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TRITON X-100 AND DMSO INDUCED CELL PERMEABILITY FOR THE RELEASE OF CAMPESTEROL AND FLAVONOIDS IN CULTURED CELLS OF *BLUMEA LACERA* (BURM. F.) DC

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ABSTRACT

Triton X-100 and Dimethyl sulfoxide (DMSO) were used as permeabilizing agent for the release of campesterol and flavonoids in cell suspension culture of *Blumea lacera*. The permeabilizing treatment with both the agents (Triton X-100 and DMSO) in *Blumea lacera* cell culture revealed interesting results indicating appropriate selection and administration of treatment protocol i.e. concentrations and treatment durations. Triton X-100 (1%) for 4 hr treatment duration favored high release of flavonoids (0.096 mg/g) (3.0 fold) as compared to control (0.032 mg/g) in suspension cultures of *Blumea lacera*. For Campesterol, 0.1% Triton X-100 treatment for 4 hr duration showed marked enhancement (2.092 mg/g, 6.0 fold) over respective control (0.346 mg/g). DMSO treatment with 0.4% concentration and 6 hr duration induced 2.5 fold release in both flavonoids (0.025 mg/g) and Campesterol (0.201 mg/g) in comparison with their respective control.

KEYWORDS: ***Blumea lacera*, Campesterol, DMSO, Flavonoids, Triton X-100.**

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INTRODUCTION

Plants are a valuable source of a wide range of secondary metabolites, which are used in pharmaceutical and agrochemical industries as drugs, food additives, pesticides, fragrances, coloring and flavoring agents. Over 80% of the approximately 30,000 known natural products are of plant origin [1-3]. Plant cell culture is a promising alternative for producing metabolites that are difficult to be obtained by extraction process or chemical synthesis due to their complex structure. Many plant products produced by cell cultures have been reported to be accumulated intracellularly. However, it may be possible to produce much higher level of product if it was secreted into the medium. This is because the product intracellularly accumulated sometimes inhibits its own synthesis by regulation mechanisms such as product inhibition and repression [4]. In such cases, it should be made possible to increase the net production of these metabolites by facilitating its removal from the site of accumulation. Extraction techniques usually require mechanical disruption of the cells, resulting in the death of the cells thus avoiding the possibility to establish semi/continuous processes[5]. Release of certain amount of betalains from cell suspension cultures of *B. vulgaris* has been achieved using electropermeabilization[6], heat treatment[7], sonication, pH gradient variation and oxygen stress[8]. However, these techniques have resulted in cell death and in low betalains recovery. Also, cell regrowth and production of metabolites of permeabilized cultures have not been achieved. Therefore it is important to develop non-destructive methods for releasing intracellular secondary metabolites and to propose efficient recovery systems [9]. Cell permeabilization has been proposed as a non-destructive approach to enhance the production of secondary metabolites and facilitate downstream processing [5, 8-10].The present experimental work was carried out in medicinal important plant *Blumea lacera*, member of Asteraceae family. *Blumea lacera* is an important medicinal plant used in various ayurvedic preparations. The major reported component of plants are Campestrol [11], the triterpenoid and prenylated phenol glycosides namely 19α-hydroxyurs-12-ene-24,28-dioate-3-O-β-D-xylopyranoside and 2-isoprenyl-5-isopropyl phenol-4-O-β-D-xylopyranoside [12], the flavonoids, 5-hydroxy-3,6,7,3',4'-pentamethoxy flavone and 5,3',4'-trihydroxy- 3, 6,7-trimethoxyflavone [13]. monoterpene glycoside, α-pinene-7β-O-β-D-2,6-diacetylglucopyranoside and flavonoides 5,4'-dihydroxy-6,7,3'-trimethoxyflavone and 3,5,4'-trihydroxy-6,7,3'-trimethoxyflavone [14]. Essential oil of the plant contains β-caryophyllene, thymol hydroquinone dimethyl ether, caryophyllene oxide, α-humulene, and E-β-farnesene [15]. Coniferal alcohol derivative is also a component of *B. lacera* [16].A wide variety of permeabilizing agents are used to enhance the accessibility of enzymes or to provoke release of intracellular stored product [17-20]. Triton X-100 was used as cell permeabilizing agent to release saponin content in *Vigna radiata* cell suspension cultures [21]. Organic solvents such as isopropanol and DMSO have been used as permeabilizing agents [22, 23]. In *Bacopa monnieri* cell culture, DMSO was as a cell permeabilizing agent for the product recovery of bioactive compound bacoside A [24]. It is reported that the viability of cells was not affected when *Catharanthus roseus* cell culture were treated with Triton X-100 and DMSO [25]. Still, different responses to permeabilizing agents have been reported based on variation in the sensitivity of cell lines to permeabilizing treatments. On the basis of the medicinal properties of *Blumea lacera* and encouraging reports of permeabilization, Triton X-100 and DMSO were used as permeabilizing agents for campesterol and flavonoids recovery in cultured cells of *Blumea lacera*.

MATERIALS AND METHODS

Cell suspension culture

Disease free, healthy and young leaf *Blumea lacera* (Burm.f.) DC. were used as explants for callus induction on MS medium supplemented with 2,4-D:6-Benzylaminopurine (1.0:0.5 mg/l). The fragile calli were used for raising cell suspension culture. The cell suspension cultures were maintained in same medium composition except agar. The cultures were incubated on a shaker, 120 rpm and 25°C under complete darkness.

Permeabilization treatment

21 days old cell suspensions were treated with Triton X-100 and DMSO separately. Triton X-100 was used in three different concentrations i.e. 0.1%, 0.5% and 1.0%. Each concentration was treated for three different treatment durations i.e. 1 hour, 2 hr and 4 hr. DMSO was used in 0.2%, 0.4% and 0.6% concentration for 2 hr, 4 hr and 6 hr treatment duration. The cultures were incubated at 25^{o}C on shaker at 120 rpm constantly during the complete period of treatment. After the treatment, the cells were separated from the suspension culture by filtration using sterilized Whatman No.1 filter paper. The suspension cultures were then dried in an oven.

Sample preparation

After treatment, the cell suspension cultures were divided into two parts. One part was dried at 20^{o}C and another part was allowed to dry at 40^{o}C. 100 mg of the cell suspension culture dried at 40^{o}C was sonicated in 2 ml methanol using SONICS Vibra Cell (VCX 130) instrument with 2 mm probe for 10 min with pulse rate operating at 10 sec on and 2 sec off and amplitude 20%. After sonication the extract was centrifuged at 5000 rpm for 5 min. the supernatant was transferred into 2 ml eppendorf tubes. This extract was used for identification and quantification of flavonoids by TLC and UV-Vis spectrophotometer respectively. For the quantification of Campesterol by HPLC, 100 mg of cell suspension culture dried at 40^{o}C was sonicated in 2 ml ethanol using 2 mm probe for 10 min with pulse rate operating at 10 sec on and 2 sec off, amplitude 20% using SONICS Vibra Cell (VCX 130) instrument. After sonication the extract was centrifuged at 5000 rpm for 5 min. the supernatant was transferred into 2 ml eppendorf tubes was used for HPLC analysis.

Identification and quantification of total flavonoid content

Identification of flavonoids was done by High Performance Thin Layer Chromatography (HTLC) and quantification of total flavonoid content was done with UV- Vis Spectrophotometer using Aluminium Chloride Colorimetric method. Minitab-1 software was used for the statistical evaluation of the data.

High Performance Thin layer chromatography (HPTLC)

The identification of flavonoid was done by TLC. TLC was performed on aluminium-backed precoated silica gel 60 F254 TLC plate (0.2 mm thickness, E. Merck). Sample solutions (10 µL in quantity) were applied as 8 mm wide bands, 8 mm apart, by the spray-on technique, by means of a Camag (Switzerland) Linomat IV sample applicator fitted with a 100-µL syringe (Hamilton, Bonaduz make, Switzerland). Plates were developed with mobile phase Ethyl acetate: formic acid: acetic acid: water (13.42:1.47:1.47:3.62). Plates were heated for 10 minutes. Visualization of the flavonoids was achieved by spraying the plates with ethanolic Polyethylene glycol (4000 MW) and estimated under UV light with a wavelength of 365 nm [26]. Yellow coloured bands of flavonoids appeared on the TLC plate.

Estimation of Total Flavonoid Content

Total flavonoids content was determined using the aluminum chloride colorimetric method [27]. Quercetin procured from Sigma-Aldrich was used as reference standard.

Quantification of Campesterol by HPLC

Centrifuged ethanol extract was filtered with 0.45 mm nylon syringe filter (Titan, Wilmington). These extracts were stored in clean HPLC vials before injection into HPLC system.Reverse phase HPLC was carried out on Agilent Technologies 1200 instrument with a series D-7000 interface with HSM Manager, L-7100 pump, L-7420 diode array detector, a rheodyne injector with a 20 mL injection loop. The method proposed by Borge Holen, 1985 was used for HPLC analysis. Column C18 ZORBAX 300SB-C18 (“Agilent Technologies”,USA) of dimensions 4.6 X 250 mm with particle size of 5µm was used. Column temperature was set at 22 ^{o}C. Detection wavelength was 206 nm. Solvent system used was Methanol: Water (99:1). The flow rate was 1.2 ml/min. and the rum was continued for 10 minutes. All the solvents used were of HPLC grade (“Fisher Scientific”, India). Standard Campesterol was obtained from Sigma-Aldrich. The 20 ppm standard solution of Campesterol was prepared in HPLC grade ethanol and 20 µl from 20 ppm solution was used for injection. Injection volume of 20 µl was kept constant for all the samples. The column was flushed after every injection by mobile phase for 5 min. to re-establish the initial conditions. Identification of Campesterol in treated cell samples was confirmed by retention time, co-chromatography with the standard and peak purity by wavelength.

RESULTS

Triton X-100 induced cell permeability for flavonoids

The flavonoids were detected as yellow band on TLC plate after derivatisation with 5% ethanolic PEG. Total flavonoid content was 0.079± 0.018, 0.086± 0.010 and 0.089± 0.017 mg/g in 0.1, 0.5 and 1% Triton X-100 concentration treated for 1 hour against 0.041± 0.009 mg/g in control (Table-1). The release of flavonoid content in 0.1%, 0.5% and 1% Triton X-100 treated samples was enhanced compared to control. In case of 2 hr treatment duration, 0.1% and 0.5% concentration indicated 0.050± 0.012 and 0.075± 0.008 mg/g flavonoid content respectively which was marginally more over the control (0.050± 0.012 mg/g). However, 1% concentration (0.032± 0.009 mg/g) showed reduced amount of flavonoid than the control. In the cell suspension cultures treated with 0.1%, 0.5% and 1% Triton X-100 for 4 hr treatment, the amount of flavonoid was 0.057± 0.019, 0.084± 0.011 and 0.096± 0.017 mg/g indicating high estimate over control (0.032± 0.011 mg/g).

Table 1
Total flavonoid content in *Blumea lacera* cell suspension cultures treated with Triton X- 100

Permeabilizing agent	Treatment duration	Concentrations (%)	Total flavonoid content (mg/g)
Triton X- 100	1hr	Control	0.041± 0.009
		0.1	0.079± 0.018
		0.5	0.086± 0.010
		1	0.089± 0.017
	2 hr	Control	0.050± 0.012
		0.1	0.075± 0.008
		0.5	0.101± 0.022
		1	0.032± 0.009
	4 hr	Control	0.032± 0.011
		0.1	0.057± 0.019
		0.5	0.084± 0.011
		1	0.096± 0.017

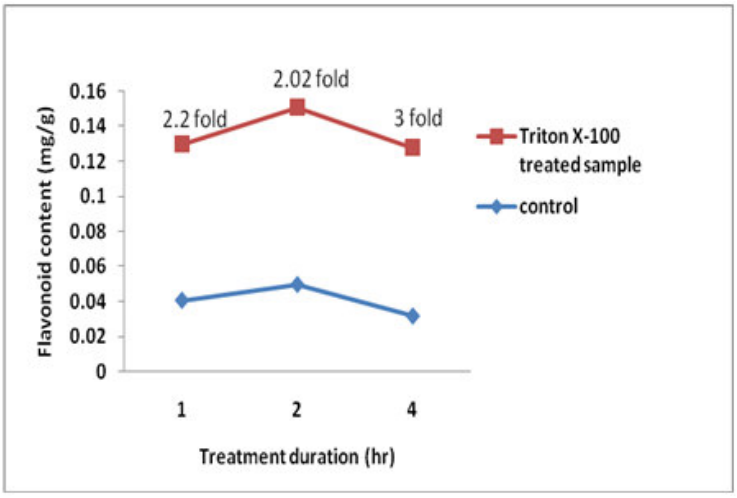

Figure 1
Comparison of flavonoid enhancement in Triton X-100 treated samples

Out of studied treatment durations, 1 hour treatment duration showed 2.2 fold enhancement over the control. In case of 2 and 4 hr treatment 2.02 and 3 fold increase was observed respectively (Fig. 1). The results indicate highest fold increase in cell suspension cultures treated with highest concentration (1%) for longest treatment duration (4 hr). The most suitable concentration and treatment duration for enhanced product recovery of flavonoid was 1% Triton X-100 for 4 hr.

Triton X-100 induced cell permeability for the product recovery of Campesterol

The HPLC analysis of studied concentrations of Triton X-100 i.e. 0.1%, 0.5% and 1% showed 2.055± 0.103, 0.330± 0.029 and 1.809± 0.075 mg/g campesterol respectively for 1 hour treatment duration against 0.937± 0.053 mg/g in control (Table-2). The 0.1% and 1% concentration indicate high yield and 05% Concentration indicate low yield for Campesterol content compared to control. For 2 hr treatment duration, 0.267± 0.031, 0.124± 0.028 and 0.093± 0.015 mg/g flavonoid content was recorded in 0.1%, 0.5% and 1% concentrations of Triton X-100 respectively, which showed marked enhancement over control (0.056± 0.011 mg/g). In case of 4 hr treatment duration variations in Campesterol content were observed. The amount of Campesterol in 0.1% and 0.5% (2.092±0.089 and 0.498±0.026 mg/g) revealed high release of compound compared to control (0.346±0.039 mg/g). However, 1% concentration (0.181±0.014 mg/g) revealed low estimate for Campesterol content.

Table 2
Campesterol content in *Blumea lacera* cell suspension cultures treated with Triton X- 100.

Permeabilizing agent	Treatment duration	Concentration (%)	Campesterol (mg/g)
Triton X- 100	1 hr	Control	0.937± 0.053
		0.1	2.055± 0.103
		0.5	0.330± 0.029
		1	1.809± 0.075
	2 hr	Control	0.056± 0.011
		0.1	0.267± 0.031
		0.5	0.124± 0.028
		1	0.093± 0.015
	4 hr	Control	0.346± 0.039
		0.1	2.092± 0.089
		0.5	0.498± 0.026
		1	0.181± 0.014

The studied treatment durations revealed marked variation in fold increase. Minimum fold increase (2.2) was observed in 1 hour treatment, moderate fold increase (4.8) in 2 hr treatment and maximum fold increase (6.0) in 4 hr treatment (Fig. 2).

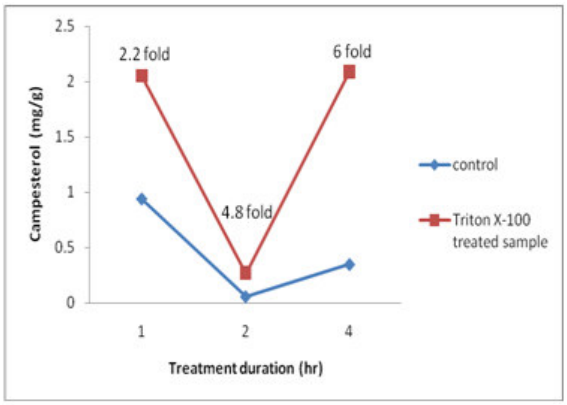

Figure 2
Comparison of campesterol enhancement in Triton X-100 treated samples

DMSO induced cell permeability for flavonoids

DMSO concentrations 0.2%, 0.4% and 0.6% showed 0.063± 0.013, 0.074± 0.021 and 0.053± 0.011 mg/g flavonoid content for 2 hr treatment duration which was slightly more than 0.040± 0.009 mg/g in control (Table-3). Higher amount of flavonoid was observed in moderate (0.4%) concentration. In 4 hr treatment duration, 0.042±0.011, 0.039±0.008 and 0.062±0.011mg/g flavonoid content was observed in 0.2%, 0.4% and 0.6% concentration in context to the control (0.028±0.008 mg/g). All the three concentrations revealed high amount of flavonoid content over control but highest amount was observed in 0.6% concentration. The flavonoid content in studied concentrations for 6 hr was 0.033±0.011, 0.063±0.013 and 0.035±0.010mg/g respectively against 0.025±0.009mg/g in control. In this treatment duration also the highest amount of flavonoid release was in 0.6% concentration.

Table 3
Total flavonoid content in *Blumea lacera* cell suspension cultures treated with DMSO.

Permeabilizing agent	Treatment durations	Concentrations (%)	Total flavonoid content (mg/g)
DMSO	2 hr	Control	0.040± 0.009
		0.2	0.063± 0.013
		0.4	0.074± 0.021
		0.6	0.053± 0.011
	4 hr	Control	0.028± 0.008
		0.2	0.042± 0.011
		0.4	0.039± 0.008
		0.6	0.062±0.011
	6 hr	Control	0.025± 0.009
		0.2	0.033± 0.011
		0.4	0.063± 0.013
		0.6	0.035± 0.010

Out of the studied treatment durations, 2 hr treatment duration showed 1.9 fold increase. The 4 and 6 hr duration revealed 2.2 and 2.5 fold increase, respectively. The results indicate that in the 2 hr and 6 hr treatment duration 0.4% concentration indicated higher product recovery whereas in case of 4 hr treatment duration 0.6% concentration showed good results. For the highest product recovery of flavonoids, 0.4% concentration of DMSO and 6 hr treatment duration was favourable (Fig.3).

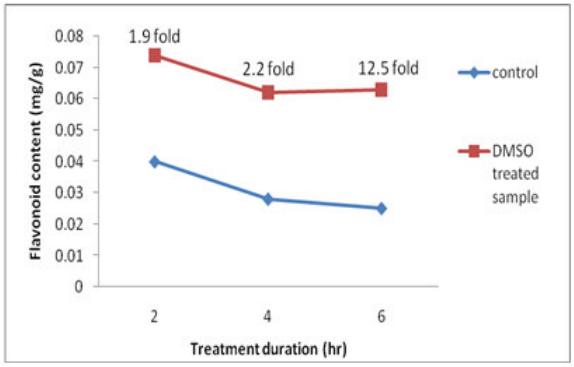

Figure 3
comparison of flavonoid enhancement in DMSO treated samples

DMSO induced cell permeability for Campesterol

The Campesterol content for 2 hr treatment duration was 0.453± 0.031, 0.277± 0.033 and 0.227± 0.021 mg/g respectively in 0.2%, 0.4% and 0.6% DMSO. Campesterol content in all the three studied concentration was less than control i.e. 1.271± 0.069 mg/g (Table-4). The same situation was observed in 4 hr treatment duration. The Campesterol content in 0.2%, 0.4% and 0.6% DMSO was 0.406± 0.023, 0.688± 0.041 and 0.688± 0.034 mg/g which was less compared to control. In case of 6 hr treatment duration, 0.087± 0.015 mg/g Campesterol content was observed in 02% concentration which revealed less yield compared to control (0.201± 0.018 mg/g). However, the Campesterol content in 0.4% and 0.6% (0.499± 0.037 and 0.282± 0.031 mg/g) indicated high yield over control. Highest amount of Campesterol was observed in 0.4% concentration treatment.

Table 4
Campesterol content in Blumea lacera cell suspension cultures treated with DMSO.

Permeabilizing agent	Treatment durations	Concentrations (%)	Campesterol (mg/g)
DMSO	2 hr	Control	1.271± 0.069
		0.2	0.453± 0.031
		0.4	0.277± 0.033
		0.6	0.227± 0.021
	4 hr	Control	0.987± 0.039
		0.2	0.406± 0.023
		0.4	0.688± 0.041
		0.6	0.688± 0.034
	6 hr	Control	0.201± 0.018
		0.2	0.087± 0.015
		0.4	0.499± 0.037
		0.6	0.282± 0.031

It appears that highest treatment duration i.e. 6 hr was favourable for the product recovery of Campesterol. About 2.5 fold increase was observed in this particular treatment duration. In case of 2 hr and 4 hr treatment the Campesterol content in DMSO treated cell suspension culture was less than control. The 0.4% concentration of DMSO and 6 hr duration favours the product recovery of Campesterol in *Blumea lacera* cell suspension cultures (Fig. 4).

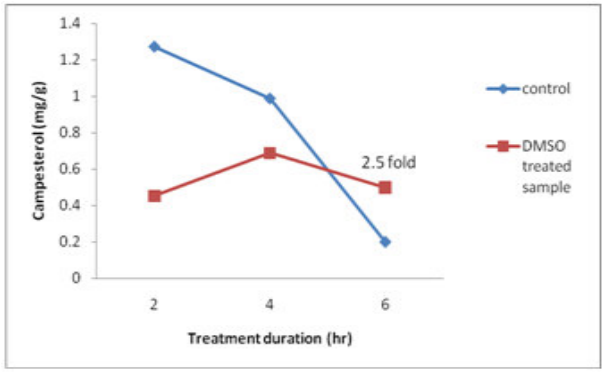

Figure 4
Comparison of campesterol enhancement in DMSO treated samples

DISCUSSION

In most cases, the products formed by plant cell cultures are stored in the vacuoles. The intracellular accumulation of product sometimes inhibits its own synthesis by regulation mechanisms. In order to release the products from vacuoles of plant cells, two membrane barriers, plasma membrane and tonoplast have to be penetrated. In such situations permeabilization of cell membranes can be enhanced using permeabilizing agents for the effective release of metabolic products. Experimental attempts have been made by many workers to permeabilize the plant cells, to maintain the cell viability and to have short time periods of increased mass transfer of substrate and metabolites to and from the cell [19, 25]. In the present experiment the impact of two cell permeabilizing agents, Triton X-100 and DMSO were studied on the product recovery of flavonoids and campesterol in cell suspension cultures of *Blumea lacera*. It was observed that low concentration of Triton X-100 (0.1%) in longest treatment duration (4 hr) favoured maximum product recovery for Campesterol in the suspension culture of *Blumea lacera*. The results indicate that for the product recovery of both the metabolites i.e. flavonoid and Campesterol 4 hr treatment duration with varying concentration was more favourable. Triton X-100 has been used for inducing cell permeability in *Catharanthus roseus* [25], *Beta* vulgaris [28]. Cell suspension cultures of *Bacopa monnieri* were treated with 0.1%, 0.5% and 1% Triton X-100 for 1, 2 and 4 hr. He reported that the studied higher concentrations (0.5 and 1.0%) of the cell permeabilizing

agent, Triton X-100 were positively more interactive at lower treatment duration for the release of bacoside – A compound. With 1 hour treatment duration, there was 57.65 and 68.37 % enhancement respectively for 0.5 and 1.0 % concentration over control. However, the lowest concentration (0.1%) of Triton X-100 does not indicate a marked change on estimates of bacoside – A release over the control [29]. The simulation of ceramide 2 bilayers in the gel phase with different concentrations of DMSO (0.00.6 mol fraction) was carried out. The higher concentration of DMSO induced the gel-phase structure to undergo a transition to the liquid-crystalline phase due to the accumulation of DMSO molecules at the headgroup region, where the molecules integrate, making hydrogen bonds with the lipid headgroups at the expense of the ceramide-ceramide hydrogen bonding. The weakening of the lateral forces and the ensuing expansion in the lipid area causes destabilization of the lipid tail packing resulting in the phase transition to the liquid-crystalline structure. The liquid-crystalline phase of ceramides is expected to be markedly more permeable to solutes than the gel-phase structure [30]. Various workers have reported the use of DMSO as cell permeabilizing agent for the product recovery. *Chenopodium rubrum* cultures which secrete amaranthin (red betacyanin pigment) when incubated with low concentrations of DMSO (5.7%) for 96 h showed no significant effect on cell viability, but a longer incubation period (196 h) was found to have a deleterious effect [31]. DMSO enhanced the release of rosmarinic acid in *Coleus blumei* cell culture without adverse effect on viability and growth of cell [32]. Cell suspension cultures were treated with 0.2%, 0.6% and 1 % DMSO for 3 hr and 6 hr. Result indicated that, the maximum release in 3 hr treatment was noticed in suspension culture treated with 1% DMSO. It was approximately 2 fold more compared to control. In case of 6 hr treatment, there was good increase in the release of bacoside A compound compared to its control in 0.2% and 1% DMSO. The dose of 1.0% DMSO was most favorable concentration for the recovery of studied metabolite in 3 hr and 6 hr treatment durations [24].

CONCLUSION

The findings in the present experimental work has contributed in understanding the extent of effectiveness of studied chemical and biological elicitors and permeabilizing agents for quantitative improvement of medicinally useful metabolites in *Blumea lacera* cell culture. The work conducted provides a practical insight to understand the suitability of appropriate permeabilizing agents, their doses and treatment durations to achieve meaningful results by targeting useful bioactive compounds in plant system of interest. The permeabilizing treatment with both the agents (Triton X-100 and DMSO) in *Blumea lacera* cell culture revealed interesting results indicating appropriate selection and administration of treatment protocol i.e. concentrations and treatment durations. Highest concentration treatment of Triton X-100 (1%) for longer treatment duration (4 hr) favored high release of flavonoids (3.0 fold) in suspension cultures of *Blumea lacera*. For Campesterol, 0.1% Triton X-100 treatment for 4 hr duration showed marked enhancement (2.092 ± 0.089 mg/g, 6.0 fold) over respective control (0.346 ± 0.039 mg/g). DMSO treatment with 0.4% concentration for 2 and 6 hr duration revealed comparatively high release of flavonoid content but 6 hr duration induced maximum release of flavonoid and Campesterol (2.5 fold each). Estimates for fold enhancement indicate Triton X-100 as a potentially better permeabilizing agent compared to DMSO (3.0 fold against 2.5 fold for DMSO) for flavonoid compound. Triton X-100 holds similar status even for the release of Campesterol indicating 6.0 fold more content against 2.5 fold by DMSO.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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