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Stem cells of *Impatiens tinctoria* A.Rich tuber and antioxidant activity of their extracts

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Abstract

The use of plant stem cell extracts as active ingredients in cosmetic formulations has gained popularity recently, highlighting the need to establish *Impatiens tinctoria* A. Rich *in vitro* cultures in order to prepare bioactive stem cell extracts. This study is aimed at establishing cell suspension culture and evaluates the antioxidant activity of the stem cell extract. In order to initiate callus and cell suspension cultures, tuber explants were inoculated onto Murashige and Skoog (MS) media containing 2,4-D (0.5–2 mg/L) and BAP (0–2 mg/L). Optimal hormone concentrations of 2 mg/L 2,4-D and 1.5 mg/L BAP were sufficient to produce callus. The obtained callus was utilized as the inoculum to start a cell suspension culture for the production of stem cell extracts. High biomass accumulation was obtained at 30 g/L sucrose concentration and 6 g inoculum size. The stem cell extract had a total phenolic content of 4.6 µgGAE/mL and a flavonoid content of 190.96 µgQE/mL. DPPH scavenging activity of 95.82% and the IC50 value of 37.54 µg/mL was detected for the stem cell extract. The study indicated that the suspension cultures of *I. tinctoria* A. Rich have the potential to produce stem cell extracts with increased flavonoids content and antioxidant activity.

Keywords**:** *Callus; cell suspension culture; stem cell extract; impatiens tinctoria; antioxidant activity*

1. Introduction

Impatiens tinctoria A.Rich is a member of the Balsaminaceae family, which is known by its vernacular names, “Balsamine” in English and “Ensosila” in Amharic (Yu et al., 2016; Degu et al., 2020). It is found in moist and shady areas of forests, along streams and shady banks, and at the edges of woods and gullies. The plant has been grown for its cosmetic uses in southern Tigray, Ethiopia (Amede, & Taye, 2015). Ethiopians have been using *I. tinctoria* tuber as a traditional folk medicine for a variety of diseases such as fungal infections, diseases of the mouth and throat, and clean wounds aseptically. Its root decoction is consumed as a purgative and to relieve stomach pains (Seboka, 2017; Degu et al., 2020).

According to Degu et al., (2021) report, the tuber extracts exhibit antifungal and antibacterial properties, indicating their therapeutic potential. Furthermore, *I. tinctoria* tuber dye is widely used by rural Ethiopian women to color their nails and hair, as well as to draw intricate patterns on their hands, palms, faces, and feet (Abebe, & Ayehu, 1993). To use it for traditional cosmetic purposes, the tuber is cleaned, pounded, cut, steeped for a minimum of 12 hours, cooked, crushed, and used as a paste to color beards, nails, and hair (Degu et al., 2020). Ethiopian women consider the tuber as a rich source of bioactive

compounds that strengthen and improve skin health. Ponded tuber juice is also used to make red ink.

Recent studies indicated that cosmetic products containing plant extracts as active ingredients have been shown to stimulate human skin stem cells for self-renewal (Caucanas et al., 2011; Lequeux et al., 2011). There were already several plant stem cell extract-containing cosmetics available on the market. These skin-care products have hydrating, moisturizing, anti-aging, anti-wrinkling, anticancer, and regenerative and wound healing effects on the skin (Adli et al., 2024; Baluchamy, & Subramanian, 2023). Many of them act by either increasing the synthesis of collagen and elastin or decreasing the degradation of collagen (Caucanas et al., 2011; Lequeux et al., 2011).

I. tinctoria's tuber is a rich source of a variety of secondary metabolites (Degu et al., 2020; Gidamo, 2023). These chemicals have antibacterial, wound healing, and antioxidant properties (Degu et al., 2021; Gidamo, 2023). The compound 2-methoxy-1, 4-naphthoquinone is the main coloring agent (Fassil, 1981; Chen et al., 2020). The lawson in henna and this compound are similar in that they both contain 1,4-naphthoquinone rings. To date, *in vitro* studies of *I. tinctoria* to enhance the production of medicinally important secondary metabolites such as phenols, flavonoids and 2-methoxy-1, 4-naphthoquinone have not been done.

With the increasing demand for natural products, most cosmetic industries prefer to incorporate botanical extracts as active ingredients. However, their limitations include scarce natural resources, slow growth of plants, seasonal harvesting, variations in active concentrations from plant to plant, harvest to harvest and extraction techniques, limited activity of the ingredient and existence of some toxic metabolites such as phytochemical allergens. Furthermore, the application of extracts obtained from intact plants for the development of botanicals-based cosmetics is hampered by oxidizing agents that make the extract turn brown (Lee et al., 2010).

In vitro cultured plant cells show fast growth with uniform biomass, improved purity, quality and efficacy of the botanical active ingredient. In addition, the *in vitro* culturing process is an eco-friendly, and a non-GMO approach that is easily acceptable by consumers. Another benefit of culturing plant cells is its capability of stimulation by UV radiation, jasmonic acid or toxic substances for higher production of active plant extracts. Furthermore, extracts from cultured cells meet the colourless requirements of cosmetic ingredients (Lee et al., 2010).

Studies in medicinal plants described the development of callus and suspension cultures. However, very little information is available in the literature regarding *I. tinctoria A*. Rich. Realizing the usefulness *I. tinctoria* and its extracts in cosmetics, this study attempted to establish callus, cell suspension cultures and describe phenolic, flavonoid content and antioxidant potential of the stem cell extract.

2. Objectives

This investigation aimed to establish cell suspension culture of *I. tinctoria* and evaluating antioxidant activity of the stem cell extracts derived from the suspension cultures.

3. Materials and methods

3.1 Plant material

Academic approval for the study and collection of the medicinal plant was given by Addis Ababa Science and Technology University, Ethiopia. The study conforms to all applicable laws and rules. *I. tinctoria* A. Rich was collected from Akaki, Ethiopia. Plant material with the voucher specimen GH-001 has been kept in Addis Ababa University (AAU) herbarium after identification.

3.2 Explant preparation

The explant was prepared first by washing with tap water, and then with distilled water. Then, they were immersed in 70% ethanol for one minute and rinsed with sterile distilled water. Then, the samples were sterilized for 15 minutes using a 5% (v/v) bleach (NaOCl) solution. To remove chemical residue from plant tissue, the samples were rinsed gently with sterile distilled water. Using a sterilized scalpel, the damaged portions were removed, and 1cm long sliced tuber explants were inoculated into the MS medium. The culture jars were kept in the growth room in the dark at room temperature (Rahiman, & Taha, 2011).

3.3 Callus induction

Callus was induced from sterile explants inoculated in MS medium containing BAP (0, 0.5, 1.0, 1.5, and 2.0 mg/L); 2, 4-D (0.0, 0.5, 1.0, 1.5, and 2 mg/L); and combinations of the hormones. The experiment used a fully randomized factorial design with three replications. After four weeks of culture incubation in the dark, the percent callus induced was recorded (Rahiman, & Taha, 2011).

3.4 Initiation of cell suspension culture

The cell suspension culture initiation was carried out according to standard protocols (Motolinia-Alcántara et al., 2023). 0.5 g of the callus formed on the explant was transferred to fresh liquid medium in 250 mL Erlenmeyer flasks containing 30 mL MS medium supplemented with 100 mg/L myo-inositol, 30 g/L sucrose, 2 mg/L 2, 4-D and 1.5 mg/L BAP. The culture was kept dark at 25°C, under constant orbital shaking at 110 rpm. The suspension culture was subcultured every 14 days. During subculturing, the biomass was diluted 1:3 ratio and clump calli were removed to get cell suspensions (Lee et al., 2010).

3.5 Plant stem cell extracts preparation

For stem cell extract preparation, cultured cells were filtered using a Büchner funnel and filter paper. The left-over medium was then removed by washing the cells with distilled water and phosphate saline buffer. Following filtering and washing, cells were freeze-dried for 24 hours at -80°C to achieve a consistent dry weight. The stem cell extract was prepared by resuspending the lyophilized cells in distilled water (10%w/v), homogenized, and centrifuged at 10,000 rpm at 4°C (Wang et al., 2023). The supernatant obtained was used as the stem cell extract of *I. tinctoria.*

3.6 Determination of total phenolic content

By using Folin-Ciocalteu method, the total phenolic content of the extract was evaluated. In a test tube containing 0.5 mL extract (10 µg/mL), 2.5 mL of a Folin-Ciocalteu reagent and 2 mL of 7.5% sodium carbonate (Na_2CO_3) was added. After vortexing, it was left to stand at room temperature in the dark for half an hour. The absorbance of the solution at 765 nm was recorded using UV-visible spectrophotometry. Gallic acid was used to prepare the standard curve and the total phenolic content of the extract was estimated and expressed as µg gallic acid equivalents (GAE) per milliliter (Gazwi et al., 2023).

3.7 Total flavonoid analysis

With the use of the aluminum chloride colorimetric method, the total flavonoid content of the *I. tinctoria* stem cell extract was determined. A 0.5 mL of the stem cell extract (10 µg/mL), 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1Mpotassium acetate, and 2.8 mL of distilled water was added to 10 mL test tube and vortexed (Kalita et al., 2013, Mehmood et al., 2022). Absorbance of the solution was recorded at 415 nm using UV-visible spectrophotometry. Quercetin was used to prepare the standard curve and the flavonoid content of the stem cell extract was estimated and expressed in µg of quercetin equivalent (QE) per milliliter.

3.8 DPPH radical scavenging activity

To evaluate DPPH scavenging capacity, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was used. One milliliter of 0.1 mM DPPH solution, and 3 mL of the stem cell extract solutions with concentrations of 0.25 µg, 0.5 µg, 1 µg, and 2 µg/mL were mixed and allowed to stand in the dark at room temperature for 30min. Absorbance of the solution was recorded at 515 nm using UV-visible spectrophotometry. L-ascorbic acid was used to prepare standard curves. The percent DPPH scavenging activity (I%) of the stem cell extract was calculated using the equation:

$$(I\ \%) = \left[\frac{(Absorbance\ of\ control - Absorbance\ of\ sample)}{Absorbance\ of\ control}\right] * 100 \quad (1)$$

where the absorbance of control is the absorbance of the control solution (containing all the reagents, except the test sample (Ayele et al., 2022). A plot of DPPH percent inhibition and log-transformed values of the extract concentrations was used to calculate the IC50 values of the stem cell extract (Gidamo, 2023).

3.9 Assay for 2-methyl-1, 4-naphthoquinone

Lyophilized samples (100 mg) were pulverized and extracted with 5 mL of methanol. Additionally, 5 mL cell-free liquid medium was twice extracted with methanol. The organic phases of the extracts were combined and dried. These dried samples were dissolved in 0.1 mL methanol. Before HPLC analysis, the solutions were filtered through a 0.45 µm filter. The reverse phase C18 (5 µm, 250 mm x 4.6 mm) column was used for HPLC analysis. Acetonitrile and water (35:65, v/v) with flow rate of 1 mL/min were used as mobile phases. The detection wavelength was set at 266 nm, and an injection volume of 20 µL was used for the analysis (Foong et a., 2020). Authentic 2-methyl-1,4-naphthoquinone was purchased from Sigma (USA). The stem cell extract's naphthoquinone content was determined using the 2-methyl-1,4-naphthoquinone calibration curve. The concentrations in the samples were estimated as the combined concentrations of naphthoquinones in cells and cell-free liquid media (Cheng et al., 2006).

3.10 Statistical analysis

The total phenolics, flavonoids, and DPPH radical scavenging activities of *I.tinctoria* stem cell extract were examined using triplicate data. Analysis of variance (ANOVA) was done using R version

4.3.1. The group means' significance was determined using the Tukeyt test at 5% significance level (Gomez, & Gomez, 1984).

4. Results

4.1 Induction of callus cultures from *I. tinctoria* A.Rich tuber

From the *I. tinctoria* A.Rich tubers, friable calli were obtained in a Murashige and Skoog (MS) medium. Maximum callus induction (100%) was obtained at 2 mg/L of 2, 4-D and 1.5 mg/L of BAP hormone concentrations (Table 1). The friable calli showed white color, and the cell mass progressively increased over time in a solid medium, allowing for the cell aggregate suspension establishment. Callus showing good growth and friable texture was selected for subsequent subculturing and went through 7 generations of subculturing. Callus induction rate of 100% was observed for the tuber explants in media fortified with 2 mg/L of 2,4-D and 1.5 mg/L of BAP (Table 1). The earliest callus induction time was 30 days, while the longest callus induction time was 44 days.

Table 1 Optimization of hormones for callus induction

Combinations of hormones		Callus indication rate (%)±SD	Fresh weight (g)±SD	Time of callus induction(day) ±SD
2,4-D(mg/L)	BAP(mg/L)			
0	0	0±0^{a}	0±0^{a}	0±0^{a}
	0.5	0±0 ^{a}	0±0 ^{a}	0±0^{a}
	1	0±0 ^{a}	0±0 ^{a}	0±0^{a}
	1.5	0±0 ^{a}	0±0 ^{a}	0±0^{a}
	2	0±0 ^{a}	0±0 ^{a}	0±0^{a}
0.5	0	0±0 ^{a}	0±0 ^{a}	0±0^{a}
	0.5	33.25 ±1.06^{c}	0.85±0.04 ^{abc}	44±3.5 ^{b}
	1	36.45±0.77 ^{abc}	0.93±0.05^{abc}	43±2.8^{b}
	1.5	36.0±1.4 ^{abc}	0.91±0.06^{abc}	40±2.1^{b}
	2	38.4 ±1.27^{abc}	0.89±0.04^{abc}	42±1.4^{b}
1	0	26.4 ±1.13^{b}	0.58±0.05^{bc}	41±1.4^{b}
	0.5	27.15±0.92 ^{b}	1.13±0.14^{abc}	42±1.4^{b}
	1	39.1 ±0.84^{abc}	0.92±0.02^{abc}	41±1.4^{b}
	1.5	46.5±1.27 ^{abcd}	0.85±0.03^{abc}	41±1.4^{b}
	2	42.5 ±1.06^{abc}	0.74±0.03 ^{abc}	38±1.4^{b}
1.5	0	39.35 ±0.63^{abc}	0.63±0.05^{cd}	39±1.4^{b}
	0.5	37.35 ±1.2^{abc}	0.77±0.08^{abc}	39±1.4^{b}
	1	41.4 ±1.69^{abc}	0.82±0.03^{abc}	37±2.12^{b}
	1.5	58.5 ±0.98^{cde}	0.94±0.56^{abc}	36±1.4^{b}
	2	69.0 ±0.84^{efg}	1.17±0.07^{cd}	34±1.4^{b}
2	0	38.0 ±0.98^{abc}	0.57±0.04^{bc}	35±0.7^{b}
	0.5	56.43 ±1.17^{bcde}	0.8±0.03^{abc}	33±1.4^{b}
	1	76.81±2.27 ^{fgh}	0.89±0.03^{abc}	33±1.4^{b}
	1.5	100 ±0.0^{h}	1.3±0.07^{d}	30±2.12^{b}
	2	91.13 ±2.03^{fg}	0.91±0.02^{abc}	32±1.4^{b}

The same lowercase letter (with in a column) indicates no significant difference in data expressed as mean ± SD at $p<0.05$, according to Tukey test

Table 2 Effect of Inoculum size on cell suspension culture and naphthoquinone production after 15 day culture

Inoculum size(g)	Fresh weight (g/L)±SD	Dry weight(g/L) ±SD	2-methoxy-1,4-naphthoquinone production(mg/L)±SD
0.5	24±0.2^{a}	0.8±0.01^{a}	0.21±0.03 ^{a}
1	55±0.4^{a}	1.4±0.05^{ab}	0.53±0.03 ^{b}
2	130±2.0^{b}	1.8±0.02^{b}	1.2±0.15 ^{c}
4	380±2.0^{c}	3.8±0.02^{c}	2.36±0.05 ^{d}
6	463±1.5^{d}	6.5±0.04^{d}	3.11±0.11 ^{e}
8	413±1.5^{c}	4.7±0.03^{c}	4.43±0.06 ^{f}

The same lowercase letter (with in a column) indicates no significant difference in data expressed as mean ± SD at $p < 0.05$, according to Tukey test.

4.2 Cell suspension culture initiation and culture optimization

In 250 mL flasks, suspension cultures were initiated by adding 0.5 g of fresh friable calli to 80 mL of MS liquid media. A net weight change of 11.03±0.54 g was observed for the cultured cells. The suspension culture obtained contains mixtures of large and small cell clumps due to uncontrolled breakdown of callus (Figure 1). The cultures remain clumpy with varying size during repeated subcultures (Figure 1). The optimal inoculum size for the suspension culture was determined by inoculating various inoculum sizes of fresh friable calli into 80 mL MS liquid medium in 250 mL flasks. Table 2 below shows the outcome for optimizing the inoculum size. From 5 to 9 times biomass increase was observed in all the tested inoculum sizes in the initial 14 days and the stationary phase was reached after 14 days. The highest biomass growth was observed for 6 g inoculum size, compared to low inoculum sizes of 0.5, 1 and 2 g (Table 2).

Culture media that had high sucrose typically produced more secondary metabolites and accumulated a lot of biomasses. Cultures were shown to accumulate higher biomass in response to an increase in sucrose concentration from 10 g/L to 60 g/L (Table 3). However, the biomass accumulation was not statistically significant at sucrose concentrations of 30 g/L and 60 g/L. On the other hand, when the concentration of sucrose was increased from 60 g/L to 100 g/L.

4.3 Total phenolic and flavonoid content of the stem cell extract

The total phenolic content in the *I. tinctoria* stem cell extracts was determined using the calibration curve of gallic acid, which is y = 0.005x + 0.038, R^2= 0.99. Here, x is the total phenolic content in µg/mL gallic acid equivalent, and y is the absorbance at 765 nm. In the plant stem cell extract, a very low total phenolic content was recorded. The stem cell extract had a total phenolic content of 4.6±0.01 µg GAE/mL.

The total flavonoid content of the stem cell extract was calculated using the quercetin calibration curve, y = 0.0015x+0.62, R^2 = 0.99, where x is the total flavonoid content in µg/mL quercetin equivalent and y is the absorbance at 415 nm. A high level of total flavonoid concentration in the stem cell extract was recorded. The total flavonoid content of the stem cell extract was 190.96±0.08 µgQE/mL.

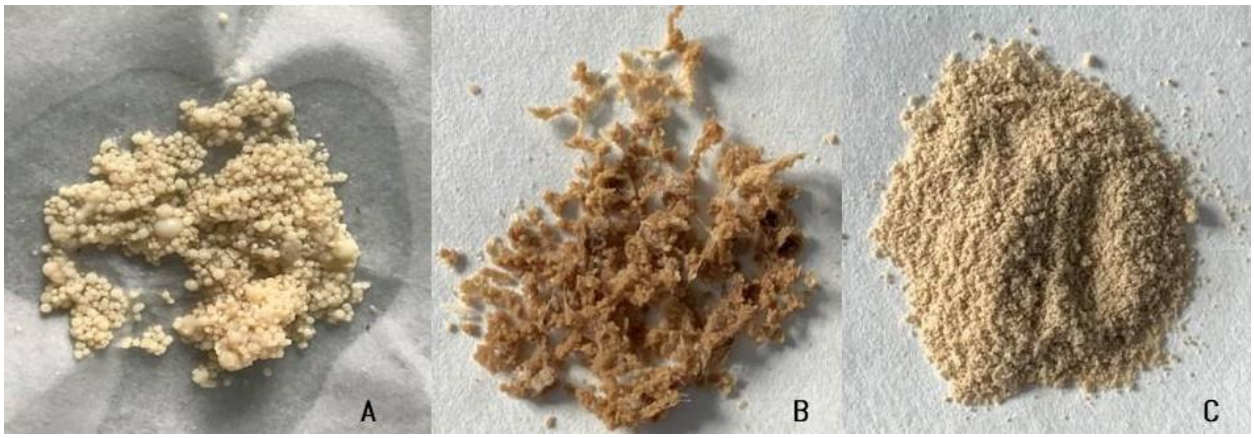

Figure 1 Cell suspensions of *I. tinctoria* A.Rich; cell aggregates (A), lyophilized cell aggregates (B), powdered cell aggregates after lyophilization (C)

Table 3 Effect of sucrose concentration on cell suspension culture and naphthoquinone production

Sucrose concentration(g/L)	Fresh weight (g/L) ±SD	Dry weight (g/L) ±SD	2-methoxy-1,4-naphthoquinone production(mg/L) ±SD
10	241±1.0^{a}	7.9±0.08^{a}	1.13±0.09 ^{a}
30	368±1.0^{b}	9.5±0.01^{ab}	3.68±0.16 ^{b}
60	385±1.5^{b}	9.8±0.01^{abc}	4.18±0.13 ^{ac}
80	326±1.5a^{ab}	7.5±0.03^{bc}	4.86±0.07 ^{c}
100	224±1.53^{a}	6.6±0.07^{c}	6.78±0.09 ^{d}

The same lowercase letter (with in a column) indicates no significant difference in data expressed as mean ± SD at $p<0.05$, according to Tukey test

4.4 DPPH radical scavenging activity

The DPPH scavenging activity of the stem cell extract from *I. tinctoria* was high. The stem cell extract had a DPPH scavenging activity of 95.82% and IC50 value of 37.54 µg/mL. Its stem cell extracts showed a concentration response relationship in terms of DPPH scavenging activity. Higher concentrations of stem cell extract are associated with an enhanced capacity for scavenging.

5. Discussion

Plant regeneration is a process carried out by plant stem cells. These cells are known to produce an unlimited number of bioactive substances. They provide a steady supply of bioextracts for the large-scale, sustainable synthesis of biomass and secondary metabolites (Bapat et al., 2023; Bouzroud et al., 2023). Proteins, lipids, carbohydrates, phytohormones, and secondary metabolites are possible components of the bioextracts. Furthermore, plant extract-derived products have demonstrated anti-aging, wound healing, anti-inflammatory, antioxidant, anticancer, and skin regenerative properties (Adli et al., 2024; Guidoni et al., 2023a). Bioextracts containing kinetin with strong antioxidant activity was reported to have skin fibroblast stimulating potential in the collagen for skin regeneration (Miastkowska, & Sikora, 2018). These extracts do not produce reactions with immune system and are regarded as safe for cosmeceutical applications. Bapat et al., (2023) in their review demonstrated a growing demand for plant stem cell extracts in the formulations of novel health care products, cosmetics, food and bioprinting. Plant extracts enriched with primary and secondary metabolites can be produced by cultivating plant cells *in vitro*. *I. tinctoria* calli and cell suspension cultures were established for the first time in this study. It was found that a 14-day suspension culture cycle produced high biomass.

Dedifferentiation, the process by which specialized cells lose their differentiated properties and become juvenile, is largely responsible for the establishment of the callus culture. The dedifferentiation process of *I. tinctoria* tuber meristematic cells resulted in the production of thicker, stiffer, swelling, and visible tissues of the explant (callus). Within 30 callus induction days, the callus induction rate was 100% on medium supplemented with 1.5 mg/L of BAP and 2 mg/L of 2,4-D. Low concentrations of BAP and high concentrations of 2,4-D encouraged the explant's active proliferation after dedifferentiation. This is because of the stimulation of proteins in the cell cycle (CDC2/CDK2 class of cyclin-dependent kinases). Furthermore, activities of CDC2/CDK2-like kinase were known to occur within the applications of auxin, cytokinin and sucrose (Richard et al., 2002). For maximum callus induction, the auxin-cytokinin optimum ratio is crucial. The production of callus in *Bletilla formosana* from seeds required 2 mg/L BA and 1 mg/L 2,4-D (Wang et al., 2023); for the development of callus in Granny Smith apple, 2 mg/L 2,4-D and 1 mg/L BA were required (Menbari et al., 2021). Similarly, Dyshlyuk et al., (2022) found that stable callus growth from *Filipendula ulmaria* seedlings was achieved using 2 mg/L 2,4-D and 0.2 mg/L kinetin. Days to callus induction in *Salvia chamelaeagnea* were also studied by Huang, & Van Staden (2002), who found that 12 days are sufficient for callus induction on 2,4-D and BAP medium. Longer durations were reported in media supplemented with 2,4-D alone. However, the requirement of 2,4-D, a potent herbicide and carcinogen, for suspension culture production is unsuitable for the pharmaceutical industry. Therefore, investigation with other favorable plant hormones for cell suspension culture production is required.

At various inoculum sizes investigated, 6 g was found to enhance biomass yield (7.3% FW). A further increase of inoculum sizes up to 8 g led to decreased culture biomass yield. The result shows that cell suspension culture growth is inhibited by both smaller and larger inoculum sizes, and that cell growth requires a specific initial cell density. From the experimental result, it was observed that the optimum inoculum size for biomass accumulation was 7.3 % (fresh weight (FW), w/v.), i. e. 6 g, which is comparable to maximum dry biomass obtained by Menbari et al., (2021) for Granny Smith apple fruit cell suspension culture. For improved biomass production in *Panax ginseng* cells suspension culture, inoculum sizes of 6 g dry weight (DW)/l and sucrose concentration of 60 g/L after 26 days of cultivation were reported (Akalezi et al., 1999; Zhang et al., 1996). The experimental result suggested that the manipulation of inoculum size was an effective method to improve cell biomass production. Literature reports also suggested that high inoculum sizes were beneficial for biomass yield (Akalezi et al., 1999).

Sucrose is the primary transport carbohydrate used by plants. It is directly involved in osmotic control and plant signalling. The enzyme invertase in the cell wall aids in the absorption of sucrose (Dantas et al., 2021). This enzyme also breaks down sucrose to produce hexoses like fructose and glucose. The resulting hexose can be allocated for the production of energy or to generate sugar signals. In this experiment, more water

absorption and biomass build up were recorded at lower sucrose concentrations (10-60 g/L). As the concentration of sucrose increased further from 80-100 g/L, the activity of the invertase reduced that tends to reduce biomass accumulation (Mello et al., 2001). This is due to saturation of the active sites of the enzyme, Moreover; it has been observed that greater sucrose content lowers cell water content and biomass accumulation (Dantas et al., 2021). However, this study revealed that higher level of sucrose concentration is important for 2-methoxy-1, 4-napththoqinone production.

In order to investigate the antioxidant activity of the stem cell extract, total phenol, flavonoids, and DPPH scavenging activity analysis was done. Total phenolic content of 4.6 µgGAE/mL was displayed by the stem cell extract of *I. tinctoria*. The Folin-Ciocalteu method normally determines reducing capacity, but not phenolic content. The reducing capacity of the phenolic compounds was determined by the number and position of the OH groups present (Platzer et al., 2021). Low total phenolic activity in this experiment is associated with the presence of reducing sugars and high protein content in the extract (Lawag et al., 2023). In addition, low molecular weight phenolic compounds such as flavonoids were predominantly produced by the cell suspension culture. Furthermore, the degrees of differentiation that exist in suspension culture are also known to influence phenolic metabolism. In highly differentiated and vacuolated cells, increase in the phenolic metabolism is reported (Meravy, 1987; Cvikrová et al., 1988).

Total flavonoid content of 190.96 µgQE/mL was recorded for the stem cell extract. The presence of high flavonoid content and high DPPH scavenging activity (95.82%) of the stem cell extract may make it a promising antioxidant. The IC50 value of the extract was 37.54 µg/mL which is lower than the previous study reported by Gidamo et al., (2023) for the methanolic extract of *I.tinctoria* tuber (44.4 µg/mL). Total phenolic content of 3.7 mgGAE/g, total flavonoid content of 1.89 mgQE/g and DPPH radical scavenging activity of 82.7% were reported for stem cell extracts of *Artemisia absinthium* (Ali et al., 2013). The wound healing and anti-aging properties of stem cell extracts derived from *in vitro* cell culture of *Coffee canephora* (Miastkowska, & Sikora, 2018; Guidoni et al., 2023b) and *Centella asiatica* (L.) (Buranasudja et al., 2021) have been reported. These anti-aging properties of the stem cell extracts were due to antioxidant activities. The antioxidant activity was due to the complexity of secondary metabolites produced during culture proliferation. Flavonoids like amentoflavone and biflavonoid from plants are known to protect cells against aging, inflammation and stress-induced disease (Bharadvaja et al., 2023). However, the flavonoid composition of the stem cell extract of *I. tinctoria* tuber and mechanisms for antioxidant activities were unknown and required further investigation.

6. Conclusion

In conclusion, *I. tinctoria* A. Rich callus and cell suspension cultures were established for the first time, and this produced a homogenous material for the synthesis of secondary metabolites. The induction and growth of calluses were greatly enhanced by the application of 2 mg/L 2,4-D and 1.5 mg/L BAP. It was possible to achieve notable increases in biomass production by manipulating the size of the inoculum and the concentration of sucrose in the culture medium. The ideal concentrations of sucrose and inoculum size were 60 g/L and 7.3% (FW, w/v), respectively. It was found that the cell suspension cultures produced more flavonoids and exhibited strong antioxidant activity. This work may help to improve and sustainably produce plant stem cells for cosmeceutical uses, as well as to further regulate the biosynthesis of secondary metabolites. For better understanding, isolation, structure elucidation and mechanism of action of the flavonoids as well as their *in vitro* and *in vivo* studies are recommended.

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Availability of data and materials: The datasets utilized and analyzed during this investigation are available upon reasonable request from the author

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