Research article

Hemolytic saponins product enhancement in *Gnidia glauca* (Fresen.) Gilg cell suspension cultures

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ABSTRACT

Introduction and Aim: *Gnidia glauca* (Fresen.) Gilg of thymelaeaceae is enlisted as an endangered plant. Hemolytic triterpenoid saponins of oleanane and ursane classes are present in *G. glauca*. The purpose of the present study is to increase the production of hemolytic triterpenoids *in vitro* using salicylic acid as an elicitor in *G. glauca* cell suspensions.

Materials and Methods: Explants were collected and surface sterilized, then inoculated onto Murashige and Skoog medium for callus culture, further establishing suspension cultures that were treated with salicylic acid. Saponins produced in the suspension culture were extracted and analyzed by HPLC and FTIR. The hemolytic activity of saponins was confirmed by spectrophotometric assay in erythrocyte suspensions.

Results: Product enhancement of hemolytic triterpenoid saponins was observed in cell suspensions treated with salicylic acid that also induced concomitant change in medium pH compared to control set of culture. Results of HPLC and FTIR analyses confirm the presence of triterpenoid saponins in suspension culture extracts. The optimal concentration of salicylic acid was found to be 200 µM for product enhancement, pH alteration. Hemolytic saponins concentration was observed to be increased with increasing concentrations of elicitor supplemented to the medium.

Conclusion: Cell suspension is a viable alternative technique to produce the phytochemicals *in vitro* using elicitors that avoids destructive harvesting of endangered plants like *G. glauca.* Results of the present study demonstrate the large scale production of hemolytic saponins through cost-effective approaches.

Keywords: Saponin; FTIR; HPLC; salicylic acid; hemolytic assay.

INTRODUCTION

Gnidia Glauca (Fresen.) Gilg of thymelaeaceae is enlisted as an endangered plant, containing hemolytic triterpenoid saponins of oleanane and ursane classes. It is also known as *Lasiosiphon eriocephalus* and is a large shrub or semi-woody herb located in certain parts of Maharashtra, Karnataka, and Tamil Nadu states in India. There are secondary metabolites present in *G. glauca* that have been evaluated to possess various biological activities. Among the secondary metabolites present in *G. glauca* are triterpenoid saponins with basic skeletons of oleanane/ursane sapogenins that possess various biological activities (1-3). Leaf extracts of *G. glauca* were used by farmers in rural Karnataka at the 'Malenadu region' for decades as a traditional practice to control bacterial blight disease in paddy (rice) caused by *Xanthomonas oryzae* (1). Blisters, swellings and contusions are frequently treated with *G. glauca* (1, 4). Significant anti-carcinogenic efficacy of *G. glauca* leaf methanol extract against *Streptococcus mutans* was evaluated (5). The anticancer activity of *G. glauca* leaf extracts has been evaluated in human lung and colon cancer cell lines (6). As the availability of *G. glauca* in the wild is scanty and endemic to only certain regions. The plant is extinct in life, enlisted and reported in endangered medicinal plants, which demands finding or development of alternative methods for its phytochemicals, to avoid destructive harvesting in the wild. One of the alternate techniques for phytochemicals production is *in vitro* cell suspensions treated with exogenous elicitors. Numerous studies have proven that elicitors get an impact on cell suspension and organ culture. Elicitors are signalling molecules that improve secondary metabolites and also give plant tolerance to biotic and abiotic stress conditions (7). Elicitor-induced signal transduction promotes the generation of secondary metabolites (8). Elicitation is the process that imposes stress leading to a corresponding defence response through secondary metabolites production (9). Despite the plant's vast medicinal properties, it is on the endangered species list since there are few publications on saponin investigations in *G. glauca*, and till-date no published reports are available on the production of saponins using the cell suspension culture method in *G. glauca*. Objective of the present study is to develop an efficient alternative method for enhancing the production of hemolytic triterpenoid

saponins of *G. glauca in vitro* through elicitation with salicylic acid (SA) in cell suspensions.

METHODS AND MATERIALS

Explant collection and establishment of callus

Fresh leaves of *G. glauca* were collected from various locations in Shivamogga district, Karnataka, India in the months of December and June. Plant was identified and authenticated by Dr. Kumarswamy Udupa, Department of Botany, Sri JCBM College, Sringeri, Chikkamagalore district, Karnataka, India. Herbarium voucher specimen (No. FSB-0982) was maintained in the Department. Leaf explants were washed under running water for 3 to 4 times to remove excess dust and soil particles. Transferred to conical flasks then washed with distilled water twice, again leaves were washed with detergent or Tween-20 (2%) solution with added 0.1% (w/v) Bavistin that acts as an antifungal agent, flasks were kept in an orbital shaker (Scigenics Biotech, India) at 110 rpm at 27℃ for 20 minutes. After that leaves were washed with double distilled water and surface sterilized with 0.1% $HgCl_2$ (W/V) for 4 minutes, followed by a quick rinse with 70% ethanol for 30 seconds. After every treatment leaf explants were rinsed in double distilled water. Explants were made into pieces (1.5-2.5 cm in size) using the sterilized blade and then inoculating it onto MS medium (10) medium with pH 5.7 for callus induction. Medium was supplemented with a combination of plant growth regulators 2iP (0.2 mg/l), picloram (0.2 mg/l) and 2,4-D (2 mg/l). A light (16 h) and dark (8 h) cycle culture regime was maintained for callus induction at 24±2 ℃. Subcultures were done every 12 days.

Establishment of suspension cultures and elicitation

White friable callus (24-day-old) was added to 50 ml of MS medium without solidifying agent in 250 ml Erlenmeyer flask at a ratio of 10 mg callus per 1 ml of medium for cell suspension establishment. Flasks were maintained in a rotatory shaker incubator at 26 ℃ set at 120 rotations per minute, and photoperiod for 24 hours. A stock solution of salicylic acid (SA) (Sigma-Aldrich, St. Louis, USA) was prepared, pH was adjusted to 5.8, diluted to different concentrations (50 µM, 100 µM, and 200 µM), and filter sterilized before use. Suspension cultures were supplemented with different concentrations of SA elicitor. Cell suspension cultures were taken out periodically (6, 12, 18, and 24 days) and subjected for phytochemical analysis. After each successive culture day cells were collected and dried to measure the growth index and subjected to total saponin content determination. While the untreated control (no SA elicitation) set was maintained in parallel. The same experiment was conducted three times.

Determination of cell growth and medium pH

After completion of each consecutive culture period (6, 12, 18, and 24 days) the fresh biomass was taken down from the liquid MS media by filtering through muslin cloth and then wiped into sterile tissue towels for absorbing excessive water content, and noted the final weight of the sample. Further, the same sample was kept in a hot air oven at 45 ±2℃, For 6 to 12 hours or up to complete evaporation of water content, then record the dry weight of the sample.

This has been done to determine the rate of cell growth (11, 12). Growth index was estimated using the formula,

$$Growth\ index = \frac{Wt\ final}{Wt\ initial} \times 100$$

where,

Wt-final, is the weight of the cell mass on the day (harvest day)

Wt-initial, is the weight of the cell mass at zero day (inoculation day)

Extraction of total saponins content and RP-HPLC analysis

Dry filtrate of cell suspension culture (1 mg/ml) was finely powdered, dissolved in 80% methanol or milli-Q (deionized) water, and filtered (0.22 µm; Hi-media, India). Filtrate was subjected to RP-HPLC (Agilent 1260) analysis using a C18 analytical column (ZORBAX Eclipse plus 4.6 × 250 mm, 5µm). Methanol: water (35:65) was the mobile phase, flow rate of 1 ml/min, column temperature maintained at 35 °C, and the injection volume was 20 µl. Analytes were detected and determined in the mobile phase stream using a diode array detector at 210 nm (DAD) on the basis of retention times of authentic standard compounds. Retention peak (Rt) of *Quillaja* bark saponin (2.152 min) (Sigma-Aldrich, St. Louis, USA) (8). Saponins present in the cell suspension culture extract were quantified using the formula,

$$\text{Saponin content} = \frac{Ht}{Hs} \times Ws \times \frac{500}{Wt\ (1-\frac{Dt}{100})}$$

where,

Ht - Height of the peak corresponds to test sample

Hs - Height of the peak corresponds to authentic standard (*Quillaja* bark saponin)

Wt – Weight of the test sample

Ws – Weight of the authentic standard (*Quillaja* bark saponin)

Dt – Test sample loss on drying

Fourier Transform Infrared Spectroscopy (FTIR) analysis

Suspension culture extract of *G. glauca* was mixed with potassium bromide (KBr moisture-free salt) at 1:7 ratio, ground using pestle and mortar until it gets properly mixed, and made into a fine pellet using hydraulic pressure. Thus, the obtained pellet of extract was analysed by FTIR spectroscopy (Bruker Alpha-P,

Germany) between 4000 and 400 cm^{-1} range. Functional groups of the sample were identified by literature studies (2).

Hemolytic assay

A hemolytic assay was carried out using 6% RBC suspension. Human blood (2 ml) was drawn, and collected into a centrifuge tube with an anticoagulant, RBC were pelleted down centrifugation at 5000 rpm for 5 min. RBC pellet was suspended in phosphate buffer-saline (4 ml PBS), and centrifuged at 3000 rpm for 5 min, this was repeated 3-4 times. For the hemolytic assay, RBC suspension (1.5 ml) was diluted in 48.5 ml PBS, an aliquot of this diluted suspension (1.5 ml) was mixed with 20 µl *G. glauca* callus saponin extract in PBS and incubated for 1 hour at 27 °C. After incubation supernatant was collected by centrifugation at 5000 rpm for 10 minutes at 5000 rpm and absorbance was measured 540 nm using a UV-visible spectrometer (Elico SL-159, India). Hemolytic activity was calculated against control, and standard saponin from *Quillaja* bark (50 µM, 100 µM, 200 µM) and calculated using the formula,

$$I_{\%} = \frac{Absorbance\ of\ test\ sample - Absorbance\ of\ negative\ control}{Absorbance\ of\ negative\ control} \times 100$$

Statistical analysis

Data were subjected to a one-way classification of ANOVA, followed by Duncan's post-hoc Multiple tests with mean values of treatment compared at $p<0.05$ using SPSS 17.

RESULTS

Influence of SA on cell growth

Callus culture and suspension cultures have been successfully established agar free liquid medium and elicited with salicylic acid (SA) for saponin production. Results of the growth index suggest that SA influenced cell growth in suspension culture significantly (Fig. 1). Cell growth was found to be increased in presence of SA throughout the culture period compared to the control set of suspension cultures, with optimal growth recorded on the 18th day in suspension culture supplemented with 200 µM SA (Fig. 1).

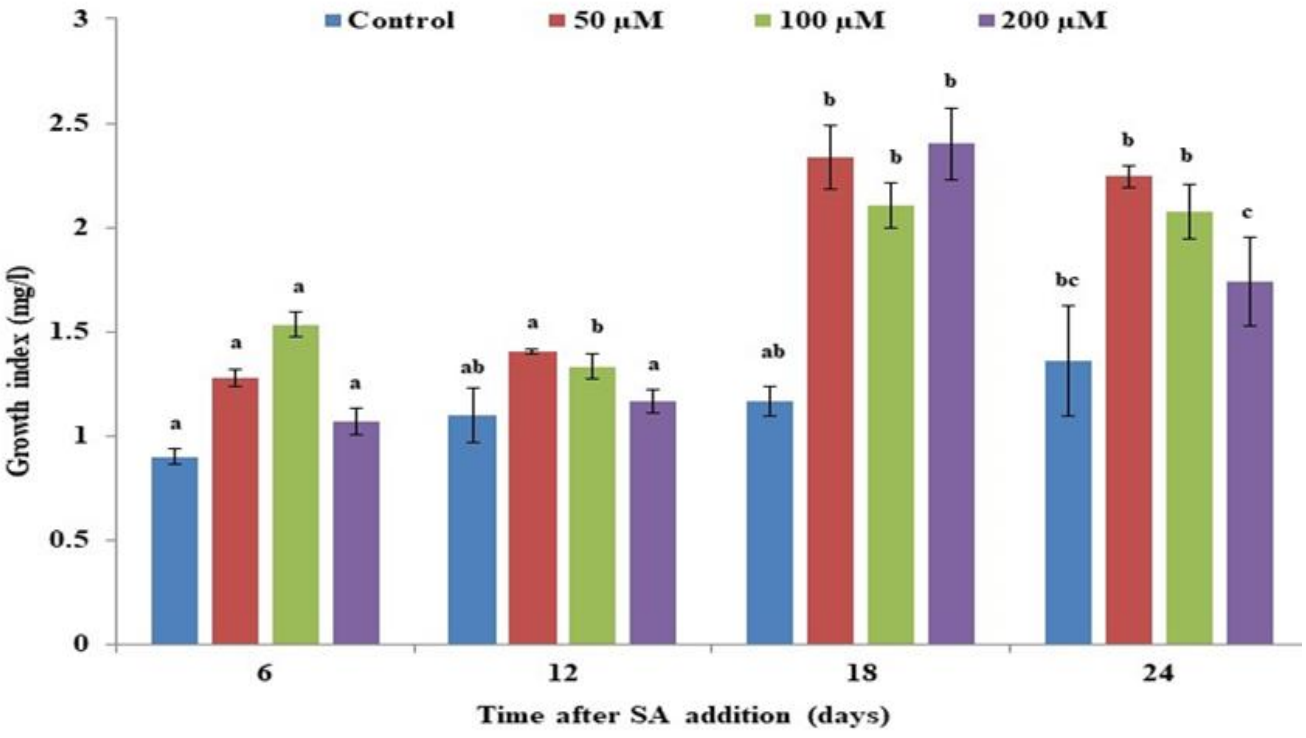

Fig. 1: Influence of salicylic acid (SA) on cell growth of *G. glauca* cell suspension

Influence of SA on medium pH

Suspension culture medium pH was observed to be affected by SA at all test concentrations throughout the culture period compared to the control set of culture (Fig. 2). Culture medium pH was observed to be decreased till day 18, thereafter a slight increase was observed till day 24. Change in medium pH was not only observed in SA-treated cultures, but also in the control set of suspension culture at all culture regimes (Fig. 2).

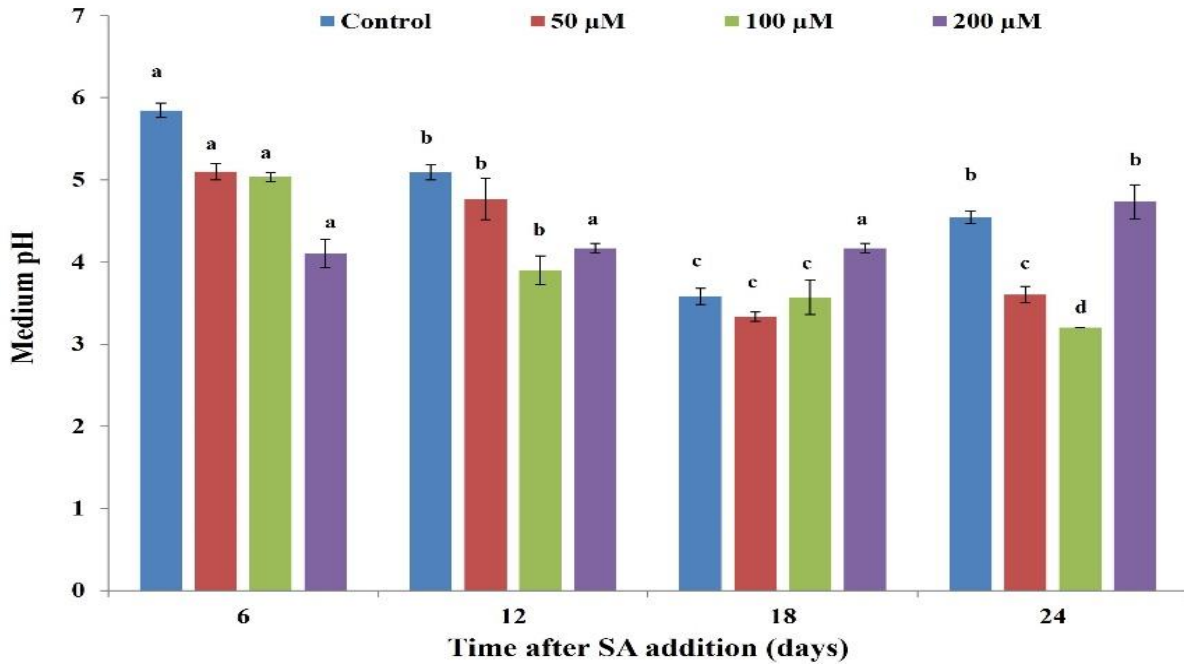

Fig. 2: Influence of SA on culture medium pH of *G. glauca* cell suspension culture

Influence of SA on saponin production

Saponin production in suspension cultures was influenced by SA at all the tested concentrations throughout the culture period. Compared to control cultures in all SA treated cultures at all concentrations an increase in saponin content was recorded (Fig. 3). Optimum production of saponins was observed on day 18th with 200 µM SA, at this concentration the product enhancement was noticed to be 3.50 folds higher than respective control (Fig. 3).

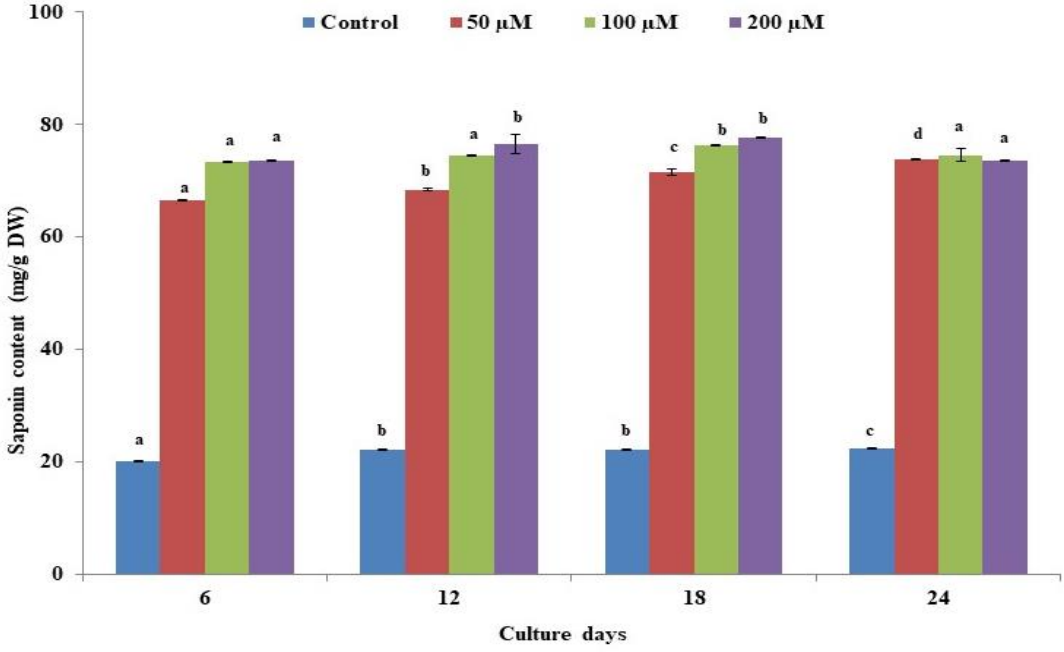

Fig. 3: Influence of salicylic acid (SA) on saponin elicitation in *G. glauca* cell suspension

Presence of triterpenoid saponins was confirmed by RP-HPLC analysis using *Quillaja* bark-saponin as standard (Fig. 4 a & b). Composition of triterpenoid saponins in the suspension culture extract was confirmed further by FTIR analysis (Fig. 5). Results confirm the presence of triterpenoid saponins in *G. glauca* cell suspension extracts (Table 1). Outcome of the FTIR spectrum of saponin extract exhibits and explains the characteristics of triterpenoids saponins functional groups that are considered fingerprint groups, such as, there are absorptions at C=C (1663.56 cm^{-1}), C=O (1349.41 cm^{-1}; 1359.75 cm^{-1}), and -OH (3468.19 cm^{-1}).

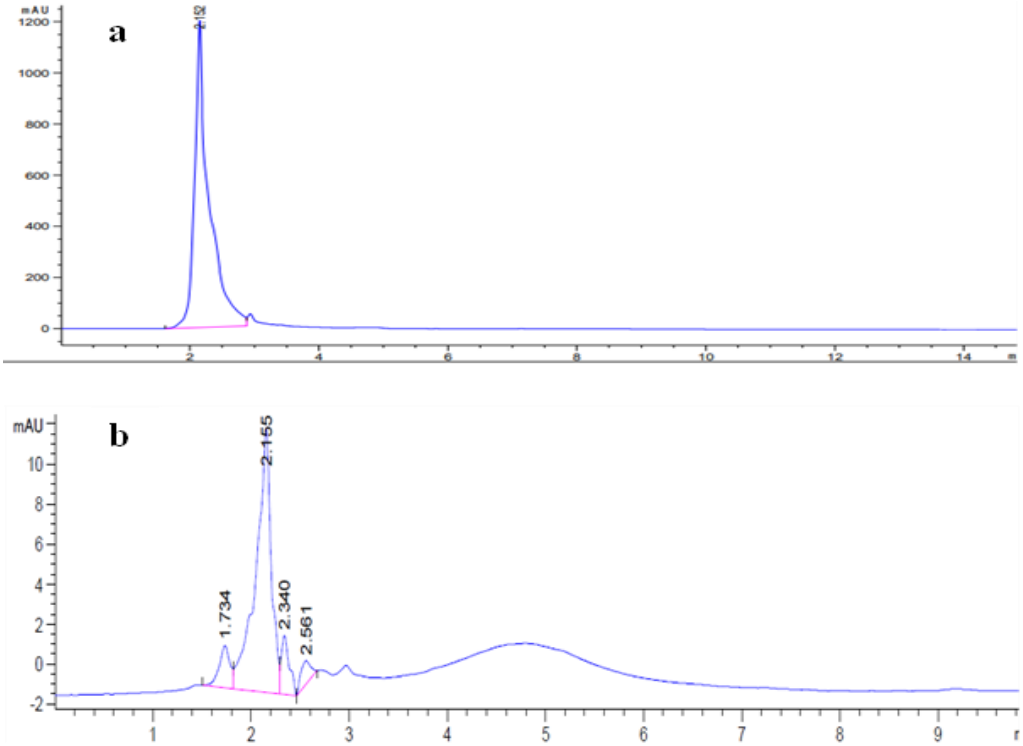

Fig. 4: (a) HPLC chromatogram of *Quillaja* bark saponin (triterpenoid standard) (b) Saponin extract of SA elicited *G. glauca* cell suspension culture

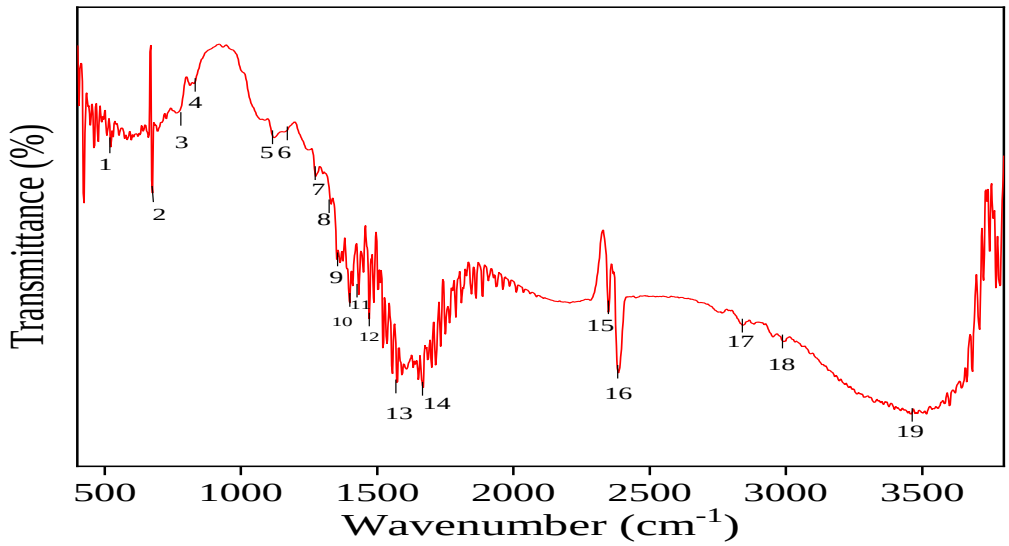

Fig. 5: FTIR spectrum of SA elicited saponin extract of *G. glauca* cell suspension culture

Table 1: Functional groups of *G. glauca* cell suspension culture saponin extract identified by FTIR analysis

Peak No.	Wavenumber in cm^{-1}	Bond	Functional groups
1	526.35(w)	--------	Fingerprint region (very complicated series of absorptions)
3	770.54 (w)	β-ring or α-CH	Heterocyclic aromatic compound
4	918.63 (w)	C-H	Alkene
5	1114.72 (w)	C-O Stretching	Alcohol
9	1349.41 (w)	O-H bending	Alcohol
10	1359.75 (w)	C-O-H bending	Carboxylic acid
13	1577.96(m)	C-C stretching	Arenes or Aromatic hydrocarbons
14	1663.56(m)	C=C stretching or O-H stretching	Alkenes and alcohols
18	2843.41 (w)	C-H stretching or O-H stretching	Carboxylic acid or Alcohol or Alkane or Aldehyde
19	3468.19(s, b)	O-H stretching	Alcohols

Hemolytic assay

The increase in hemolytic activity seems to be a reliable marker of cytotoxicity against healthy erythrocytes. In the present study, saponin extract of *G. glauca* cell suspension extract was tested for its hemolytic activity in erythrocyte suspension. Results confirm the hemolytic activity of saponins present in the extract, as the absorbance of the test sample was increased compared to respective control at 540 nm (Fig. 6).

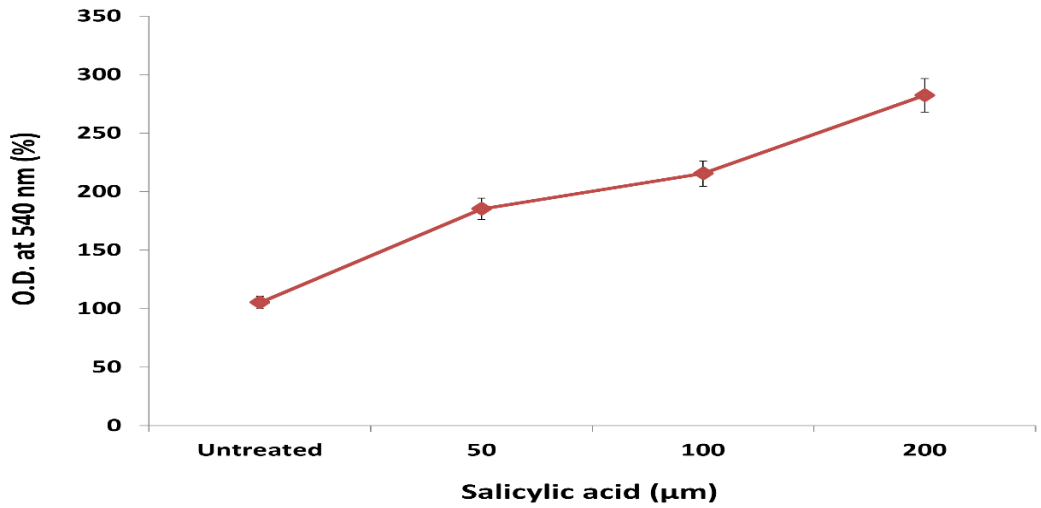

Fig. 6: Absorption spectrum of hemolytic assay performed using SA elicited saponin extract of *G. glauca* cell suspension culture

DISCUSSION

Elicitation of plant cell suspension cultures is an alternative method for enhancing the production of plant secondary metabolites. Triterpenoid saponins are a sub-group of phytochemicals that are well known for therapeutic properties. Some of the triterpenoid saponins exhibit intrinsic hemolytic activity. Among the triterpenoid saponins oleanane type of saponins possess a stronger hemolytic activity than that of ursane, dammarane, and other types of saponins (3).

Presence of oleanane saponins in *G. glauca* suspension cell suspensions has been identified by HPLC (Fig. 4 a & b) and FTIR (Fig. 5) analyses. As *G. glauca* is an endangered plant, to avoid the destructive harvesting of the plant from its wild environment in the present study elicitor enhancement of saponins has been evaluated using salicylic acid. It is critical to standardize the elicitor dosage and culture periods for secondary metabolite production due to the minimal growing lag within cells (8). In the published literature, it has been suggested that suspension cultures treated with elicitors produce increased secondary metabolites in the cell suspension method (8, 9). It has been suggested that elicitor-mediated product enhancement involves standardization of elicitor concentration, culture period, culture parameters (pH, nutrient medium, growth regulators, light conditions, shearing force), and choosing the type of cell culture are all the factors that are to be considered for standardization. Measurement of the culture growth stage is a significant parameter in the elicitor-induced production of phytochemicals, which is a highly difficult process (13). In the present study, it has been investigated to understand the SA-mediated growth index changes and pH changes in suspension cultures. Results of the investigations confirm that SA influences cell growth in *G. glauca* cell suspension culture at all the concentrations tested compared to the respective control set of cultures, optimal growth index was observed at 200 µM on the 18th day (Fig. 1). However, medium pH was decreased at the optimal growth index period and SA concentration (Fig. 2). The same concentration of SA (200 µM) and at 18th day maximum amount of saponin production by elicitation (Fig. 3). This indicates that a regular elicitor dosage influences saponin production and cellular proliferation in *G. glauca* cell suspensions. These results suggest a positive correlation between elicitor (SA) dosage and cell growth, with higher elicitor doses reducing cell growth (Fig. 1 & 3). A strongly negative correlation between secondary metabolite production on cell growth was reported (14, 15), and exogenous SA enhances the production of saponin (16). A cell suspension study is the most efficient method for increasing the secondary metabolites in the *in vitro* method (17). A higher elicitor dosage inhibits secondary metabolites production (18).

Spectral analysis results by FTIR in the present study confirm the presence of hydroxyl groups, carboxyl groups that are characteristic functional groups of triterpenoid saponins, with a strong absorption peak at 3468 cm^{-1}, 2843 cm^{-1} respectively. These results are similar to the IR spectrum of saponins reported (3). There is C=C absorption and C=O represents the characteristics of the triterpenoids saponins of oleanane class in *G. glauca* cell suspension cultures. These results of FTIR analysis and interpretation of

absorption data are confirmed with the results reported for standard saponin of *Quillaja* bark (2, 13, 19, 20).

Saponins' amphipathic characteristics allow them to interact with cell membranes as surface-active molecules, breaking the membrane. Saponins have intrinsic hemolytic activity, and when combined with water can produce a significant amount of foam. Saponins have intrinsic hemolytic activities. Erythrocytes are lysed by various toxins, metal ions, and other causes. The presence of a free carboxylic acid group in position 28 of the triterpene aglycone is found to affect hemolytic activity on human blood cells, oleanolic acid aglycone shows considerable hemolytic effects (21). The majority of oleanane-type sapogenins are more hemolytic than ursane and dammarane sapogenins (3). Although the mechanism underlying saponin-mediated hemolysis remains unknown, it has been proposed that saponins interact with cholesterol in erythrocyte membranes, generating pores that destabilize the membrane. This activity causes hemoglobin and other components to be liberated into the surrounding fluids (22).

Mesenchymal stem cells (MSCs) isolated from bone marrow hemolysis have therapeutic applications in human ailments such as cancer and Covid-19 infection. Bone marrow hemolysis has been recommended to be a better method for bone marrow MSCs isolation from small amounts of bone marrow compared to gradient centrifugation which requires a large amount of bone marrow (23). Hemolysis of bone marrow is achieved by treating it with commercially available buffers that may cause protein denaturation (24). As *G. glauca* triterpenoid saponins possess hemolytic activity, these saponins may be used for the isolation of MSCs of human bone marrow. The perspective of the present study of product enhancement of hemolytic saponins of *G. glauca* could be considered as a viable alternative method and upgraded for the large-scale production of hemolytic saponins.

CONCLUSION

Elicitors have a significant impact on the capacity to induce plant defense responses and induce secondary metabolite production. Salicylic acid is a signalling compound that elicits phytochemicals production. Results of the current study suggest that, SA at 200 µM has induced increased saponins production in cell suspensions of *G. glauca* after 18 days of incubation, without affecting the growth index but lowering medium pH. Triterpenoid saponins present in the *G. glauca* suspension culture extract with hemolytic activity. Product enhancement of *G. glauca* saponins elicited by SA is 200 µM 3.50 fold higher than respective controls. Hemolytic triterpenoids of *G. glauca* could be exploited for mesenchymal stem cell isolation by the hemolysis of bone marrow, and the present method could be upgraded for production of triterpenoid saponins at large scale.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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