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

ORIGINAL ARTICLE

Growth stage-specific Accumulation of Cardiac Glycosides in Two Variants of *Calotropis gigantea* (L.) W.T. Aiton

Pankaj Singh[1], Yatendra Singh[1], Sanjeev Kanojiya[1,2], Vineeta Tripathi[1,2], Dipak Kumar Mishra[1,2]

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

Submitted: 15-Nov-2021 Revised: 28-Feb-2022 Accepted: 29-Mar-2022 Published: 07-Jul-2022

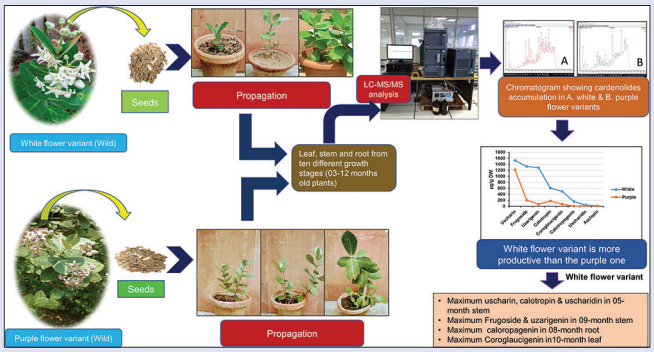
ABSTRACT

Background: *Calotropis gigantea* (L.) W.T. Aiton, belonging to the family Apocynaceae, is a source of many bioactive cardiac glycosides. Accumulation of secondary metabolites in plants depends upon various external and internal factors, including the plant age or growth stage. **Objectives:** The main aim of this study was to identify the correct variant and growth stage of *C. gigantea* for the maximum yield of targeted metabolites. **Materials and Methods:** In the present experiment, three different tissues, that is, leaf, stem, and root, of 3–12-month-old white flower variant (WFV) and purple flower variant (PFV) of *C. gigantea* were investigated to find out the growth stage-specific accumulation pattern of five cardiac glycosides (CGs) and their three genins. **Results:** Highest concentrations of calotropin, uscharin, and uscharidin were found in the stem of five-month-old WFV. Frugoside and uzarigenin were at peak levels in the stem of a nine-month-old member of the same variant. Calotropagenin and coroglaucigenin were at maximum levels in the root and leaf of 8- and 10-month-old members, respectively, of this variant too. The only CG accumulated at maximum level in the root of 11-month-old PFV was asclepin and its quantity was comparable to 8-month-old WFV root. **Conclusion:** It was observed that the WFV was superior to PFV, considering the accumulation of most of the CGs. Therefore, it can be concluded that the specific tissues of 5–10-month-old members of WFV are optimum for an economic yield of respective CGs.

Key words: Calotropin, *Calotropis gigantea*, cardenolides, coroglaucigenin, LC-MS analysis

SUMMARY

- WFV is superior to PFV, considering the accumulation of most of the CGs.
- Maximum quantities of calotropin, uscharin, and uscharidin are found in the stem of 5-month-old WFV.
- Maximum quantities of frugoside and uzarigenin are found in the stem of 9-month-old WFV.
- Maximum quantity of calotropagenin is found in the root of 8-month-old WFV.
- Maximum quantity of coroglaucigenin is found in the leaf of 10-month-old WFV.



Abbreviations: CG: cardiac glycoside; **TDQ:** triple quadrupole; **UPLC:** ultra performance liquid chromatography; **ESI:** electrospray ionization; **DW:** dry weight; **WFV:** white flower variant; **PFV:** purple flower variant

Correspondence:

Dr. Dipak Kumar Mishra,
Botany Unit, Sophisticated Analytical Instrument Facility and Research, CSIR-Central Drug Research Institute, Lucknow, Uttar Pradesh - 226 031, India.
E-mail: dk_mishra@cdri.res.in

DOI: 10.4103/pm.pm_525_21

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

INTRODUCTION

Cardiac glycosides (CGs) are well known for the treatment of congestive heart failure, and there are several CG drugs like digoxin, digitoxin, and ouabain that are presently available in the market. Recently, CGs have been explored as an antiproliferative agent for treating various cancer types. Asclepin, calotropin, coroglaucigenin, and uscharidin are known for their cytotoxic activity against HepG2 and Raji cancer cell lines.[1] Calotropagenin and frugoside have revealed cytotoxic activity against breast cancer cell line (MCF7), oral epidermal carcinoma (KB), and small cell lung cancer (NCI-HI87).[2] Uscharin has shown acute toxicity against HepG2, HCT116, and A549.[3] Uzarigenin has demonstrated anticancer activity against human lung adenocarcinoma A549.[4] Presently, a semisynthetic cardenolide viz. UNBS1450, developed from 2″-oxovuscharin, a derivative of voruscharin from *Calotropis procera,* and PBI-02504, a supercritical CO_2 extract of *Nerium oleander,* Phase I and Phase II clinical trials, respectively, for the treatment of cancer.[5,6] Various plants like *Digitalis purpurea*, *Convallaria majalis*, *Strophanthus kombe*, *C. procera*, *Calotropis gigantea,* and so on and animals including *Bufo marinus* are rich sources of CGs. *C. gigantea* contains diverse CGs like calotropin, uscharin, uscharidin, frugoside, and so on. The genins include calotropagenin and uzarigenin.[7]

There are two major wild variants, that is, white flower variant (WFV) and purple flower variant (PFV), of *C. gigantea* available in nature [Figure 1]. Both the variants are up to 10 ft tall and highly branched shrubs or small trees. Despite many similarities between these two variants, taxonomists treat these as two different forms, mainly due to their contrasting floral characters.[8] Several

Cite this article as: Singh P, Singh Y, Kanojiya S, Tripathi V, Mishra DK. Growth stage-specific accumulation of cardiac glycosides in two variants of *Calotropis gigantea* (L.) W.T. Aiton. Phcog Mag 2022;18:353-9.

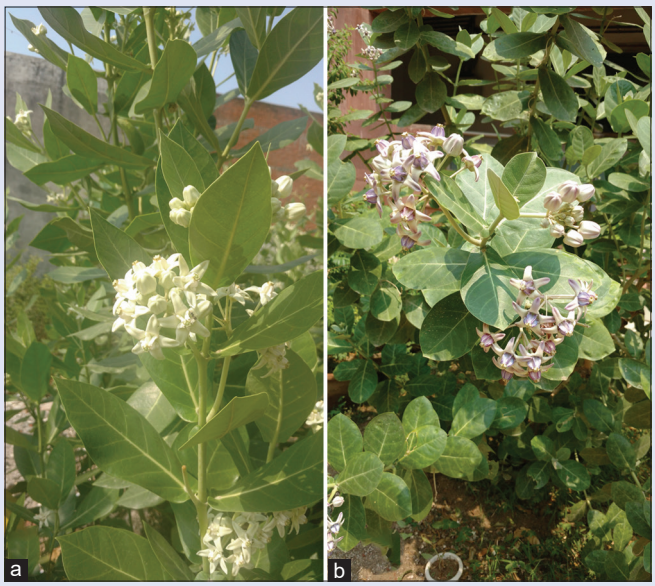


Figure 1: *Calotropis gigantea* plant: (a) white flower variant; (b) purple flower variant

micro-morphological, micrometric, and molecular differences have been observed between these two variants.[8,9] Micro-morphological studies on both variants revealed that WFV has broad sepals and petals compared to PFV. Powder microscopy analyses have depicted calcium oxalate crystal structure variation in both variants. WFV has prismatic and rhomboidal crystals, whereas rosette crystals are observed in PFV.[8] Significant divergence between WFV and PFV was observed using Random Amplified Polymorphic DNA (RAPD) approach with 10 random primers. Further, cluster analysis of these two variants, based on the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) Jacob coefficient, confirms their genetic polymorphism.[9] Expressed Sequence Tag-Simple Sequence Repeat (EST-SSR) markers also provided a vast range of gene polymorphisms between WFV and PFV.[10] The plant is a member of the Apocynaceae family and is widely distributed in subtropical countries like Cambodia, China, India, Indonesia, and Pakistan, in tropical Africa, and so on. It is commonly available in many states of India, including Assam, Bengal, Bihar, Karnataka, Maharashtra, and Uttar Pradesh. Ethnomedicinally, the plant is essential in India, as various tribes of Sikkim, Bengal, and Orissa use it as an abortifacient and antifertility agent.[11] The other ethnobotanical uses of this plant include curing asthma,[12] dropsy, and syphilis.[13] In modern pharmacology also, the plant has shown many biological activities like antidiarrheal, antioxidant, anti-inflammatory, analgesic, antipyretic, and so on.[7]

Plant age or its different growth stages are known to have a significant impact on the accumulation of various secondary metabolites, which have been reported in many plants by several workers from time to time. It was observed that the phenolics, hypericin, and flavonoid concentration of *Hypericum prunatum* reached their maximum levels during the flowering stage.[14] Similarly, the highest concentrations of rosmarinic acid, ursolic acid, and oleanolic acid in *Pronella vulgaris* were found at the complete flowering stage, and their quantities within leaves were more elevated than in stem.[15] Our present study aims to determine and compare the growth stage-specific accumulation of selected CGs and their genins in three different parts of *C. gigantea* WFV and PFV.

MATERIALS AND METHODS

Ethanol and methanol absolute Ethanol from Merck (Darmstadt, Germany) and HPLC grade methanol from Merck Limited (Mumbai, India). Soilrite and vermiculite were purchased from Nirmal Nursery (Lucknow, India). Seeds of *C. gigantea* WPV and PFV were collected from Lucknow (Uttar Pradesh) and Midnapore (West Bengal), respectively. Both the variants were identified and authenticated by the angiosperm taxonomist of Central Drug Research Institute (CSIR-CDRI), Lucknow, Uttar Pradesh, India. The herbarium specimens of these two variants have been deposited at CSIR-CDRI's Herbarium (acronym "CDRI") bearing the voucher specimen numbers 25200 and 25201, respectively.

Seed germination and plant establishment

Seeds were sown in seedbeds containing soilrite and vermiculite mixture (3:1 ratio). Seeds were germinated within 10–15 days. After achieving 8–12 cm height (in 30–40 days), plantlets were transferred to earthen pots and maintained under controlled conditions. A monthly collection of plant parts like leaf, stem, and root was started in triplicates from 3-month-old seedlings and continued with 12-month-old mature plants.

Sample preparation

All the collected plant materials (leaf, stem, and root) were chopped and placed in a conventional oven at 40°C. After drying, the plant materials were powdered and stored in airtight containers. Later, 500 mg of each sample was extracted with 10 ml ethanol and kept on a shaker for 48 h. Thereafter, each extract was sonicated and filtered through Whatman filter paper. Excess solvent was removed with a rotary evaporator (Rotavapor R-210; Buchi, Flawil, Switzerland) at 40°C, and samples were dissolved in 2 ml methanol to identify the CGs through Liquid Chromatography-mass spectrometry (LC-MS).

LC-MS analysis

The LC-MS analysis was performed on a Waters TQD triple quadrupole mass spectrometer (Waters, Milford, USA) equipped with Waters H-Class Acquity ultra performance liquid chromatography (UPLC) system and electrospray ionization (ESI) source. The UPLC column used was Water BEH C-18 100 × 2.1 mm, 1.7 µm, and dual-mode (±). LC-ESI-MS experiments were performed after injecting 2 µl samples by the autosampler. The chromatographic separation and identification of CGs were carried out as per our previously reported analysis method.[16] LC-ESI-MS/MS spectra of identified CGs and their genins are provided. Apart from this, quantitative analysis of CGs was done with the area under extracted ion chromatogram (EIC) *m/z* 405 $[M + H]^+$ calotropagenin, 537 $[H + H]^+$ frugoside, 391 $[M + H]^+$ coroglaucigenin, 533 $[M + H]^+$ calotropin, 375 $[M + H]^+$ uzarigenin, 575 $[M + H]^+$ asclepin, 531 $[M + H]^+$ uschardin, and 570 $[M + H\text{-}H_2O]^+$ uscharin, and they were quantified with respect to digitoxin as the reference standard (*m/z* 783 $[M + NH_4]^+$).

Preparation of standard solution

To prepare 1 mg/ml stock solution of digitoxin, which served as the reference standard for relative quantitative analysis, 1 mg of digitoxin was dissolved in 1 ml of methanol (HPLC grade). After that, a series of the same solution with lower concentrations (1–400 µg/ml) was prepared and filtered through a 0.22 µm syringe filter (Millipore) before being used for LC-MS.

Calibration curve of digitoxin and semi-quantitative analysis of CGs content

The calibration curve of digitoxin was drawn by plotting values of different concentrations (1–400 µg/ml) of digitoxin standard versus their peak areas. The regression equation obtained from this curve was ultimately used to quantify the cardenolides' content in all the experimental samples. Cardenolides' concentrations (µg/ml) were calculated as "x" values by putting the peak areas of the experimental samples ("y" values) in the equation. The x values were obtained in µg/250 mg dry weight (DW) of the sample because 500 mg of samples was reconstituted in 2 ml methanol during sample preparation. Finally, all the cardenolide content was expressed as µg/g DW of samples.

Data analysis

All results were calculated as mean ± standard error (SE). The analyses were carried out in three replications for each staging experiment. The results obtained were presented as mean ± SE.

RESULTS

Identification of CGs

Based on our previously established method,[16] five CGs and three genins were identified in both the variants of *C. gigantea* through LC-MS analysis. The characteristic MS/MS fragmentation of genins and their chromatographic retention time were used to identify both CGs and independent genins. The list of CGs and genins with their retention time, molecular weight, and characteristic MS/MS fragmentation is given in Table 1. Semi-quantitative estimation of CGs was carried out with the area under EIC *m/z* 405 $[M + H]^+$calotropagenin, 537 $[H + H]^+$ frugoside, 391 $[M + H]^+$ coroglaucigenin, 533 $[M + H]^+$ calotropin, 375 $[M + H]^+$ uzarigenin, 575 $[M + H]^+$ asclepin, 531 $[M + H]^+$ Uscharidin, and 570 $[M + H\text{-}H_2O]^+$ uscharin, and they were quantified with respect to digitoxin as the reference standard. The calibration curve of digitoxin was obtained by plotting a graph for different concentrations (1–400 µg/ml) of digitoxin standard versus their peak areas with R^2 0.993.

Accumulation of CGs

WFV was found to be superior to PFV, considering accumulation of most of the detected CGs [Table 1]. Uscharin was accumulated at a maximum level in the stem of 5-month-old members of both variants. In WFV, it was quantified as 1532.58 µg/g DW, whereas in PFV it was 1222.27 µg/g DW. The roots of 4- and 7–10-month-old members and the leaf of the 12-month-old member of WFV accumulated the metabolite in moderate concentrations (899.85–1168.71 µg/g DW). Similarly, the leaves of 4-, 5-, 7-, and 12-month-old members and the roots of 6- and 11-month-old members of PFV also produced the same metabolite in moderate concentrations (805.13–1128.57 µg/g DW). Frugoside was found to be the second major CG, considering its accumulation in this plant. Its maximum accumulation (1329.2 µg/g DW) was observed in the stem of the 9-month-old WFV. In other parts of different age groups of this variant, the compound had accumulated in very meagre to moderate quantity (7.25–769.29 µg/g DW). In PFV, its accumulation was restricted, and the maximum concentration (209.79 µg/g DW) was observed in the stem of 7-month-old members. Calotropin concentration was observed to be, to some extent, higher in the stem of immature plantlets and roots of mature members in both the variants. Its maximum quantity (607.13 µg/g DW) was found in the stem of 5-month-old WFV, followed by the roots of 12-, 10-, 8-, and 9-month-old members (548.39, 533.38, 526.98, and 503.48 µg/g DW, respectively). In PFV, its concentration was comparatively less and the maximum quantity (~180 µg/g DW) was found in the root of 12- and 11-month-old members. Like calotropin, maximum accumulation of uscharidin (49.4 µg/g DW) was noticed in the stem of 5-month-old WFV, followed by the root of a 4-month-old member (44.10 µg/g DW). The root of 8-month-old (39.70 µg/g DW) and the stem of 9-month-old (36.70 µg/g DW) members showed their moderate accumulation. PFV could accumulate this metabolite in a lesser quantity, and its maximum concentration (24.95 µg/g DW) was observed in the root of an 11-month-old member, which was followed by the roots of 6-month-old (22.40 µg/g DW) and 10-month-old (21.10 µg/g DW) members. Contrary to other CGs, both the variants of *C. gigantea* were found to be almost equally capable of accumulating asclepin in the roots of their mature individuals. Maximum accumulation was observed

Table 1: List of cardiac glycosides/genins with their retention time, molecular weight, and characteristic MS/MS fragmentation

Name of cardiac glycoside/genin	RT	MW	Characteristic MS/MS fragmentation of genin (*m/z*)	Loss of sugar unit (Da)
Calotropagenin	6.95	404	387, 369, 351, 341, 323	-
Frugoside	7.81	536	391, 373, 355, 337, 325	−146
Coroglaucigenin	8.17	390	391, 373, 355, 337, 325	-
Calotropin	8.88	532	387, 369, 351, 341, 323	−146
Uzarigenin	10.20	374	375, 357, 339, 321	-
Asclepin	10.34	574	341, 323	−188
Uschardin	10.42	530	387, 369, 351, 341, 323	−144
Uscharin	11.59	587	387, 369, 341, 323	−201

MW=molecular weight, RT=retention time

Table 2: Comparative analysis of maximum CG accumulation in WFV and PFV

Name of CG	Accumulated quantity of CG (µg/g DW)					
	WFV			PFV		
	Plant part	Growth stage (age in months)	Quantity (µg/g DW)	Plant part	Growth stage (age in months)	Quantity (µg/g DW)
Uscharin	Stem	5	1532.58	Stem	5	1222.27
Frugoside	Stem	9	1329.29	Stem	7	209.79
Calotropin	Stem	5	607.13	Root	12	182.67
Uscharidin	Stem	5	49.48	Root	11	24.95
Asclepin	Root	8	19.19	Root	11	20.10

CG=cardiac glycoside, DW=dry weight, PFV=purple flower variant, WFV=white flower variant

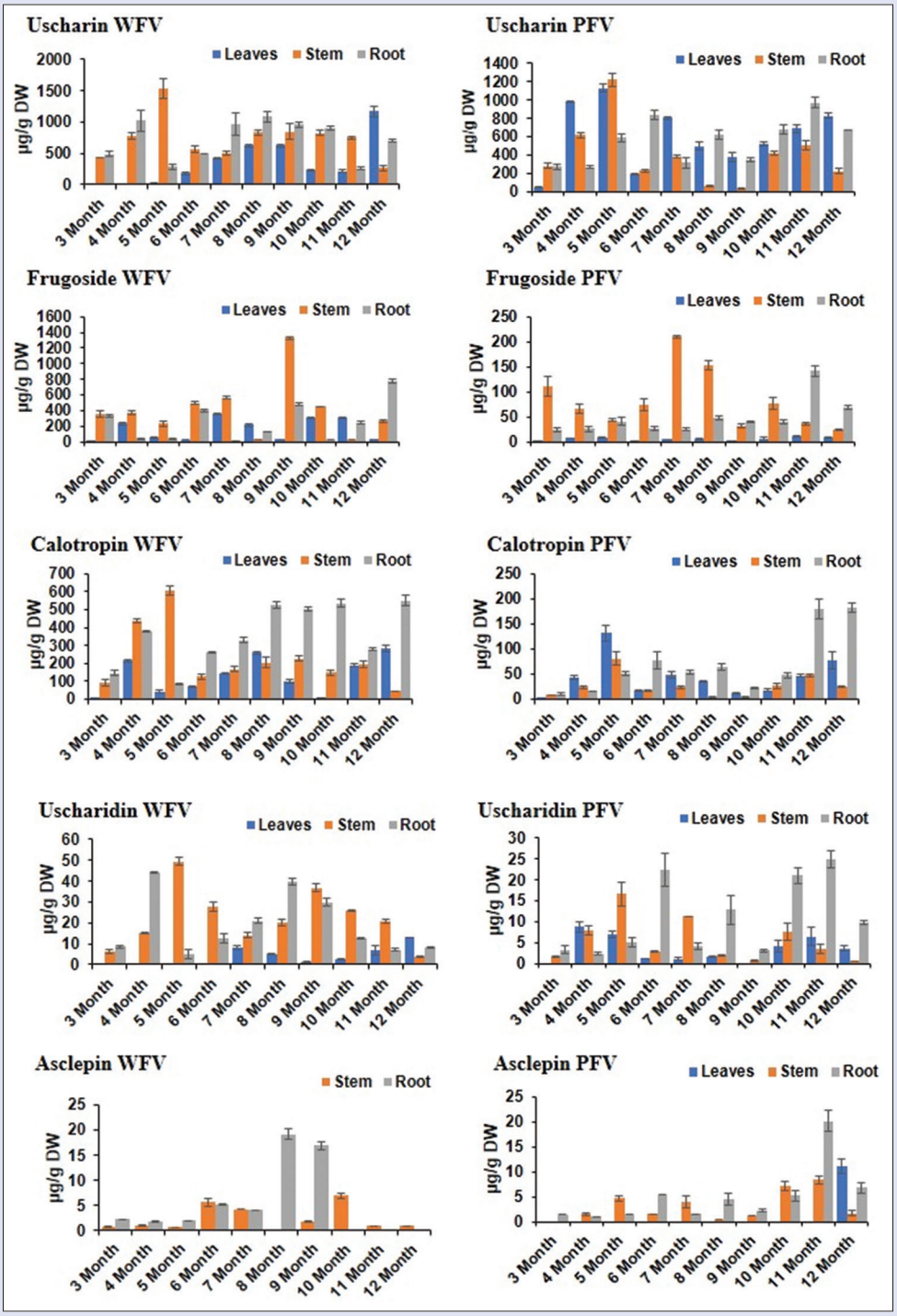


Figure 2: Accumulation of CGs at different stages of *Calotropis gigantea* white and purple flower variants. Each value is the mean of three replicates with standard error. DW = dry weight, PFV = purple flower variant, WFV = white flower variant

in the 11-month-old (20.10 µg/g DW) and 8-month-old (19.19 µg/g DW) members of PFV and WFV, respectively. Considering leaf, the metabolite was interestingly beyond the traceable limit in WFV, and in PFV, it could be detected only in the 12-month-old mature plants [Figure 2 and Table 2].

Accumulation of genins

Accompanying five CGs, three genins, namely, uzarigenin, calotropagenin, and coroglaucigenin, were detected from the growth stage-specific different parts of both *C. gigantea* variants [Table 1]. Like CGs, WFV was found to be far superior to PFV, considering the accumulation of these genins. PFV could accumulate these genins in a very meager quantity. Maximum accumulation of *uzarigenin* (1288.58 µg/g DW) was observed in the stem of 9-month-old WFV. Leaf of its 4- and 7-month-old members could accumulate this genin in moderate quantity (684.46 and 684.08 µg/g DW, respectively). Its maximum accumulation (~70 µg/g DW) in PFV was found in the stem of 7- and 4-month-old members, although it was very less in comparison to WFV.

Coroglaucigenin, on the other hand, was found to be accumulated at a maximum level (~500 µg/g DW) in the leaves of 10- and 7-month-old members of WFV. The root of 11-month-old and the stem of 6- and 9-month-old individuals of the same variant showed its moderate accumulation (380.31–445.13 µg/g DW). PFV could accumulate this metabolite at the maximum level (79.87 µg/g DW) in the root of the 11-month-old member, which was followed by the stem of 10-month-old member (73.70 µg/g DW). *Calotropagenin* accumulation was observed at the maximum level (162.06 µg/g DW) in the root of an 8-month-old member of WFV. Other parts of all the growth stages of this variant showed very little accumulation of the same. In PFV, the metabolite was found to be accumulated at a maximum level (10.51 µg/g DW) in the leaves of 5-month-old plant, but in very meager quantity [Figure 3 and Table 3].

DISCUSSION

Growth or developmental stages of plants have a major impact on the biosynthesis and accumulation of various secondary metabolites. Braga *et al.*[17] explored the effect of such a factor on the accumulation of cardenolides in *Digitalis lanata*. It was observed that six cardenolides, namely, lanatoside A, lanatoside B, glucoevatromonoside, glucogitoroside, glucodigifucoside, and digitoxin, were accumulated at a maximum level in the 12-month-old members (pre-flowering stage) of this plant, whereas highest concentrations of several other cardenolides

Table 3: Comparative analysis of maximum genin accumulation in WFV and PFV

Name of genin	Accumulated quantity of genin (µg/g DW)					
	WFV			PFV		
	Plant part	Growth stage (age in months)	Quantity (µg/g DW)	Plant part	Growth stage (age in months)	Quantity (µg/g DW)
Uzarigenin	Stem	9	1288.58	Stem	7	73.15
Coroglaucigenin	Leaf	10	505.25	Root	11	79.87
Calotropagenin	Root	8	162.06	Leaf	5	10.51

DW=dry weight, PFV=purple flower variant, WFV=white flower variant

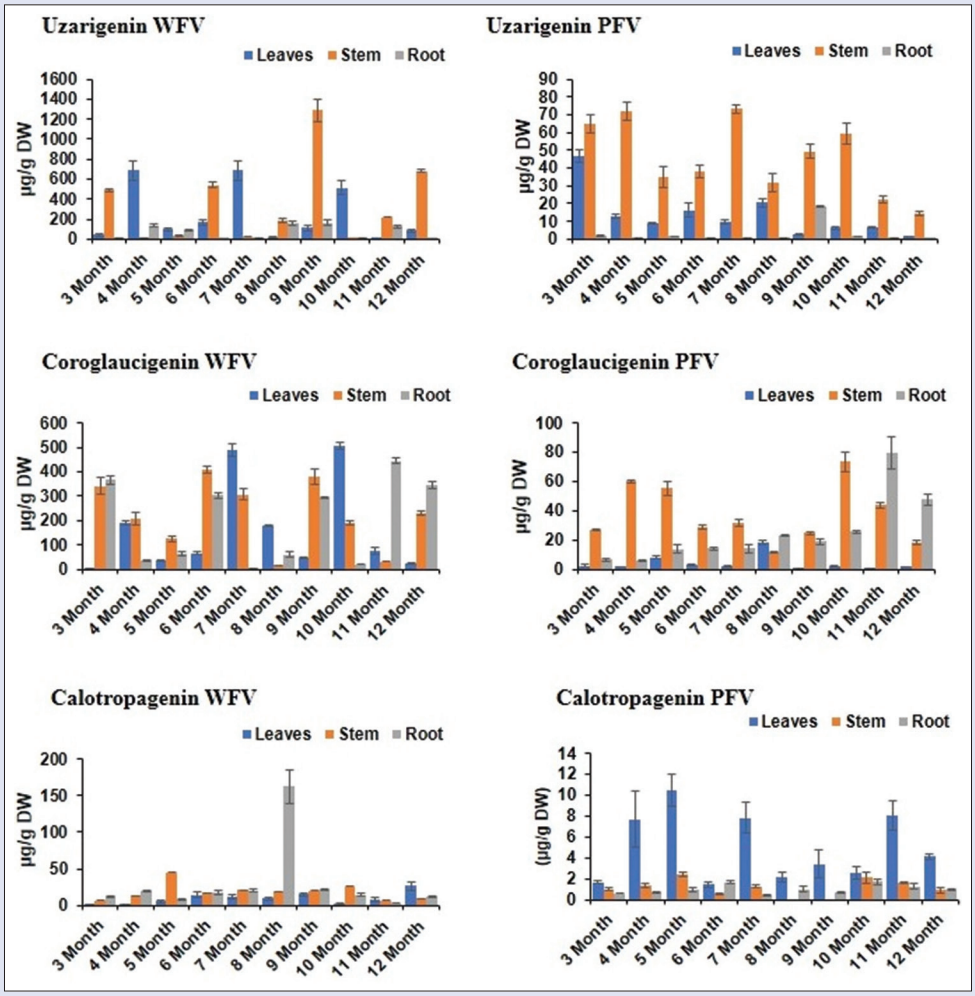


Figure 3: Accumulation of genins at different stages of *Calotropis gigantea* white and purple flower variants. Each value is the mean of three replicates with standard error. DW = dry weight, PFV = purple flower variant, WFV = white flower variant

like lanatoside C + digoxin, odorobioside G, and digitalinum verum were noticed in the 18-month-old plant (post-flowering stage). In our present study, we also observed such type of growth stage-specific variations of detected CGs in different vegetative parts of *C. gigantea* variants.

Growth stage-specific variations of secondary metabolites in plants might be due to differential activation/inactivation of enzymes necessary for biosynthesis.[15] Developmental stages of plant influence the expression of genes, which regulate the initiation and differentiation of cellular organelles and promote the production and storage of secondary metabolites.[18] According to Bennett,[19] certain enzymes or groups of enzymes are activated at specific stages of plant development due to the ordered expression of respective genomes, which are responsible for particular secondary metabolites' biosynthesis. Specific regulatory mechanisms and transport routes also govern secondary metabolites' biosynthesis. Therefore, their synthesis and accumulation are observed differentially in various tissue or organs such as stem, root, or leaf.[20] The storage and transportation of CGs in different parts of a plant normally depends upon the types of CGs, that is, primary (with glucose at the end of the sugar chain) or secondary (without glucose). In *Digitalis*, it was found that the secondary CGs are converted into primary type by a specific enzyme and transported to cell vacuoles for storage purpose. The primary CGs were again converted into secondary type and transported to various tissues of plants, such as the latex of nearly all milkweeds.[21] However, this mechanism of storage and transportation of CGs has not been confirmed in the family Apocynaceae, although tissue specificity in cardenolide expression in this family has been observed.[22-25] Nelson *et al*.[26] found that a relative concentration of four cardenolides in *Asclepias eriocarpa* was gradually increased from roots to leaves to stems to latex. It was noticed that the structure and polarity of individual compounds present in different parts of *A. eriocarpa* were varied. This might be due to diverse selection pressures by herbivores or differential regulation of CGs to plant parts. Physiological and developmental constraints might also be other reasons for the same.[21]

Further, it has been stated that, the species- and tissue-specific biosynthesis and accumulation of secondary metabolites were determined by genetic factors of the medicinal plants.[20] Our study found that WFV is more potent than PFV, considering CGs accumulation. In general, stem was found to be better source for the accumulation of major CGs in both the variants. This study has opened further scopes to discover the molecular mechanism behind such an accumulation pattern. Also, the results of this work will help the natural product chemists or industries by providing necessary information regarding specific plant parts or plant ages for economic isolation of targeted CGs.

CONCLUSION

From the present study, it can be concluded that WFV of *C. gigantea* is a better choice for the maximum yield of most of the selected CGs. However, plant age or growth stage and plant part are also essential for the same. For example, for calotropin, uscharin, and uscharidin, stem of 5-month-old members and for frugoside and uzarigenin, stem of 9-month-old members of WFV are the best. Whereas, for asclepin and calotropagenin, roots of 8-month-old member of WFV should be preferred. Leaves of 10-month-old individuals of WFV can be selected for the highest yield of only coroglaucigenin.

Acknowledgements

We sincerely thank the Sophisticated Analytical Instrument Facility (SAIF), CSIR-Central Drug Research Institute, for the analytical facilities. The CDRI communication no of this paper is 10388.

Financial support and sponsorship

This work was supported by the project GAP0257 of Department of Science and Technology (DST), India, and GAP0180 of Department of Biotechnology (DBT), India. P. S. and Y. S. are thankful to Council of Scientific and Industrial Research (CSIR), New Delhi, for fellowships.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. 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.
2. Seeka C, Sutthivaiyakit S. Cytotoxic cardenolides from the leaves of *Calotropis gigantea*. Chem Pharm Bull (Tokyo) 2010;58:725-8.
3. Jacinto SD, Chun EA, Montuno AS, Shen CC, Espineli DL, Ragasa CY. Cytotoxic cardenolide and sterols from Calotropis gigantea. Nat Prod Commun 2011;6:803-6.
4. 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.
5. 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;78:1-10.
6. ClinicalTrials.gov. Efficacy and safety study of PBI-05204. In: Patients With Stage IV Metastatic Pancreatic. Identifier NCT02329717 [Internet]. U.S.: National Library of Medicine; 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT02329717. [Last accessed 2019 Jun 16].
7. Joshi H, Gururaja MP, Suares D. *Calotropis gigantea* R. Br. (Asclepiadaceae): A review. Int J Pharmacol Res 2011;3:10-4.
8. Priya TA, Manimekalai V, Ravichandran P. Intra specific genetic diversity studies on calotropis gigantea (L) R. Br.-using RAPD markers. Eur J Biotechnol Biosci 2015;3:7–9.
9. Anagha R, Harisha CR, Rabinarayan A, Switu J. Micro-morphological and micrometric evaluation of white and purple variety flowers of calotropis gigantea Linn. R. Br IJUPBS 2014;3:289–302.
10. Srivastava P, Pandey T, Pandey A, Kumar J, Mishra DK, Dikshit A, *et al.* Identification and sequence-based validation of the EST-SSR markers from calotropis procera. Plant Mol Biol Report 2020;38:430–40.
11. Tarafder CR. Ethnogynaecology in relation to plants-II. Plants used in abortion. J Econ Tax Bot 1983;4:507-16.
12. Saxena HO. Observations on the ethnobotany of Madya Pradesh. Bull Bot Surv India 1986;28:149-8.
13. Jain SK, Tarafder CR. Medicinal plant-lore of the Santals. Econ Bot 1970;24:241-78.
14. Toker Z. Variation of total hypericin, phenolic and flavonoid compounds in Hypericum triquetrifolium during its phenological cycle. Pharm Biol 2009;47:285-8.
15. Chen Y, Zhu Z, Guo Q, Zhang L, Zhang X. Variation in concentrations of major bioactive compounds in Prunella vulgarisL. related to plant parts and phenological stages. Biol Res 2012;45:171-5.
16. 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.
17. Braga FC, Kreis W, Récio RA, De Oliveira AB. Quantitative evaluation of cardenolides in a Brazilian Digitalis lanata cultivar and perspectives of its industrial use. Quim Nova 1997;20:481-5.
18. Broun P, Liu Y, Queen E, Schwarz Y, Abenes ML, Leibman M. Importance of transcription factors in the regulation of plant secondary metabolism and their relevance to the control of terpenoid accumulation. Phytochem Rev 2006;5:27–38.
19. Bennett JW. Secondary metabolism in microorganisms, plants, and animals. Martin Luckner. Q Rev Biol 1992;67:355-6.
20. Li Y, Kong D, Fu Y, Sussman MR, Wu H. The effect of developmental and environmental factors on secondary metabolites in medicinal plants. Plant Physiol Biochem 2020;148:80–9.
21. Agrawal AA, Petschenka G, Bingham RA, Weber MG, Rasmann S. Toxic cardenolides: Chemical ecology and coevolution of specialized plant–herbivore interactions. New Phytol 2012;194:28-45.

22. Isman MB, Duffey SS, Scudder GGE. Variation in cardenolide content of the lygaeid bugs, Oncopeltus fasciatus and Lygaeus kalmii kalmii and of their milkweed hosts (Asclepias spp.) in central California. J Chem Ecol 1977;3:613-24.
23. Malcolm SB. Disruption of web structure and predatory behavior of a spider by plant-derived chemical defenses of an aposematic aphid. J Chem Ecol 1989;15:1699-716.
24. ABE F, Yamauchi T. Cardenolide glycosides from the roots of Apocynum cannabinum. Chem Pharm Bull 1994;42:2028-31.
25. Rasmann S, Agrawal AA. Latitudinal patterns in plant defense: Evolution of cardenolides, their toxicity and induction following herbivory. Ecol Lett 2011;14:476-83.
26. Nelson CJ, Seiber JN, Brower LP. Seasonal and intraplant variation of cardenolide content in the California milkweed, Asclepias eriocarpa, and implications for plant defense. J Chem Ecol 1981;7:981-1010.