# Micropropagation of critically endangered herb of Himalyan region: *Trillium govanianum* for in vitro shoot proliferation and production of diosgenin

**Gupta Rolika, Sharma Deepak and Sood Hemant***
Department of Biotechnology and Bioinformatics, JUIT, Waknaghat, Solan, H.P. 173234, INDIA
*hemant.sood@juit.ac.in

***Abstract***
*Trillium govanianum (Trilliaceae) often called as nag chhatri is an indigenous species of Himalayas. This herb is widely used in various traditional medicines and curing many ailments. As per its esteemed importance in the market, it leads to its increase in the economic demand upto Rs 15000-20000/Kg. From many years, T. govanianum have been collected from the wild leading to destruction of its natural habitat. As T. govanianum is a critically endangered herb so as to alleviate the pressure of its extinction and to promote micro propagation technique, we examined the past methods of in vitro propagation in Trillium plants and conducted our own pilot study.*

*We report our observation on in vitro micro propagation for shoot proliferation in T. govanianum and also checked the amount of its secondary metabolite i.e. diosgenin. Combination of 10 different media were prepared out of which 1 mg/L TDZ +0.5 mg/L KN + 0.5 mg/L IBA was observed to be finest media for shoot proliferation. Diosgenin content was accounted through the HPLC in the in vitro grown plants of T. govanianum and it was obtained 0.89µg/mL. Extensive use of in vitro techniques will provide different mode for the conservation of extinct species.*

**Keywords:** Trillium govanianum, Conservation, Diosgenin and Trilliacea.

## Introduction

Medicinal herbs had been used in clearly all culture as a source of drugs. Assurance of the protection, pleasant and efficacy of medicinal plant life and herbal products are now a matter of concern in industrialized and in developing countries[5,6]. The extensive use of herbal medicine and healthcare arrangements is described in the Bible and the Vedas. Medicinal herbs had been used for thousands of years to taste and conserve meals, to treat fitness problems and to prevent ailments[24].

As per National Medicinal Plants Board, India is one of the most affluent nations in terms of biodiversity. It has 15 agro-climatic zone and 17000-18000 types of flowering plants out of which 7000 species are evaluated to have medicinal utility[2,3]. As per the pharmaceutical importance of the medicinal herbs, current study was conducted for micropropagation of critically endangered *T. govanianum*[18,19].

*Trillium* as an important herb is popular for its medicinal and ornamental utility. *T. govanianum* is one of the critically endangered herb belongs to the family of Trilliaceae. *Tr. govanianum* is commonly called as Nagchhatri, teenpatra and satva, it is a perennial herb of Himalayan region distributed in India, Nepal, China and Bhutan at an altitudinal range from 2700 m-4000 m[10,26].

Trillium grow from horizontally oriented rhizome and it produce characteristic 3 leaves in a loop at the peak of the stem with purplish bloom at the middle with round fruit at the top along with numerous seeds. The herb is 30 cm in stature and blossoming has been accounted for in the long stretch of May and July pursued by seedlings in the long stretch of October[13,25]. Rhizome which is present beneath the surface of the ground was the main source for trade as it contains trillarin which on its hydrolysis yield 5.9% diosgenin[17,23] which is a cortico steroid harmone and used in numerous ailments. Diosgenin as an important secondary metabolite is produced through steroid biosynthetic pathway[7,9] and this herb also contains other important steroidal saponin as Borassoside E, Govanoside A and pennogenin[15,20].

*T. govanianum* is a therapeutic herb and its roots are used for various ailments as anticancer, anti-inflammatory and as anti aging agent[12,15]. It is used in various remedies and pharmacological examination. This herb has proved its significance as antibacterial, antifungal, analgesic and antioxidant activity along with its rhizome used to cure diarrhoea, loose bowels, ulcerous injuries and sexual disorders[4,11]. Remarkable work has been done on this herb, diosgenin which is an important metabolite of this herb was found to be almost triple in content as compared to other plants like *Dioscorea* and *Trigonella*, so this herb is an alternative source for production of this important metabolite[23,26].

It was reported that hydrolyzed extract of *T. govaninanum* indicated huge anticancer action against MCF – 7 cell lines[20] and also showed its anti fertility and contraceptive potential in female wistar rats. Due to its extensive pharmaceutical importance and high commercial usage i.e. Rs 15,000-20,000/ kg this herb is under the verge of extinction[3,6]. Due to its medicinal importance for the sake of money people uprooted the plantlets before the maturation of seeds this

leads to serious threat. That is why, unlawful activity and exchanging of this highly prized herb is restricted by Indian Government Law.

Due to the natural surrounding calamity and deforestation various therapeutic herbs are confronting danger of eradication[2,4]. A considerable lot of the plants are not ready to recover normally because of absence of recovery capacity, low seed germination feasibility, bacterial, fungal diseases, extreme natural habitats and environmental conditions. As the villagers of the native place uprooted the roots before the setting of seeds, so degrading their natural habitat is leading to verge of extinction. Illegal massive collection of this herb to meet the industrial demand is a source of the income to the villagers. This illegal smuggling of the herb was banned by the Government so as to stop the over exploitation of the plant[26]. The rate of vegetative propagation of *T. govanianum* is slow and it is under the verge of extinction so, *in vitro* micro propagation of shoots can be an alternative mode for multiplication of this herb and source of conservation program.

Micropropagating the plants in the tissue culture conditions is the promising approach for the maintainance of medicinal herbs[7,8]. Effect of different media on tissue culture production of *T. reliquum* and *T. persistens* was also reported. Combinations of growth harmones like auxin and cytokinin resulted in production of some of the offshoots from both the specie[11].

## Material and Methods

**Selection of mother plant and procedure of extraction:** Plants of *T. govanianum* were obtained from HFRI Shimla. These plants were further stored at greenhouse in Jaypee University of Information Technology, Waknaghat under maintained conditions at 27 ±˚C.

**Establishing sterile culture:** There are no reports of cultivation through conventional methodology as availability of seeds and plants is the biggest concern, so the best alternate is *in vitro* propagation which provides whole year production and platform for its large scale production of *in vitro* grown plantlets. To accomplish this, highly reproducible protocols are required so this study was undertake to develop *in vitro* methodology for the micropropagation of *T. govaninaum*. To establish sterile cultures from the vegetative plant, it required many efforts for successful result. Explant is thoroughly washed to remove all the soil so as to reduce the chances of contamination.

Plants of *T. govanianum* were maintained in green house, fresh rhizomes of the plant were washed under running tap water to remove the dirt and soil further they are washed with autoclaved water. *Trillium* cultures were aseptically established in Laminar Air Flow using rhizomatous buds with different parts like roots, buds and rhizomes. The explants collected were washed with different detergent solutions for surface sterilization and further rinsed with distilled water in laminar air flow (Table 1).

**Media Preparation:** After surface sterilization of the explants, these explants were inoculated in Erlenmeyer flasks containing 50 mL Murashige and Skoog (MS) medium with 0.3% sucrose and 0.8% w/v agar and different concentrations of Plant Growth Regulators (PGR's). Explants were grown in MS media supplemented with TDZ, IBA and KN (Table 2). After 10 days of initiation of shoots, it was again subcultured into fresh media, multiple sub culturing was done to maintain arising shoots and the experiment was repeated thrice. These explants were stored in the optimized culture conditions in plant tissue Lab at 15 ± ˚C under white fluorescent light in 3000 lx intensity with 40% humidity and 16 hr light and 8hr of dark photoperiod. Explant cultured on to the media without any growth regulators is considered as control. Every treatment was repeated thrice (Fig. 1).

**Sample preparation and procedure of extraction:** *In vitro* grown plants of *T. govanianum* which were maintained in the plant tissue Lab at 15 ± ˚C under white fluorescent light in 3000 lx intensity with 40% humidity and 16 hr light and 8hr of dark photoperiod were used for extract preparation to quantify the amount of bioactive compound i.e. Diosgenin through HPLC. Different parts of the *in vitro* grown plant like proliferated shoots and buds were washed in running tap water, shade dried and grinded under liquid nitrogen and further subjected to (30:70 $H_2O$:Methanol) Soxhlet extraction for about 72 hrs. Extract of the plant was filtered out and concentrated under rotatory evaporator followed by lyophilisation. Extracts were further quantified by HPLC and rest of the extract was stored at 4˚C for further use.

**Chromatographic conditions for HPLC:** Quantification was carried out with reverse-phase HPLC (Agilent 1200 series) equipped with HPLC pump, DAD photodiode array detector range from 190 to 800 nm. Diosgenin was estimated with a Zorbax-Eclipse XBD C-18, 3.5-lm column (4.6 9 150 mm). Solvent system used in gradient mode to run the samples was 0.2% formic acid with HPLC-grade water and methanol in a ratio of 30:70 (v/v). The column was eluted in the gradient mode with a flow rate of 0.8 ml/min. Diosgenin was detected at an absorbance of 230-nm wavelength. The required time period of analysis was 30 min at 25˚C with injection volume of 10 µL. At the end of each run, the column was rinsed with pure solvents. The compound was recognized by measuring retention time and comparison of UV spectra with the authentic standard from ChromaDex Inc[23].

## Results

**Use of sterilants on plant material:** To establish sterile cultures of *T. govanianum*, different explants of the plant were used. After removing from the pots plants were washed with distilled water and variety of sterilizing agent was used at different concentrations to make sterile cultures of plant.

**Micropropagation:** The plants were micropropagated with MS medium supplemented with different concentration of harmones. Plants supplemented with growth hormone at concentration of 1 mg/L TDZ + 0.5 mg/L IBA + 0.5 mg/L KN with 9gram agar, 30 gram sucrose at pH 5.6 for 1 litre showed best results. Plants were incubated in the culture room at 15 ± ˚C under white fluorescent light in 3000 lx intensity with 40% humidity and 16 hr light and 8hr of dark photoperiod. Data on the shoot proliferation was recorded on regular basis with sub culturing after 30 days.

**Quantification of diosgenin through HPLC:** Diosgenin is a steroidal saponin mainly procures from the *T. govanianum* rhizomes.[24] Diosgenin is an important bioactive constituent reports to produce from steroid biosynthetic pathway.[26] It is one of the principal raw materials for steroid drug development. It is used for commercial synthesis of steroidal drugs as progesterone, testosterone and glucocorticoids[25]. Rhizomes of field grown *T. govanianum* contain a high measure of diosgenin (5.99%) obtains from hydrolysis of hydroalcoholic extract[23]. In our study, diosgenin content was found to be 0.89 µg/mL and it was reporting in the *in vitro* grown plants of *T. govanianum* (Fig. 2).

## Discussion

On the basis of successful shoot proliferation, MS Media containing composition of growth regulator i.e. 1mg/L TDZ + 0.5mg/L IBA + 0.5mg/L KN was the best media for growth of shoots. This media was reported with shoot multiplication in *T. govanianum*. As reported by Gagliardo et al,[7] they used 2M media for growth and multiplication in *T. persistans* and *T. reliquum* where they reported maximum number of 9 shoots and 24 roots and 21 shoots and 24 roots so we have also investigated different media formulations using various growth regulators for growth of *T. govanianum*. *In vitro* propagation of other trillium species were also reported as in *T. erectum* and *T. grandiflorum* here they used stem and leaf explants for in vitro production of mini rhizomes for production of roots and shoots[15,16].

**Table 1**
**Effect of sterilants on sterilization of explants of *T. govanianum***

| S.N. | Sterilizing Agent | Concentration % | Duration | Explant Used | % of contamination |
|---|---|---|---|---|---|
| 1. | Bavistin | 0.5 | 2-3 min. | **Buds** | 10 |
| 2. | Mercuric chloride | 0.1 | 30 sec. | | 10 |
| 3. | Tween 20 | 0.5 | 15 min. | | 30 |
| 4. | Tween 80 | 0.2 | 10 min. | **Roots** | 30 |
| 5. | Sodium hypochlorie | 0.5-5 | 5 min. | | 10 |
| 6. | Calcium hypochlorite | 8-10 | 5-20 min. | | 20 |
| 7. | Hydrogen peroxide | 4-12 | 5-10 min. | **Rhizome** | 10 |
| 8. | Ethyl Alcohol | 70-90 | 3-5 min. | | 40 |
| 9. | Silver Nitrate | 1 | 10 min | | 50 |
| 10. | Benzalkonium chloride | 0.1-1 | 5-10 min. | | 50 |

**Table 2**
**Effect of different concentration of growth regulators for multiplication of *T. govanianum***

| S.N. | Medium | No. of shoots | No. of days required for shoot formation | Status of explants |
|---|---|---|---|---|
| 1. | MS + 2mg/L 2,4-D + 1mg/L BAP | - | 40 | No growth |
| 2. | MS + 2mg/L 2,4-D + 1mg/L TDZ | - | 50 | Swollen bud |
| 3. | MS + 3mg/L IBA + 1mg/L KN | - | 55 | Swollen bud |
| 4. | MS + 1mg/L TDZ + 0.5mg/L IBA + 0.5mg/ L KN | **2** | **35** | **Bud proliferated** |
| 5. | MS + 1mg/L BAP + 0.5mg/L NAA | - | 30 | No growth |
| 6. | MS + 1mg/L TDZ + 0.5mg/L BAP + 0.5mg/ L KN + 0.5mg/ L $GA_3$ | **2** | 30 | Sub culturing |
| 7. | MS + 1mg/L TDZ + 0.5 mg/L KN + 0.5 mg/ L $GA_3$ | - | 25 | No growth |
| 8. | MS + 1mg/L TDZ + 0.5 mg/L BAP + 0.5 mg/ L $GA_3$ | - | 40 | No growth |
| 9. | MS + 2 mg/L 2,4-D + 1 mg/L TDZ + 1 mg/L KN | - | 45 | Swollen bud |
| 10. | MS + 3 mg/L IBA + 1 mg/L KN + 0.5 mg/ L NAA | - | 30 | No growth |

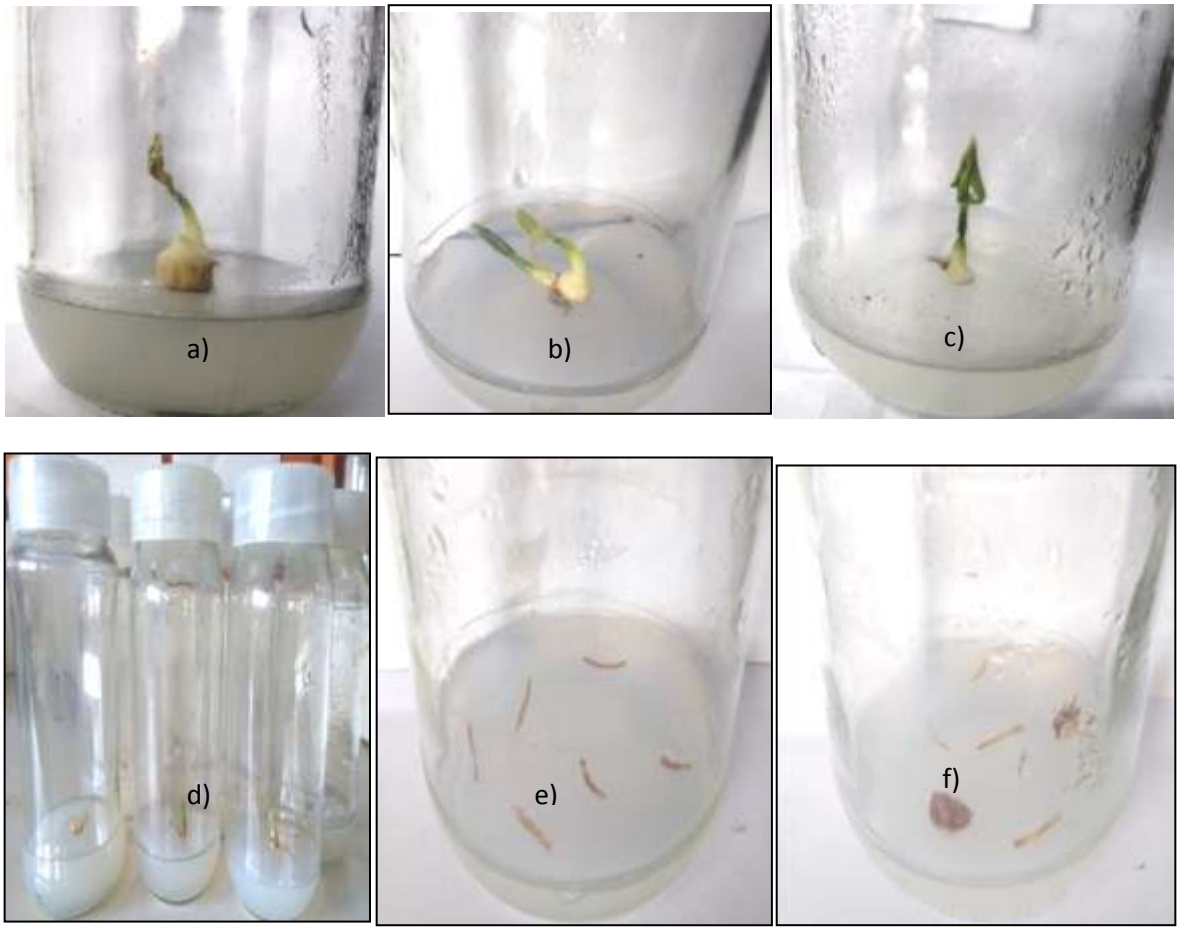


**Fig. 1: Different phases of in vitro shoot proliferation of *T. govanianum* a) and b) initiation of shoot proliferation in 35 days c) and d) Different stages of shoot development e) and f) root and rhizome bud used as explants in different media.**

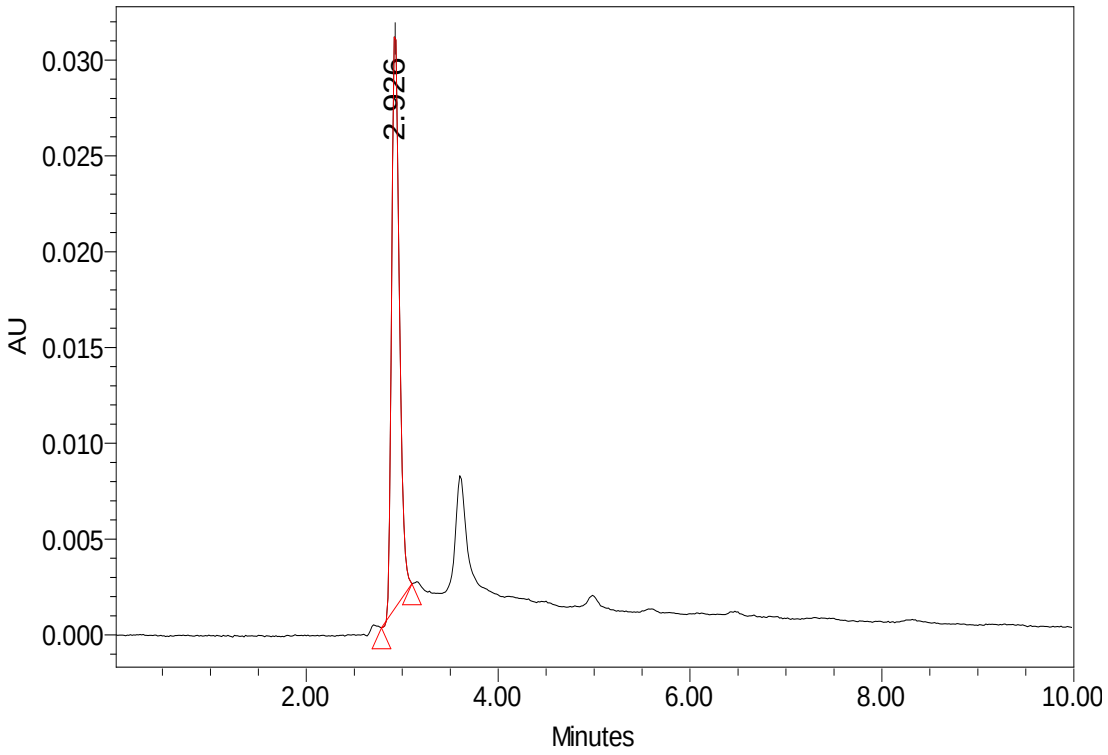


**Fig. 2: HPLC Chromatogram of Diosgenin Standard**

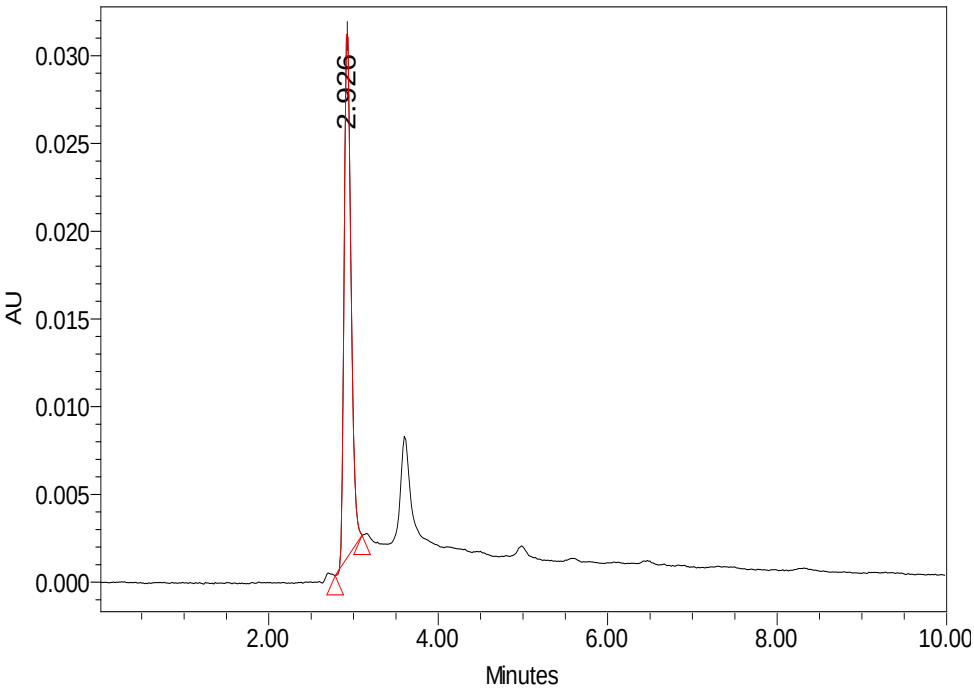


**Fig. 3: HPLC chromatogram *in vitro* grown plant of *T. govanianum***

Different concentrations of growth harmones cytokinin and auxin resulted in shoot proliferation. In our study, diosgenin content was found to be 0.89 µg/mL and it was reported in the in vitro grown plants of *T. govanianum* (Fig. 2). Till now quantified amount of Diosgenin was only reported in the field grown plants. As plants like yams as well as tuber of *Trigonella* and *Dioscorea* were significant source of diosgenin but the amount quantified from them was not satisfactory so *T. govanianum* plant can be good alternative for diosgenin.

As other species are not commercially available so this herb can be the best alternative to introduce new drug in the market. As in our finding sample size was less, so more number of shoots can be used for future purpose for well established root system too. Removal of the decayed plants and further sub culturing of the plant helps in good number of shoots. As this experiment was based on pilot study more work was required for further conformation but this will help in development of future protocols for the propagation of *T. govanianum*.

As *T. govanianum* is an endangered herb so *in vitro* micro propagation can be an excellent alternative source as the conservation of the herb is very important due to the destruction of its natural habitat and to meet up with the industrial demand. More work and attention is needed to confirm the pilot scale results but we hope these results help in future to establish more laboratory protocols to conserver this beautiful herb from extinction.

## Conclusion

The current study reports the standard protocol for shoot proliferation in *T. govanianum* and *in vitro* propagation of plant using rhizomatous buds. The normalized convention in the current examination could be utilized for the mass proliferation of such profoundly significant therapeutic plant and could refute the danger to the wild presence while simultaneously will meet the pharmaceutical needs.

## Acknowledgement

The authors wish to thank Jaypee University of Information Technology, Waknaghat for giving the research facility to do this work.

## References

1. Abbasi A.M. and Khan M.A., Ethnobotanical study of wound healing herbs among the tribal communities in Northern Himalaya Ranges District Abbottabad, Pakistan, *Pak J Bot*., **6**, 3747-3753 **(2010)**

2. Biota of North America Program county distribution map **(2013)**

3. Chauhan N.S., Medicinal and aromatic plants of Himachal Pradesh, Indus publishing company, New Delhi **(1999)**

4. Diarra S.T. and Wang J., Ethylene treatment improves diosgenin accumulation in in vitro cultures of Dioscorea zingiberensis via up-regulation of CAS and HMGR gene expression, *Electronic Journal of Biotechnology*, **16(5)**, 6-6 **(2013)**

5. Favel A. and Kemertelidze E., Antifungal activity of steroidal glycosides from Yucca gloriosa *L*. Phytotherapy Research, *An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, **19(2)**, 158-161 **(2005)**

6. Fukuda I. and Freeman J.D., Trillium channellii, sp. nov.(Trilliaceae), in Japan and T.camschatcense Ker Gawler,

correct name for the Asiatic diploid Trillium, *Novon*., **Volume,** 164-171 **(1996)**

7. Gagliardo R. and Labarthe M., In vitro propagation of Trillium species, *Native Plants Journal*, **13(1)**, 56-63 **(2012)**

8. Gates R.R., A systematic study of the North American genus Trillium its variability and its relation to Paris and Medeola, *Annals of the Missouri Botanical Garden*, **4(1)**, 43-92 **(1917)**

9. Ismail M. and Shah M.R., Govanoside A, a new steroidal saponin from rhizomes of Trillium govanianum, *Steroids*, **104**, 270-275 **(2015)**

10. Khan K.M. and Nahar L., Cytotoxicity of the roots of Trillium govanianum against breast (MCF7), liver (HepG2), lung (A549) and urinary bladder (EJ138) carcinoma cells, *Phytotherapy Research*, **30(10)**, 1716-1720 **(2016)**

11. Murasnige T. and Skoog F., A Revised Medium for Rapid Growth and Bio Agsays with Tohaoco Tissue Cultures, *Physiologia Plantarum*, **15**, 473-497 **(1962)**

12. Nakano K. and Nohara T., 18-Norspirostanol derivatives from Trillium tschonoskii, *Phytochemistry*, **22(4),** 1047-1048 **(1983)**

13. Naoumkina M.A. and Modolo L.V., Genomic and coexpression analyses predict multiple genes involved in triterpene saponin biosynthesis in Medicago truncatula, *The Plant Cell*, **22(3),** 850-866 **(2010)**

14. Newman D.J. and Cragg G.M., The influence of natural products upon drug discovery, *Natural Product Reports*, **17(3),** 215-234 **(2000)**

15. Pence V.C. and Soukup V.G., Plant regeneration from Trillium spp. In vitro, *Hort Science*, **21,** 1211-1213 **(1986)**

16. Pence V.C. and Soukup V.G., Factors affecting the initiation of mini-rhizomes from Trillium erectum and T. grandiflorum tissues in vitro, *Plant Cell, Tissue and Organ Culture*, **35(3),** 229-235 **(1993)**

17. Rahman S. and Ismail M., GC/MS analysis, free radical scavenging, anticancer and β-glucuronidase inhibitory activities of Trillium govanianum rhizome, *Bangladesh Journal of Pharmacology*, **10(3),** 577-583 **(2015)**

18. Ramakrishnan R. and Periyasamy R., In Vitro Propagation and Conservation of Useful Endangered Medicinal Plants with Anticancer Activity, *J Mol Biol Biotech.*, **2(3),** 8 **(2017)**

19. Rani S. and Rana J.C., Ethnomedicinal plants of Chamba district, Himachal Pradesh, India, *Journal of Medicinal Plants Research*, **7(42)**, 3147-3157 **(2013)**

20. Sagar A. and Thakur L., Studies on Endophytes and Antibacterial activity of Trillium govanianum Wall. ex D. Don, *Studies*, **2(1),** 63-67 **(2017)**

21. Samant Pant S. and Singh M., Medicinal plants in Himachal Pradesh, north western Himalaya, India, *The International Journal of Biodiversity Science and Management*, **3(4)**, 234-251 **(2007)**

22. Samant S.S. and Dhar U., Medicinal Plants of Indian Himalaya: Diversity Distribution Potential Values, Nainital: Gyanodaya Prakashan **(1998)**

23. Sharma S. and Sharma A., Efficient hydroalcoholic extraction for highest diosgenin content from Trillium govanianum (nag chhatri) and it's in vitro anticancerous activity, *Asian J Pharm Clin Res*., **9(4)**, 386-392 **(2016)**

24. Singh P. and Singh G., Spatial transcriptome analysis provides insights of key gene (s) involved in steroidal saponin biosynthesis in medicinally important herb Trillium govanianum, *Scientific Reports*, **7,** 45295 **(2017)**

25. Ur Rahman S. and Adhikari A., Beneficial effects of Trillium govanianum rhizomes in pain and inflammation, *Molecules*, **21(8),** 1095 **(2016)**

26. Vaidya K. and Ghosh A., De novo transcriptome sequencing in Trigonella foenumgraecum L. to identify genes involved in the biosynthesis of diosgenin, *The Plant Genome*, **6(2),** 1-11 **(2013)**.

(Received 10th October 2020, accepted 05th December 2020)