The cells were cultivated in 500 ml flasks, each containing 150 ml of MS medium supplemented with different concentrations of plant growth regulators and Ascorbic acid serving as an antioxidant agent (see Table 1). The medium's pH was adjusted to 5.8 prior to autoclaving. Cultures were incubated on an orbital shaker set at a rotational speed of 120 rpm, maintained at 22°C in darkness. Subculturing of suspension cultures occurred 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	Auxins	Antioxidants
M1	BA (0.5-1.5 mg L^{-1})	2,4-D (0.5-3 mg L-1)	Ascorbic acid (50-100mg L-1)
M2	KN (0.2-1 mg L^{-1})	2,4-D (2-4 mg L^{-1})	Ascorbic acid (50-100mg L-1)
M3	BAP (0.5-2.5 mg L^{-1})	NAA (0.5-1mg L^{-1})	Ascorbic acid (50-100mg L-1)
M4	(TDZ) (0.5-2 mg L^{-1})	NAA (0.5-1 mg L^{-1})	Ascorbic acid (50-100mg L-1)

2.5 Enhancing PTOX Production in Cell Suspension Cultures through Elicitor Treatment

The study investigated the impact of various elicitors—sodium chloride, sodium alginate, salicylic acid, and chitosan on the enhanced production of podophyllotoxin (PTOX) in cell suspension cultures of *P. hexandrum*. Three different concentrations of each elicitor were evaluated to determine the optimal concentration for maximizing podophyllotoxin yield (Table. 2). These concentrations were systematically adjusted to identify the most effective concentration that would boost PTOX production without causing adverse effects. Calli were subcultured every 15 days and transferred to fresh media. Following 45 days of growth, the calli underwent treatment with elicitors for a duration of 24 to 48 hours. Elicitors were introduced into the culture media to stimulate podophyllotoxin production in the callus cells. The cultures were then maintained at 25°C with agitation at 100 rpm in darkness. The treated callus cultures were closely monitored and subjected to analysis to evaluate the efficacy of the elicitors in enhancing PTOX production, utilizing the HPLC method.

Table 2: Table 2: Various Elicitors for Enhancing PTOX Yield in Callus Culture

Treatments	Elicitors	Concentration in 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

2.6 Extraction and Analysis of Podophyllotoxin via HPLC

2.6.1 Sample Extraction and Preparation

Soft callus masses were dried at 45°C for 48 hours, followed by weighing, crushing, and grinding using a mortar and pestle. The ground samples (~0.5g dry weight) were soaked in ethanol for 24 hours, then filtered and evaporated to obtain solid residue. This residue underwent sonication for approximately 15-30 minutes to improve podophyllotoxin (PTOX) extraction. Subsequently, the extract was centrifuged to separate the supernatant, containing PTOX, from the solid residue.

2.6.2 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 the PTOX standard in methanol. Subsequently, a series of calibration solutions with different concentrations (ranging from 10 µg/mL to 100 µg/mL) were prepared by diluting appropriate volumes of the stock solution with methanol.

2.6.3 Generating the Calibration Curve:

The calibration solutions underwent injection into the HPLC system, and the resulting chromatograms were recorded. The HPLC system was equipped with 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 comprising water: acetonitrile: methanol (40:30:25) served as the eluent, with a flow rate set at 1 mL/min (milliliters per minute). All samples were injected with a volume of 20 µL (microliters), and detection occurred at a wavelength of 254 nm (nanometers). The retention time for PTOX was approximately 8 minutes. Subsequently, a calibration curve was constructed by plotting the peak area of PTOX against its concentration in the calibration solutions. The peak area was chosen as the parameter for the calibration curve due to its superior sensitivity and accuracy compared to peak height.

2.6.4 High Performance Liquid Chromatography (HPLC) Testing

The solid residue obtained from sample extraction was dissolved in methanol to achieve a final concentration of 1 mg/mL (milligrams per milliliter). Then, a 20 µL (microliter) aliquot 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 minutes. Quantification of PTOX in the callus sample involved comparing its peak area to a calibration curve. This curve was constructed by plotting the peak area of PTOX against its concentration in calibration solutions, which comprised five points ranging from 0.1 to 1.0 µg/mL.

The calibration curve served as the basis for determining the concentration of PTOX in the callus sample. Results were reported as PTOX content in the callus tissue in µg/g (micrograms per gram). Precision and accuracy were evaluated by analyzing replicates of standard solutions and callus samples. Based on three replicates, the mean and standard deviation of PTOX content in the callus tissue were determined to be 0.56 ± 0.03 µg/g.

3. Results and Discussion

In this experimental investigation, cell suspension cultures on MS media were subjected to various elicitors, including sodium alginate (NaAlg), sodium chloride, salicylic acid, and chitosan, at different concentrations to enhance PTOX production. The findings demonstrate that elicitors were significantly more effective in stimulating the highest yield of PTOX compared to the control **(**Table 3 and Figure 1**)**. These elicitors were administered to the calli during the exponential growth phase, specifically in the third week, to facilitate the biosynthesis of PTOX.

3.1 The Influence of Chitosan on Podophyllotoxin Production in Cell Suspension Cultures

The results indicate a significant enhancement in podophyllotoxin content within the callus cultures upon treatment with chitosan, with the most substantial increase (6.4-fold) observed at a concentration of 150 mg L-1 (619.3 µg/g DW) (Table 3 and Figure 1). However, higher chitosan concentrations (200 mg L-1) demonstrated a detrimental effect on podophyllotoxin production, suggesting a dose-dependent response. The optimal concentration of chitosan was identified as 50 mg L-1, resulting in a 6.4-fold increase in podophyllotoxin content compared to the control.

Furthermore, PTOX accumulation was influenced by the growth phase of the culture, with the highest levels recorded during the exponential phase and the lowest during the stationary phase. Chitosan treatments displayed varying PTOX yields, with the highest mean observed at 150 mg L-1 (619.33 µg/g DW). These findings suggest that an intermediate concentration of chitosan proves more effective in enhancing PTOX production compared to lower or higher concentrations.

Hence, the study underscores chitosan's potential as an effective elicitor for stimulating podophyllotoxin biosynthesis in *P. hexandrum* callus cultures. It emphasizes the importance of carefully determining the optimal concentration and timing of chitosan application to achieve maximum yield.

The data obtained from this study align with previous research indicating the positive impact of chitosan on podophyllotoxin production across various plant species (Ferri & Tassoni, 2011). For instance, Giri et al. (2016) observed a 3.1-fold increase in podophyllotoxin content in P. peltatum callus cultures upon treatment with chitosan at 100 mg L-1. Similarly, Nandy et al. (2021) demonstrated a 3.6-fold enhancement in podophyllotoxin content in *P. hexandrum* cell suspension cultures with chitosan at 0.5%. Ahmad et al. (2007) reported a 2.9-fold increase in podophyllotoxin content in L. album cell suspension cultures treated with chitosan at 100 mg/L. These studies collectively suggest that chitosan serves as a universal elicitor for podophyllotoxin production in various plant systems.

Additionally, Singh et al. (2011) found that chitosan at 100 mg/L led to a 3.7-fold increase in podophyllotoxin content compared to the control, while salicylic acid at 100 µM resulted in a 50% decrease, underscoring the efficacy of chitosan as an inducer of podophyllotoxin production.

Sharma et al. (2016) investigated the effects of chitosan and methyl jasmonate on podophyllotoxin production in *P. hexandrum* callus. They observed a 2.9-fold increase in podophyllotoxin content with chitosan at 100 mg/L compared to the control. Although the precise mechanism by which chitosan enhances podophyllotoxin production remains unclear, it is believed to involve the activation of defense-related genes and enzymes associated with podophyllotoxin biosynthesis. Chitosan may induce defense responses and secondary metabolite production in plants by mimicking pathogen attack or wounding stress (Singh et al., 2011; Bhattacharyya et al., 2012). According to Rizwan et al. (2021), chitosan induces the expression of genes encoding phenylalanine ammonia-lyase (PAL), a key enzyme in the lignan biosynthetic pathway leading to podophyllotoxin formation (Govindaraju et al., 2018). These findings suggest that chitosan may enhance podophyllotoxin production by upregulating the lignan biosynthetic pathway in plant cells.

3.2 The Influence of Sodium Chloride (NaCl) on Podophyllotoxin Production in Cell Suspension Cultures

NaCl emerged as the second most effective elicitor for PTOX production, yielding 583.66 µg/g DW at an optimal concentration of 50 mg/L (Table 3). NaCl can induce osmotic stress in plant cells, potentially stimulating the accumulation of PTOX and other secondary metabolites. However, concentrations exceeding 100 mg/L exhibited a negative impact on both growth and PTOX production, suggesting a threshold level of salt tolerance.

NaCl treatments were administered to the cell suspension culture at three different concentrations (25 mg/L, 50 mg/L, 100 mg/L). Results from the data (refer to Table 3 and Figure 1) demonstrated that sodium chloride at 100 mg/L enhanced PTOX content by 5.3-fold compared to the control calli. Furthermore, the PTOX contents in suspension cells varied with different concentrations and exposure

times (24 hours). Specifically, PTOX content significantly increased with higher concentrations of NaCl, ranging from 25 mg/L to 100 mg/L.
NaCl treatments also exhibited variability in PTOX yield, with the highest mean observed at 50 mg/L (583.66 µg/g DW). Interestingly, a concentration of 100 mg/L NaCl resulted in a lower PTOX yield, indicating a non-linear response to NaCl concentration. The induction of PAL in *P. hexandrum* cultures by NaCl suggests its potential as an applicable elicitor, as PAL serves as a precursor required for lignan biosynthesis (Kadkade, 1982; Yan et al., 2019).

3.3 The Influence of Salicylic Acid on PTOX Production in Cell Suspension Cultures
The cell suspension culture of *P. hexandrum* leaf-derived calli exhibited robust initiation (Figure 3.A), followed by strategic elicitation with a gradient of Salicylic Acid concentrations ranging from 5 to 15 mg/L, resulting in induced PTOX yield (Table 3 and Figure 1). Maximum PTOX production (401.66 µg/g DW) was attained at 15 mg/L Salicylic Acid concentration. Notably, careful HPLC analysis revealed a striking 4-fold increase in PTOX accumulation within the 24-hour timeframe of the cell suspension culture experiment, compared to the parallel control cell suspension culture (Fig. 1). Salicylic acid treatments demonstrated a similar trend, with the highest mean observed at 15 mg/L (401.66 µg/g DW).
These results suggest that moderate concentrations of salicylic acid may be more effective in eliciting PTOX production in *P. hexandrum* callus cultures. This observation underscores the potency of Salicylic Acid in orchestrating a pronounced metabolic response in *P. hexandrum* cells, extending its influence beyond mere metabolite accumulation to encompass the broader growth and viability spectrum within the suspension culture system.
Salicylic Acid (SA) has been shown to increase secondary metabolites such as PTOX in plant cell cultures. SA serves as a plant defense signal, activating secondary metabolite synthesis. Studies by Khawar et al. (2012) demonstrated that SA boosted taxol, another secondary metabolite, in Taxus chinensis cells, suggesting its potential to enhance PTOX in *P. hexandrum* cells.
Furthermore, research by Zanetti et al. (2000) utilized supercritical fluid technology to produce SA–alginate microparticles and tested them on Linum album hairy roots, resulting in a 1.8-fold increase in PTOX with 0.5% (w/w) SA–alginate. SA stimulates enzymes and genes for secondary metabolites in plants, as shown by Li et al. (2013) who demonstrated SA-induced artemisinin genes in Artemisia annua, suggesting its ability to activate PTOX genes in *P. hexandrum* cells. Yousufzadi et al. (2010) investigated the effects of SA, which improved PTOX in L. album cells by 3-fold with 10 µM for 3 days. SA also upregulated PAL, CCR, and CAD genes, initiating PTOX synthesis, and 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, comparing them with Cinnamic Acid (CA) and Jasmonic Acid (JA), other potential elicitors. They found that CA had the best effect on PTOX production, followed by SA and JA.
In conclusion, the utilization of Salicylic Acid as an elicitor to enhance the production of Podophyllotoxin in *P. hexandrum* cell suspension cultures holds significant promise.

3.4 The Influence of Sodium Alginate (NaAlg) on PTOX Production in Cell Suspension Cultures
The results presented in Table 3 and Figure 1 demonstrate a significant increase in PTOX production in the 24-hour cell suspension culture treated with sodium alginate at different concentrations compared to the control (2.7 µg/g DW) cell suspension culture. Sodium alginate treatments exhibited a rising trend in PTOX yield with higher concentrations, with the highest mean observed at 50 mg/L (423.00 µg/g DW), suggesting a positive correlation between sodium alginate concentration and PTOX production. The optimal PTOX yield (423 µg/g DW) was achieved with a sodium alginate concentration of 20 mg/L in suspension culture, strongly indicating the effective induction of PTOX biosynthesis by sodium alginate application. 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) 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 utilization of plant cell suspension cultures for producing valuable secondary metabolites such as PTOX is gaining traction, particularly for its pharmaceutical applications, notably in cancer treatment. Zhao et al. (2020) highlight the potential of cell culture methods for sustainable and controlled PTOX production. NaAlg, derived from brown seaweeds, is notable 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 described study, 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), the effect of sodium alginate (NaAlg) and calcium chloride ($CaCl_2$) on the development of synthetic seeds of *P. peltatum* was evaluated. 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., 2011). In conclusion, the utilization of Chitosan as an elicitor in *P. hexandrum* cell suspension culture has shown significant effectiveness in enhancing PTOX production. While Chitosan, NaCl, NaAlg, and Salicylic acid all influence PTOX yield in *P. hexandrum* callus cultures, their optimal concentrations vary. The remarkable 5.0-fold increase observed in the 24-hour cell suspension culture underscores the potential of this approach for achieving higher yields of PTOX, a compound of considerable pharmaceutical importance. This study contributes to the expanding body of research aimed at enhancing secondary metabolite production through plant cell culture techniques and highlights the promising role of sodium alginate as a valuable elicitor in such systems.

Table 3: Elicitation of PTOX Content Influenced by Various Elicitors in Callus Suspension Culture of *P. hexandrum*

Elicitors	**Treatments (mg/L)**	**Podophyllotoxin 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	5	383.00
Salicylic acid	10	392.00
Salicylic acid	15	401.66

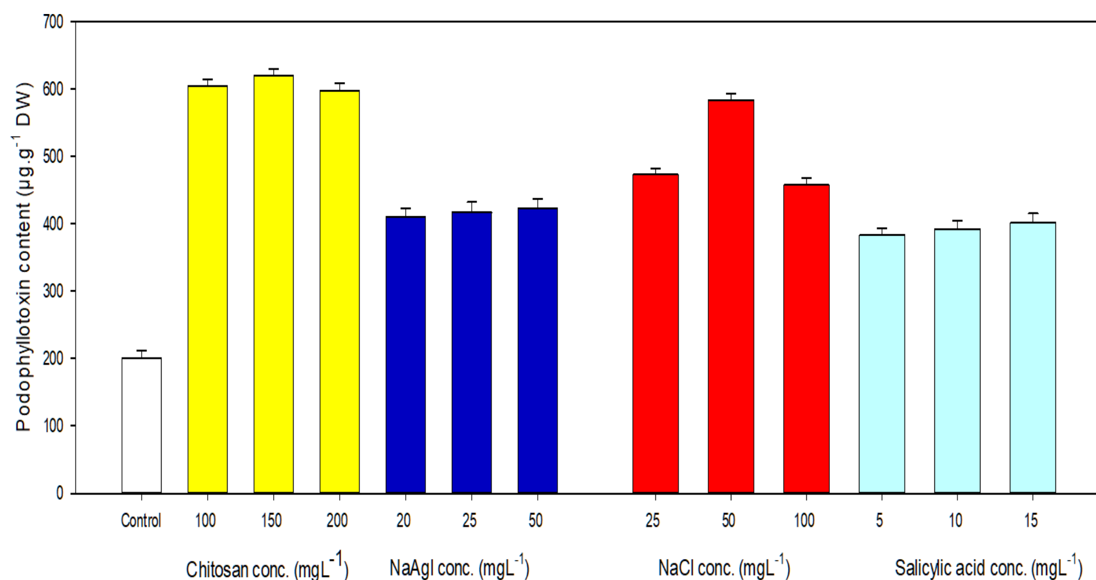

Figure 1: Effect of Chitosan, NaAl, NaCl and Salicylic Acid on PTOX Production in

P. hexandrum Rhizome-Derived Callus Cultures

Figure 2.

A: Callus culture induced from explants on MS medium supplemented with 1mg L-1 BA and 1.5mg L-1 2,4-D,
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).

4. Conclusion

P. hexandrum Royle, renowned for its PTOX content, holds immense significance in cancer treatment due to the efficacy of its derivatives like etoposide and teniposide against various cancer types.

However, the natural growth limitations and slow propagation of *P. hexandrum* present substantial challenges, hindering the meeting of society's demands for its valuable secondary metabolite, PTOX. Consequently, there is a pressing need for biotechnological interventions to boost its production. This study aimed to augment PTOX production in cell suspension cultures of *P. hexandrum* using different elicitors. Results identified chitosan as the most effective elicitor, followed by NaCl, NaAlg, and salicylic acid. Optimal concentrations for elicitors were determined as 50 mg/L for chitosan, 50 mg/L for NaCl, 15 mg/L for salicylic acid, and 20 mg/L for NaAlg. Chitosan emerged as the most potent compound in stimulating PTOX production in callus suspension cultures of *P. hexandrum*, yielding the highest PTOX content (619.33 µg/g DW) at 150 mg/L. However, higher concentrations of chitosan adversely affected growth. PTOX accumulation varied with the growth phase of the culture, with the exponential phase showing the highest levels. The NaAlg-treated cell suspension culture exhibited a notable 5.0-fold increase in PTOX compared to the control, indicating the promising potential of this approach for enhancing PTOX production. These findings underscore the efficacy of elicitors, particularly chitosan, in enhancing PTOX production in *P. hexandrum* cultures, opening avenues for improved biotechnological strategies in podophyllotoxin synthesis and ensuring a steady and sufficient supply of PTOX for the treatment of cancers, bacterial and viral infections.

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