Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Standardization of Enrichment Protocols for Some Medicinally Important Cardenolides within *in vitro* Grown *Calotropis gigantea* Plantlets

Pankaj Singh[1], Yatendra Singh[2], Amar Jeet[1], Renu Nimoriya[1], Sanjeev Kanojiya[2,3], Vineeta Tripathi[1,3], Dipak Kumar Mishra[1,3]

[1]Division of Ethnobotany, CSIR-Central Drug Research Institute, [2]Sophisticated Analytical Instrument Facility, CSIR-Central Drug Research Institute, Lucknow, [3]Faculty, Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, Uttar Pradesh, India

Submitted: 03-10-2018 Revised: 13-11-2018 Published: 06-03-2019

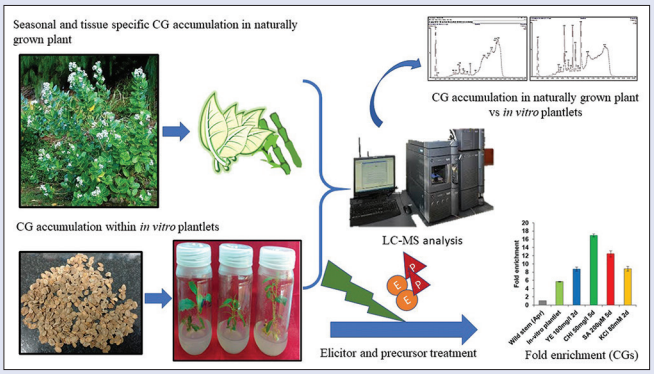
ABSTRACT

Background: *Calotropis gigantea* (L.) Dryand, belonging to the family *Apocynaceae* is a source of many bioactive cardenolides. However, inadequate accumulation of these cardenolides within this plant restricts their economic isolation. Due to multiple chiral centers, these metabolites could not be chemically synthesized so far. **Objective:** The main objective of this study was to develop protocols for enriched production of targeted bioactive cardenolides within *in vitro* grown plant system. **Materials and Methods:** In our study, we established *in vitro* plantlets from the seeds of naturally grown plants. Various biotic and abiotic elicitors, as well as precursors with different concentrations and incubation period, were used for the same. **Results:** It was observed that the *in vitro* grown control plantlets could biosynthesize a higher quantity of seven bioactive cardenolides than the naturally grown plants, whereas only coroglaucigenin was in less quantity. All the eight cardenolides could further successfully be enriched through our experiments. Uscharidin could be enriched significantly to a maximum level (~17-fold) followed by calotropagenin (~11-fold), uzarigenin (~8.5-folds), calotropin (4.5-fold), frugoside (~4-fold), uscharin (3.8-fold), asclepin (~2-fold), and coroglaucigenin (~1.5-fold) when they were compared to their maximum accumulation in naturally grown plants. For effective quantitative calculation of natural abundance of cardenolides within naturally grown plant, their seasonal variations were carried out using ultra high-performance liquid chromatography-mass spectrometry. **Conclusion:** From the above results, it can be concluded that the *in vitro* grown plantlets are the better choice than the naturally grown plants for enriched production of cardenolides. Elicitors were found more effective than precursors for the same.

Key words: Calotropagenin, calotropin, cardenolides, enrichment, uscharidin, uzarigenin

SUMMARY

- Protocols for the enrichment of Uschardin (~17 fold), calotropagenin (~11 fold), uzarigenin (~8.5 fold), calotropin (4.5 fold), frugoside (~4 fold), uscharin (3.8 fold), asclepin (~2 fold) and coroglaucigenin (~1.5 fold) were standardized.
- Control *in vitro C.* gigantea plantlets biosynthesized higher quantity of cardenolides in compare to wild plant.
- Elicitor treatment was found to be more effective than precursor feeding for CGs enrichment.



Abbreviations used: CGs: Cardiac glycosides; CH: Cholesterol; CHI: Chitosan; DDW: Double distilled water; MJ: Methyl Jasmonate; PG: Progesterone; SA: Salicylic acid; SQ: Squalene; YE: Yeast extract.

Correspondence:

Dr. Dipak Kumar Mishra,
Division of Ethnobotany, CSIR-Central Drug Research Institute, Sector 10, Jankipuram Extension, Sitapur Road, Lucknow - 226 031, Uttar Pradesh, India.
E-mail: dk_mishra@cdri.res.in
DOI: 10.4103/pm.pm_507_18

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Calotropis gigantea (L.) Dryand, belonging to the family *Apocynaceae*, is a widely distributed species. In Indian traditional systems of medicine, various parts of this species are used as an analgesic, anti-inflammatory, antispasmodic, and antitumor agents. The plant is also used to treat various other diseases such as ascites, asthma, dysentery, elephantiasis, jaundice, leprosy, syphilis, and ulcer.[1,2] There are several phytoconstituents belonging to the category of alkaloids, glycosides, flavonoids, tannins, saponins, sterols, and triterpenoids have been isolated from different parts of this species. The major cardiac glycosides (CGs) identified from this plant are calotropin, calotoxin, uscharin, voruscharin, uscharidin, uzarigenin, syriogenin, proceroside, and frugoside.[2] For a long period, CGs are well-known as anti-arrhythmic agents, and there are several drugs such as digoxin, digitoxin, and ouabain are presently in market.

Cite this article as: Singh P, Singh Y, Jeet A, Nimoriya R, Kanojiya S, Tripathi V, *et al*. Standardization of enrichment protocols for some medicinally important cardenolides within *in vitro* grown *Calotropis gigantea* plantlets. Phcog Mag 2019;15:264-9.

However, in recent years, anti-proliferative activity of several CGs against various tumor cell lines has also been reported. Frugoside and calotropagenin isolated from leaves of *C. gigantea* had shown cytotoxic activity against small cell lung cancer (NCI-HI87), oral epidermal carcinoma, and breast cancer cell line (MCF7).[3] Coroglaucigenin, calotropin, uscharidin, and asclepin also showed cytotoxic activity against two cancer cell lines HepG2 and Raji.[4] Whereas, uzarigenin showed anticancer activity against human lung adenocarcinoma A549[5] and uscharin exhibited extreme toxicity to A549, HCT116, and HepG2 with IC50 values of 0.003, 0.013, and 0.018 µg/mL, respectively.[6] Frugoside and calotropin, isolated from root bark of *Calotropis procera,* showed potent growth inhibitory activity against A549 non-small cell lung cancer, U373 glioblastoma, and PC-3 prostate cancer cell lines.[7] At present, a semisynthetic cardenolide namely. UNBS1450 derived from 2″-oxovuscharin, a derivative of voruscharin, extracted from *C. procera* and a supercritical CO_2 extract of *Nerium oleander* namely PBI-02504 are under Phase I and Phase II clinical trial respectively for the treatment of cancer.[8,9] Cardenolides are also effective against cystic fibrosis and provide neuroprotection against ischemic stroke.[10] The main source of cardenolides is green plants such as *Digitalis* spp., *Strophanthus* spp., *Calotropis* spp., and *N. oleander.* However, inadequate accumulation of these metabolites within these plants restricts their economic isolation. Chemical synthesis of such compounds is also equally difficult due to their multiple chiral centers. Therefore, enhanced biosynthesis of cardenolides within *in vitro* system can open an alternative option for their constant and bulk production. In the earlier work, biosynthesis of 03 CGs and 02 genin moieties was done through callus culture from *C. gigantea* plant.[11] Here, efforts were made to develop *in vitro* plantlets for the biosynthesis of more bioactive CGs and to enrich them to a significant level. As a result, we could successfully develop the standardized protocols for the enriched bioproduction of 5 bioactive CGs and their 3 genins within *in vitro* grown *C. gigantea* plantlets.

MATERIALS AND METHODS

Seeds of *C. gigantea* plant were collected from CSIR-CDRI campus, Lucknow, Uttar Pradesh, India. The plant was identified and authenticated by Dr. D. K. Mishra, an angiosperm plant taxonomist of CSIR-Central Drug Research Institute, Lucknow, Uttar Pradesh, India. The herbarium specimen of this plant has been deposited at CSIR-CDRIs internationally recognized Herbarium (Acronym "CDRI") bearing the voucher specimen number 25200. All the macro and micronutrients for tissue culture media, sucrose, agar, as well as NaOH were procured from Hi-Media Laboratories (Mumbai, India) and the Extran detergent from Merck Specialties (Mumbai, India). Elicitors such as yeast extract (YE), salicylic acid (SA), and KCl purchased from Hi-Media Laboratories (Mumbai, India). Other elicitors such as methyl jasmonate (MJ), chitosan (CHI), and all the precursors such as cholesterol (CH), progesterone (PG), and squalene (SQ) were purchased from Sigma-Aldrich (now Merck). Absolute ethanol and high-performance liquid chromatography grade methanol were procured from Merck.

Establishment of *in vitro* plants

At first, seeds were washed thoroughly in running tap water with mild detergent, followed by savlon treatment and again washing with double distilled water (DDW). Thereafter, seeds were surface sterilized with 0.2% $HgCl_2$ for 2 min under laminar hood and washed with DDW for three times before inoculation in MS basal medium. The medium pH was adjusted to 5.8 with 0.1 N HCl or NaOH and was supplemented with sucrose 3% (w/v) and agar 0.8% (w/v). Seeds were germinated within 1 week, and after around 2 weeks, the plantlets were transferred into culture bottle for further growth [Figure 1]. Their number was further increased through micropropagation technique.

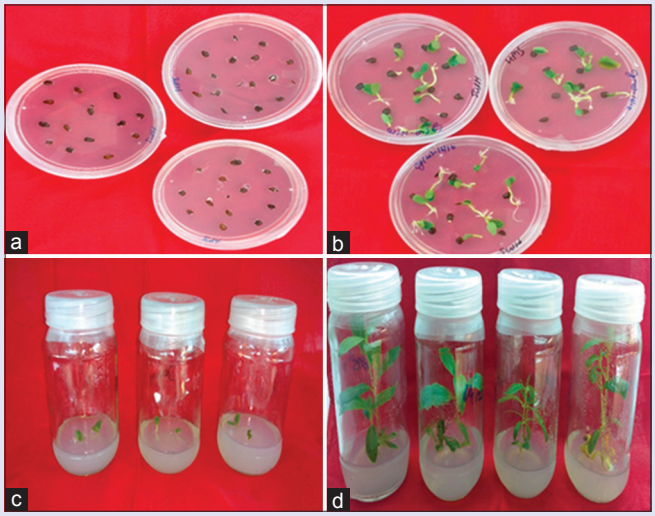


Figure 1: Establishment of *in vitro* plants from seeds. (a) Inoculation of seeds in MS hormone free media. (b) Germination of seed and seedlings development. (c) Seedlings transferred to culture bottles. (d) Full grown *in vitro* plantlets

Table 1: List of elicitors and precursors used for the enrichment of cardiac glycosides

Precursors/elicitors	Concentration	Incubation period
MJ	50, 75, 100 mg/l	1, 2 and 5 days
YE	50, 100, 150 mg/l	2, 5 days
Chitosan	50, 150 mg/l	2, 5 days
SA	50, 100, 150, 200, 250 µM	2, 5 days
KCl	80, 120, 160 mM	2, 5, 10 days
Cholesterol	100, 200, 300 mg/l	2, 5 days
Progesterone	100, 200, 300 mg/l	2, 5 days
Squalene	1, 2, 3 mM	2, 5 days

SA: Salicylic acid; YE: Yeast extract; MJ: Methyl jasmonate

Elicitation and precursor feeding for the enrichment of cardenolides

All the elicitors and precursors except MJ were dissolved in media for their application on plants, whereas MJ was applied through spray on aerial parts. Different elicitors and precursors with their specific concentrations and incubation period are mentioned Table 1. After a specific incubation period, plant materials from all the experimental sets were harvested, and samples were prepared for liquid chromatography-mass spectrometry (LC-MS) analysis.

Harvesting and extraction of *in vitro* plants and seasonal plant materials from naturally grown plant

Whole plants from all the experimental sets were harvested after the scheduled incubation period. To study seasonal variation of CGs, three mature wild *C. gigantea* plants were selected from Lucknow, and two vegetative parts namely leaf and stem were collected in every month from January 2017 till December 2017. All the samples were dried at 40°C in a conventional oven, homogenized into fine powder using a mortar and pestle and stored in airtight plastic vials. 500 mg of this fine powder was extracted with 10 mL ethanol and kept into continuous shaking for 48 h followed by sonication and filtration of the sample through Whatman

filter paper. Excess solvent was removed by rotary evaporator at 40°C and samples were dissolved into 2 ml methanol for the identification of CGs through LC-MS.

Liquid chromatography-mass spectrometry analysis

The LC-MS analysis was performed on a Waters TQD triple quadrupole mass spectrometer (USA). It was equipped with Waters, H-Class Acquity UPLC system and ESI source. The UPLC column used was Water BEH C-18 100 mm × 2.1 mm, 1.7 µm and dual mode (±) LC-ESI-MS experiments performed after injecting 2 µl samples by the autosampler. The chromatographic separation and identification of CGs were carried out as per our previous established analysis method.[12,13] Extracted ion chromatogram [Figure S1] and LC-ESI-MS Spectra of identified CGs and their genins [Figure S2] are provided in Supplementary material.

Data analysis

All results were calculated as mean ± standard error differences between means were tested for statistical significance using the Student's *t*-test at $P \leq 0.05$.

RESULTS

Seasonal variation of cardenolides accumulation in naturally grown plants

Four CGs namely uscharin, frugoside, calotropin, and uscharidin as well as 3 genins namely coroglaucigenin, uzarigenin, and calotropagenin could be detected in seasonal samples of naturally grown *C. gigantea* plant out of total 8 that were biosynthesized within *in vitro* plant [Table 2]. Only asclepin could not be detected by us in naturally grown plant sample. It was observed that the general trend of most of the metabolites to become accumulate in maximum quantity during winter, spring, and summer season, whereas April is the most favorable month for their maximum accumulation. Uzarigenin, the only metabolite, which was found accumulated at maximum level during February. The stem was the most preferred plant part for the accumulation of all the metabolites with compare to leaf [Figure 2].

Enrichment of cardenolides

Uscharidin was biosynthesized 5.5-fold more within the *in vitro* plantlets with compare to their maximum accumulation in naturally grown plant. Elicitation with CHI at 50 mg/l and SA at 200 µM, the metabolite was enriched to a significant level, i.e., around 17 and 12.6-fold, respectively in 5 days' incubation period. KCl at 80 mM concentration and YE at 100 mg/l were also found good enough for the enrichment of this compound up to around 9-fold in 2 days [Figure 3a]. Calotropagenin was biosynthesized 5.3-fold more within the *in vitro* plantlets with compare to their maximum accumulation in naturally grown plant, which was increased 10–11.2-fold with the help of CHI at 100 mg/l, MJ at 75 mg/l, and YE at 100 mg/l in 5 days' incubation period. SQ, the other elicitor could enrich this metabolite more than 6-fold at 1 mM concentration in same days [Figure 3b]. Uzarigenin was biosynthesized 3-fold more within *in vitro* plantlets with compare to their maximum accumulation in naturally grown plant. However, after elicitation with YE at 100 mg/l and SA at 100 µM, the metabolite was enriched more than 5 and 8.6-fold respectively in 5 days' incubation period. The two other elicitors, i.e., MJ and CHI could enrich this metabolite 5.6- and 4.4-fold, respectively, at 100 mg/l concentration in 2 days. Precursors such as CH and PG at 100 mg/l were also effective to enrich the compound around 5-fold in 2 and 5 days, respectively [Figure 3c]. Calotropin was biosynthesized 1.6-fold more within the *in vitro* plantlets with compare to their maximum accumulation in naturally grown plant. SA acid was observed the most active among all elicitors and precursors, which could

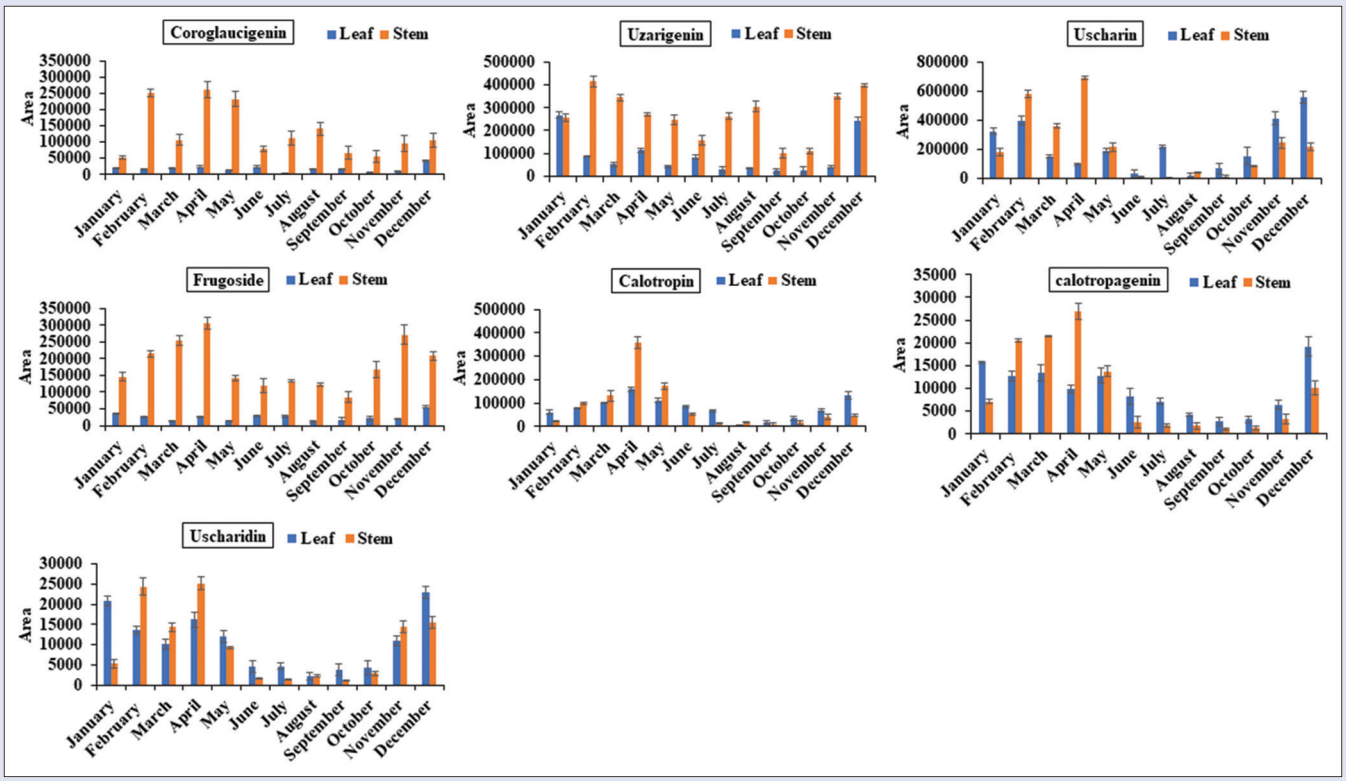


Figure 2: Seasonal variation of cardiac glycosides in naturally grown mature plants

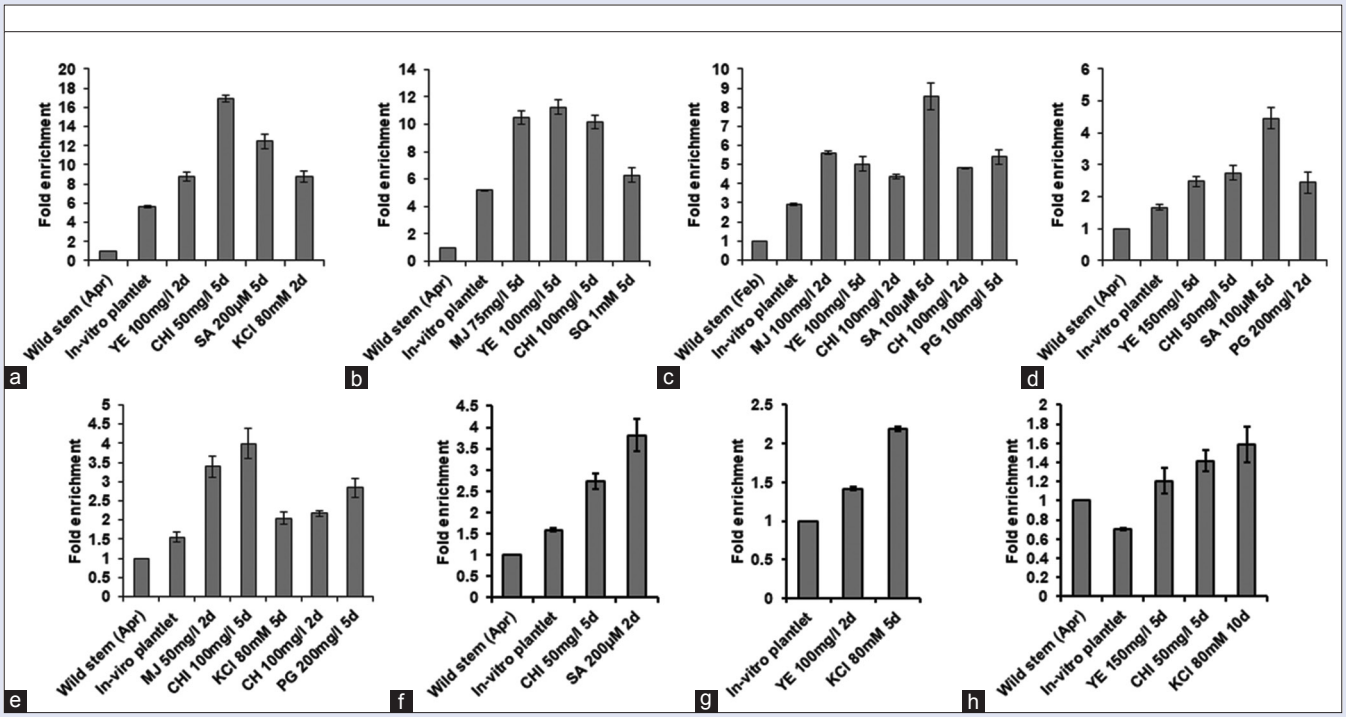


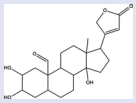
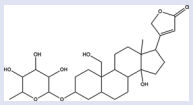
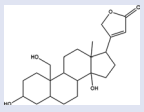
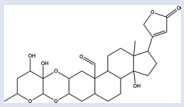
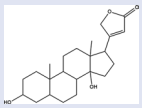
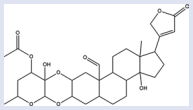
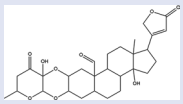
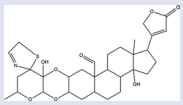
Figure 3: Maximum enrichment of cardenolides with the specific concentration and incubation period of particular elicitors and precursors. (a) uscharidin, (b) calotropagenin, (c) uzarigenin, (d) calotropin, (e) frugoside, (f) uscharin, (g) asclepin, (h) coroglaucigenin. Fold change values are significant $P \leq 0.05$ (Student's *t*-test)

enrich its production up to 4.5-fold at 100 µM concentration and 5 days' incubation period. YE and CHI enriched this metabolite 2.4–2.8-fold at 150 mg/l and 50 mg/l, respectively, in same days. PG, the only precursor, could also enrich it around 2.5-fold at 200 mg/l concentration and in 2 days [Figure 3d]. Frugoside was biosynthesized 1.4-fold more within the *in vitro* plantlets with compare to their maximum accumulation in naturally grown plant. CHI at 100 mg/l could enrich this compound around 4-fold in 5 days' incubation period whereas PG at 200 mg/l and MJ at 50 mg/l enriched it 2.8–3.4-fold in 5 and 2 days, respectively. The abiotic stress KCl at 80 mM and the other precursor CH at 100 mg/l enriched this metabolite more than 2-fold in 5 days [Figure 3e]. Uscharin was biosynthesized 1.6-fold more within the *in vitro* plantlets with compare to their maximum accumulation in naturally grown plant. The metabolite was enriched up to 3.8 and 2.7-fold with SA at 200 µM and CHI at 50 mg/l concentration in 2 and 5 days, respectively [Figure 3f]. Asclepin could not be detected by us from naturally grown samples, and hence, its enrichment was compared with the *in vitro* grown control plantlets. KCl at 80 mM concentration was found the most effective, which could enrich it more than 2-fold in 5 days' incubation period whereas 1.4-fold enrichment was taken place by YE at 100 mg/l concentration in 2 days [Figure 3g]. Coroglaucigenin is the only metabolite, which was biosynthesized in less quantity (0.7-fold) within the *in vitro* plantlets with compare to naturally grown sample. With the help of KCl at 80 mM concentration and in 10 days' incubation period, the compound could be enriched up to 1.6-fold. The other two elicitors, i.e., YE at 150 mg/l and CHI at 50 mg/l enriched this metabolite 1.2–1.4-fold in 5 days [Figure 3h]. The complete enrichment data of the biosynthesized CGs with all the concentrations and incubation period of elicitors and precursors, which are not given here, provided in Supplementary material [Tables S1-S8].

DISCUSSION

Plant secondary metabolites are the result of plant environment interaction, which help them in adapting or acclimatizing adverse environmental conditions. Biosynthesis and accumulation of secondary metabolites largely depend on the external biotic and abiotic factors affecting plants. Some previous workers have already described effects of several external factors such as temperature, radiation, and availability of water on the production of secondary metabolites in different plants.[14-18] From our studies on seasonal variations of selected CGs in *C. gigantea* plant, it was observed that the CGs accumulation pattern was remarkably varied in different seasons and maximum accumulation of most of the CGs took place in the month of April. It can be explained by the fact that every season have different quantum of water, temperature, and radiation. These climate changes not only alter abiotic factors but also manipulate different biotic factors of the surrounding environment, which exert plethora of abiotic and biotic stress on the plants and leads to biosynthesis of specific secondary metabolites for either direct defense or induction of signaling in response to abiotic/biotic stress condition. It is well-known fact that the secondary metabolites are armamentaria used by plants to fight battle for survival and propagation. Their main role is to protect plant either from the extreme environmental condition or from predators (herbivores), pathogens, or competitors. In *C. procera,* CGs have been reported to accumulate in response to wounding.[12] Defense signaling induced due to herbivory or wounding in different plants is often mediated by MJ,[19] and probably, CGs accumulation follows the same route and ultimately provide defense to the mother plant.[20] MJ leads to the rapid release of α-linolenic acid from the lipid pool of the plant cell and elicits secondary metabolites production.[21] SA induces SAR (systemic acquired resistance), which ultimately elicit secondary metabolites production. Such as MJ and SA, YE and CHI are also inducing the biosynthesis of defence-related secondary metabolite

Table 2: List of metabolites biosynthesized within *in vitro* plant

Cardenolides	MW	RT	Structure
Calotropagenin	404	5.53	
Frugoside	536	6.95	
Coroglaucigenin	390	7.05	
Calotropin	532	7.74	
Uzarigenin	374	9.22	
Asclepin	574	9.62	
Uscharidin	530	9.62	
Uscharin	587	10.05	

MW: Molecular weight, RT: Retention time

by mimicking herbivory/pathogen attack through MJ and SA mediated signaling pathway.[22] Salinity or salt stress (inorganic salts such as NaCl, KCl, and $MgCl_2$) generates the ROS production that activates various transcription factor involved in the biosynthesis of various secondary metabolites.[23] Considering these basic principles, these elicitors were used for the enrichment of bioactive CGs within *in vitro* grown plantlets. In major cases, it was observed that the *in vitro* grown control plants accumulated more CGs in comparison to the mature naturally grown plants. This is probably due to the age of the *in vitro* grown plantlets. These plantlets were much younger (1–3 months old) when compared with the mature naturally grown plant (3–4 years old). Our results are in accordance with the previous study done on *C. procera,* where authors had shown that younger plants accumulate more CGs in comparison to mature plants.[12]

In our experiment, MJ was found to increase the accumulation of 03 CGs within the *in vitro* grown plants. Sun *et al*. also reported that cardenolide content in hairy roots of *C. gigantea* was increased by 2-fold with 50 mg/l concentration of MJ treatment in 10 days when compared to control hairy roots.[24] MJ is well known to influence the accumulation of some other secondary metabolites in different plants.[22] MJ-induced enhanced production of alkaloids along with their precursors and enzymes was reported in *Cathranthus roseus* and *Cinchona ledgeriana* seedings.[25] YE, an other biotic elicitor, was also observed to increase the accumulation of six different CGs within *in vitro* grown *C. gigantea* plants. A similar effect of YE on CGs enrichment was reported in hairy root cultures of *C. gigantea*.[24] YE elicitation resulted in the enrichment of several indole alkaloids namely. vinblastine and vincristine about 22.74% and 48.49%, respectively in germinating embryo and *in vitro* raised leaves of *Catharanthus roseus*.[26] Echitamine was also reported to be enriched more than 2-fold in the callus, derived from the *Alstonia scholaris* leaves.[27] Another biotic elicitor CHI was found effective in increasing the accumulation of 07 CGs in the *in vitro* grown plants of our studies. Like YE, CHI also increased CGs accumulation in hairy root cultures of *C. gigantea*.[24] CHI induced production of simple coumarins (pinnarin and rutacultin), furanocoumarins (bergapten, isopimpinelin, psoralen, xanhotoxin), and dihydrofuranocoumarins (chalepin and rutamarin) was reported in *Ruta graveolens*.[28] SA acts as stress signalling molecule and activate different signal transduction cascade that help plants to combat different biotic and abiotic stresses. In our studies, SA treatment enriched 04 CGs significantly. This finding is in accordance with the results obtained in shoot cultures of *Digitalis purpurea*, where SA elicitation could enrich 02 CGs namely. digitoxin and digoxin. SA elicitation leads to accumulation of hyoscyamine (3.30-fold) and scopolamine (4.0-fold) compared to control root culture of *Datura metel* L.[29] Osmotic or salt stress could efficiently improve the secondary metabolite biosynthesis in *in vitro* as well as *in vivo*.[30] Salt stress lead to ROS production in plant cell, which is countered by several mechanisms such as flavonoids generation, H_2O_2 detoxification, and OH-radicle.[31,32] In our experiment, KCl elicited the production of uscharidin significantly, i.e., around 9-fold and other 03 CGs namely asclepin and frugoside and coroglaucigenin to a certain extent. Similar type of works has also been reported for some other compounds in different plants. Compact callus cultures of *Catharanthus roseus* treated with 03 and 04 g/l KCl promoted catharanthine and serpentine production by 3–4-fold over the control.[30] A study on the plant *Digitalis purpurea* suggested that KCl at 80 mM treatment resulted in the accumulation of digitoxin by 7.75-fold.[33]

In plants, CGs are synthesized through pregnenolone probably by precursor and intermediate of terpenoids and steroid biosynthetic pathways.[12] The concentration of precursors or metabolic intermediates affects the biosynthesis of products. We studied the effect of different precursors namely CH, PG, and SQ on the biosynthesis of CGs. SQ is the starting point of steroid biosynthetic pathway and through series of chemical events, it forms different types of phytosterols up to pregnenolone. On SQ feeding, we observed a slight increase in calotopagenin biosynthesis in compare to its control. It is because within plant system, SQ has many fates and as a result, its effect might be diluted. It is believed that CH may be involved in CGs biosynthesis through pregnenolone. Therefore, we fed *in vitro* grown plants with CH and observed certain increase in the concentration of uzarigenin and frugoside. Wickramasinghe *et al.* first demonstrated transformation of exogenously supplied CH into cardenolides.[34] The addition of CH in the medium improved the accumulation of both digitoxin and digoxin in shoot culture of *Digitalis purpurea*.[33] PG, on the other hand, showed certain increase in the biosynthesis of CGs such as uzarigenin, frugoside and calotropin like Patil *et al*., where increased accumulation of digitoxin and digoxin was reported in shoot culture of *Digitalis purpurea* through this precursor feeding.[33]

CONCLUSION

From the above observations, it can be concluded that the *in vitro* plantlets are a better performer than naturally grown plants for CGs biosynthesis. For the enrichment of CGs, elicitor application was more effective than precursor feeding, and elicitors were very much concentration specific along with their incubation period for the same.

Acknowledgement

P. S., Y. S., A. J. and R. N. are thankful to CSIR, DBT, and UGC, New Delhi, for fellowships. We sincerely thank SAIF, CSIR-CDRI, for the analytical facilities. The manuscript bears CDRI Communication No 9787.

Financial support and sponsorship

This study was funded by DST project GAP0257 and DBT project GAP0180.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Chopra RM, Nayar SI, Chopra IC. Glossary of Indian Medicinal Plants. New Delhi: Council of Scientific & Industrial Research; 1956. p. 309-11.
2. Joshi H, Gururaja MP, Suares D. *Calotropis gigantea* R. Br. (*Asclepiadaceae*): A review. Int J Pharm Res 2011;3:10-4.
3. Seeka C, Sutthivaiyakit S. Cytotoxic cardenolides from the leaves of *Calotropis gigantea*. Chem Pharm Bull (Tokyo) 2010;58:725-8.
4. Li JZ, Qing C, Chen CX, Hao XJ, Liu HY. Cytotoxicity of cardenolides and cardenolide glycosides from *Asclepias curassavica*. Bioorg Med Chem Lett 2009;19:1956-9.
5. Zhang J, Ponomareva LV, Nandurkar NS, Yuan Y, Fang L, Zhan CG, *et al.* Influence of sugar amine regiochemistry on digitoxigenin neoglycoside anticancer activity. ACS Med Chem Lett 2015;6:1053-8.
6. Jacinto SD, Chun EA, Montuno AS, Shen CC, Espineli DL, Ragasa CY, *et al.* Cytotoxic cardenolide and sterols from *Calotropis gigantea*. Nat Prod Commun 2011;6:803-6.
7. Ibrahim SR, Mohamed GA, Shaala LA, Moreno L, Banuls Y, Kiss R, *et al.* Proceraside A, a new cardiac glycoside from the root barks of *Calotropis procera* with *in vitro* anticancer effects. Nat Prod Res 2014;28:1322-7.
8. Juncker T, Schumacher M, Dicato M, Diederich M. UNBS1450 from *Calotropis procera* as a regulator of signaling pathways involved in proliferation and cell death. Biochem Pharmacol 2009;78:1-0.
9. National Library of Medicine (U.S.). Efficacy and Safety Study of PBI-05204 in Patients with Stage IV Metastatic Pancreatic Adenocarcinoma. Identifier NCT02329717; 29 September, Updated 2016. Available from: https://www.clinicaltrials.gov/ct2/show/NCT02329717. [Last accessed on 2018 Jul 27].
10. Prassas I, Diamandis EP. Novel therapeutic applications of cardiac glycosides. Nat Rev Drug Discov 2008;7:926-35.
11. Tripathi PK, Awasthi S, Kanojiya S, Tripathi V, Mishra DK. Callus culture and *in vitro* biosynthesis of cardiac glycosides from *Calotropis gigantea* (L.) ait. *In Vitro* Cell Dev Biol Plant 2013;49:455-60.
12. Pandey A, Swarnkar V, Pandey T, Srivastava P, Kanojiya S, Mishra DK, *et al.* Transcriptome and metabolite analysis reveal candidate genes of the cardiac glycoside biosynthetic pathway from *Calotropis procera*. Sci Rep 2016;6:34464.
13. Kanojiya S, Madhusudanan KP. Rapid identification of calotropagenin glycosides using high-performance liquid chromatography electrospray ionisation tandem mass spectrometry. Phytochem Anal 2012;23:117-25.
14. Ramakrishna A, Ravishankar GA. Influence of abiotic stress signals on secondary metabolites in plants. Plant Signal Behav 2011;6:1720-31.
15. Arbona V, Manzi M, Ollas CD, Gómez-Cadenas A. Metabolomics as a tool to investigate abiotic stress tolerance in plants. Int J Mol Sci 2013;14:4885-911.
16. Graham DA, Patterson BD. Responses of plants to low, nonfreezing temperatures: Proteins, metabolism, and acclimation. Annu Rev Plant Physiol 1982;33:347-72.
17. Jakobsen HB, Olsen CE. Influence of climatic factors on emission of flower volatiles *in situ*. Planta 1994;192:365-71.
18. Shulaev V, Cortes D, Miller G, Mittler R. Metabolomics for plant stress response. Physiol Plant 2008;132:199-208.
19. Farmer EE, Ryan CA. Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. Plant Cell 1992;4:129-34.
20. Joubert JP. Cardiac Glycosides Toxicants of Plant Origin. Vol. 2. Boca Raton, Florida: Peter R. Cheeke Press; 1989. p. 61-96.
21. Memelink J, Verpoorte R, Kijne JW. ORCAnization of jasmonate-responsive gene expression in alkaloid metabolism. Trends Plant Sci 2001;6:212-9.
22. Sharma M, Sharma A, Kumar A, Basu SK. Enhancement of secondary metabolites in cultured plant cells through stress stimulus. Am J Plant Physiol 2011;6:50-71.
23. Owolabi IO, Yupanqui CT, Siripongvutikorn S. Enhancing secondary metabolites (emphasis on phenolics and antioxidants) in plants through elicitation and metabolomics. Pak J Nutr 2018;17:411-20.
24. Sun J, Xiao J, Wang X, Yuan X, Zhao B. Improved cardenolide production in *Calotropis gigantea* hairy roots using mechanical wounding and elicitation. Biotechnol Lett 2012;34:563-9.
25. Aerts RJ, Gisi D, De Carolis E, De Luca V, Baumann TW. Methyl jasmonate vapor increases the developmentally controlled synthesis of alkaloids in *Catharanthus* and *Cinchona* seedlings. Plant J 1994;5:635-43.
26. Maqsood M, Abdul M. Yeast extract elicitation increases vinblastine and vincristine yield in protoplast derived tissues and plantlets in *Catharanthus roseus*. Rev Bras Farmacogn 2017;27:549-56.
27. Singh SK, Joshi T, Kanojiya S, Tripathi V, Mishra DK. Callus culture and *in vitro* biosynthesis of echitamine from *Alstonia scholaris* (L.) R. Br. Plant Cell Tissue Organ Cult 2015;120:367-72.
28. Orlita A, Sidwa-Gorycka M, Paszkiewicz M, Malinski E, Kumirska J, Siedlecka EM, *et al.* Application of chitin and chitosan as elicitors of coumarins and fluoroquinolone alkaloids in *Ruta graveolens* L. (common rue). Biotechnol Appl Biochem 2008;51:91-6.
29. Ajungla L, Patil PP, Barmukh RB, Nikam TD. Influence of biotic and abiotic elicitors on accumulation of hyoscyamine and scopolamine in root cultures of *Datura metel* L. Indian J Biotechnol 2009;8:317-22. Available from: https://pdfs.semanticscholar.org/bf02/b802fef1bbbd07b4fdfe6e6a4fa79c3113b6.pdf [Last accessed on 2018 Dec 26].
30. Zhao J, Hu Q, Guo YQ, Zhu WH. Effects of stress factors, bioregulators, and synthetic precursors on indole alkaloid production in compact callus clusters cultures of *Catharanthus roseus*. Appl Microbiol Biotechnol 2001;55:693-8.
31. Blokhina O, Virolainen E, Fagerstedt KV. Antioxidants, oxidative damage and oxygen deprivation stress: A review. Ann Bot 2003;91:179-94.
32. Shao HB, Chu LY, Lu ZH, Kang CM. Primary antioxidant free radical scavenging and redox signaling pathways in higher plant cells. Int J Biol Sci 2007;4:8-14.
33. Patil JG, Ahire ML, Nitnaware KM, Panda S, Bhatt VP, Kishor PB, *et al. In vitro* propagation and production of cardiotonic glycosides in shoot cultures of *Digitalis purpurea* L. By elicitation and precursor feeding. Appl Microbiol Biotechnol 2013;97:2379-93.
34. Wickramasinghe JA, Hirsch PC, Munavalli SM, Caspi E. Biosynthesis of plant sterols. VII. The possible operation of several routes in the biosynthesis of cardenolides from cholesterol. Biochemistry 1968;7:3248-53.