World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org
SJIF Impact Factor: 6.129

DETECTION OF PODOPHYLLOTOXIN FROM CALLUS CULTURE OF *PODOPHYLLUM HEXANDRUM*

Ahmad Rizwan[1*], Sharma Saurabh[1], Sharma Satish K[2], Kumar Vishal[1], Bari Darakhshan G.[1] and Verma Chhavi[1]

[1]Vivek College of Technical Education, Bijnor (UP) India.
[2]Glocal University Saharanpur (UP) India.

***Corresponding Author: Ahmad Rizwan**
Vivek College of Technical Education, Bijnor (UP) India.

Article Received on 11/04/2021 **Article Revised on 01/05/2021** **Article Accepted on 21/05/2021**

ABSTRACT

Podophyllum hexandrum Royle is an endangered medicinal plant. Rhizomes of *Podophyllum hexandrum* Contain several lignans which possess antitumour activity. Podophyllotoxin is the most active cytotoxic natural product. It is used as starting compound for the synthesis of anticancer drug etoposide and teniposide, which are used in the treatment of various types of cancer. Besides this, it also shows antiviral activities. Availability of Podophyllotoxin from plants has its limitations because of its intense collection from nature and lack of organized cultivation. The chemical synthesis of Podophyllotoxin is considered to be very complicated. The use of biotechnological approaches for the production podophyllotoxin using tissue cultures is an attractive alternative for production of Podophyllotoxin The present paper discuss *In Vitro* propagation of *podophyllum hexandrum*. Callus cultures have been established from root explants of aseptically grown *Podophyllum hexandrum* seedlings. A fully defined MS medium supplemented with Naphthalene acetic acid and 6-benzylaminopurine were effective for both initiation and sustained growth of callus tissue. The relative proportion of callus were markedly influenced by presence of plant growth regulators .The relative proportions of Podophyllotoxin in Cultivated root and callus was estimated by HPLC and HPTLC.

KEYWORDS: *Podophyllum hexandrum*, Tissue culture, Podophyllotoxin, HPTLC, HPLC.

INTRODUCTION

Podophyllum hexandrum Royle (Berberidaceae) also known as the Indian podophyllum is a perennial herb, growing on the lower slopes of the Himalayas in scrub and Forest from Afghanistan eastwards to central china.[1, 2, 3, 4]

The rhizomes of *Podophyllum hexandrum* are known to contain several lignans which are dimerisation product of Phenypropanoid pathway intermediates linked by central carbons of their side chain.[5,6,7] The lignans occurring in Podophyllum posses anti-tumor properties, Podophyllotoxin being the most active cytotoxic contains 4.3% Podophyllotoxin on a dry weight basis. Its insecticidal and phytotoxic activities are also reported.[8,9] However these lignans are too toxic for the treatment of neoplastic disease in humans. Nevertheless, Podophyllotoxin is used as starting compound for the chemical synthesis of etoposide and teniposide both being applied successfully as antitumor agents.[10,11] Their cytotoxic action is based on inhibition of topoisomerase II, while Podophyllotoxin acts as an inhibitor of the microtubule assembly. These semi synthetic analogues are indicated for small lung cell cancer, testicular cancer, neuroblastoma, hepatoma and other tumor disease.[12]

The limited availability of *Podophyllum hexandrum* plant due to its long juvenile phase and poor fruit setting ability as well as the time consuming collection of the plants results in shortage of Podophyllum resin. Moreover, because of the non-optimal yield after extraction, Podophyllotoxin is an expensive starting compound for the chemical synthesis of its derivatives. Therefore, the biotechnological production of Podophyllotoxin using plant cell culture derived from *Podophyllum hexandrum* may be an attractive alternative.[13,14,15]

Extensive literature survey reveals that the tissue culture of *Podophyllum hexandrum* detected lignan in callus tissue. Podophyllotoxin content are prone to changes due to environment factors, type of fertilizers applied and stages of harvest.[16,17,18] These changes could be

controlled by *in-vitro* culture of the *Podophyllum hexandrum* for the synthesis of lignan Podophyllotoxin. Hence in the present study experiments were carried out to investigate the production and level of Podophyllotoxin in the callus culture of *Podophyllum hexandrum*.[19,20]

Optimization for establishment of static culture of *Podophyllum hexandrum* was standardized. Presence of Podophyllotoxin in callus culture and root extract was estimated by HPLC and HPTLC.

This study demonstrates the potentiality of static culture in production of Podophyllotoxin.

MATERIAL AND METHODS

Seeds and Seedlings

Mature fruits of *Podophyllum hexandrum* Royle were collected during the month of October from G.B Pant Institute of Himalayan Environment and Development Kosi-Katarmal, Almora and High altitude plant physiology research Center, Srinagar .Seeds were separated from pulp, washed under running tap water for 20 min.dried under shade and stored at 4˚c until used. Seeds were cut with a scalpel blade, removing a section of seed coat with two incisions around the seed hilum region, and then maintained in dark, sterile conditions on moist filter paper at 27˚C until emergence of the radical. Germinated seeds was placed on solid nutrient agar slabs (full strength MS medium, 0.8% agar, P^H 5.8) in sterile culture tubes and transferred to growth room conditions, with diffuse light day/night regime (16/8 hr) as well as in the dark.

Callus initiation and growth

Aseptically germinated seed embryo were washed with double distilled sterile water and surface was disinfected with aqueous solution of sodium hypochlorite for 8 min. followed by repeated washing (4 times) in sterilized double distilled water under aseptic conditions so as to remove traces of sodium hypochlorite. The explants 3- 4 mm were carefully excised and transferred into MS media with various concentrations of BAP (0.5-2.5µM), NAA (0.5-5µM) and GA_3 (0.5µM-1µM). Specific growth regulator combinations referred to below.

Table 1: Effect of different supplement on growth of callus of *Podophyllum hexandrum.*

TREATMENT OF SUPPLEMENT(µM)				TYPES OF RESPONSE		
S.NO.	NAA	BA	GA_3	CALLUS	COLOUR	NATURE
1	0	1.5	0	+ +	Brownish	Compact
2	0	2.0	0	+	Brownish	Compact
3	1.5	0	0	++	Brownish	Compact
4	2.0	0	0	+	Brownish	Compact
5	0	0	0.5	+	Brownish	Compact
6	0.5	2.5	0	+++	Greenish	More Friable
7	1.0	2.0	0	+++	Greenish	More Friable
8	1.5	1.5	0	++	Greenish yellow	Less friable
9	2.0	1.0	0	++	Greenish yellow	Less friable
10	1.0	0	0.5	+++	Greenish	More Friable
11	1.5	0	0.5	+++	Greenish	More Friable
12	0.5	0	0.5	+ +	Greenish yellow	Less friable

(+): Poor callusing (+ +): Average callusing (+ + +): Good callusing (-): No response

The P^H of the medium was adjusted to 5.8. The cultures were maintained at 25±2°c in 16 hr. light and 8 hr. dark cycle and were transferred to fresh MS medium after a period of fourteen(14) days.

Lignan extraction

Obtained calli and plant root were extracted with ethanol. The lignan extract was redissolved in analytical grade methanol prior to analysis and analysis of the calli and Plant root was carried out to identify and compare the Podophyllotoxin and Resin content in the callus with cultivated *Podophyllum hexandrum* root extracts.

HPLC Analysis

HPLC was carried out using a Column: C-18 Bonda pack (250 X 4.6 mm) A solvent system of Acetonitrile: Water: methanol: n-heptane (30:40: 25: 5) was used, with a flow rate of 1.2ml/min, and UV detection at 280 nm. Peak areas were assessed by integrator.

Retention time for authentic podophyllum lignans were as follows: standard Podophyllotoxin (6.810), root extract (6.890), and for callus extract (6.800).

HPTLC Analysis

Quantitative and Qualitative analysis was carried out using HPTLC method. Callus extracts, and plant extract identification was carried out using TLC method. Sample extract compared with standard Podophyllotoxin. HPTLC was carried out using a silica gel GF254 Precoated plate of 0.2mm thickness as a stationary phase. A Mobile Phase of Acetonitrile: Water (4: 6) was used and UV detection at 210 nm. Peak areas were assessed by integrator.

The extract made from the roots of *Podophyllum hexandrum* contains a mixture of potent pharmacologically active compounds. The major components are resin Podophyllotoxin. The extract is standardized on the basis of Podophyllotoxin.

RESULT AND DISCUSSIONS

In present study experiments were carried out on tissue culture of (*Podophyllum hexandrum* (Berberidaceae). Tissue culture study starting from aseptic seed germination, standardization of media, callus initiation and growth study followed by Extraction, and estimation of Podophyllotoxin content in the callus cultures, and from cultivated roots of the plant.

Seed and Seedlings

Establishment of a routine protocol for tissue culture of *Podophyllum hexandrum* proved to be Difficult due to erratic seed germination, problems with the sterilization of explants, and the poor response of most plants to the culture regimes tested. Sterilization of explants, especially root material from soil-grown plants was unsuccessful[11,12], and germination of seeds under aseptic Conditions proved the most satisfactory approach.[13] Germination was dependent on storage Conditions and appropriate Pregermination treatment. A successful procedure was developed.

Involving aseptic seed germination, light day/night regime (16/8 hr) condition at 27°C. For aseptic germination of seeds they were treated with 70% ethanol for 15 seconds followed by 3% sodium hypochlorite for 5 minutes. They were then washed several times with sterile double distilled water to remove excess of sterilants. Various methods were taken up for the aseptic germination. The result showed that seed germination was within 6 days in petridish method. The sprouted seedling in the immature stage showed better proliferation and growth in M.S. basal medium with 100 µM solution of Gibberrllic acid is better whereas it remained stagnated if allowed to stay in cotton bed method for long.

For callus initiation, full strength MS (Murashige and skoog) gave better results[14,15] and was therefore adopted for further studies. Root explants from seedlings derived from single plant were tested on hormone grids. No individual auxin or cytokinin initiated callus at the concentrations tested, With the exception of NAA where some response was observed at 0.5-5µM after four weeks. High concentrations of the BAP (2.5µM) in the presence of NAA (0.5µM) enhanced callus initiation. Development of a friable callus was particularly associated with BAP in combination with NAA.

Optimum growth response was achieved with combined GA_3 NAA treatment, which appeared independent of GA_3 concentration in the range tested (0.5-1.0µM), but dependent on the NAA concentration(0.5-1.0µM). A similar high response was observed in the presence of BAPconcentration (0.5-1.0µM). Higher concentration of BAP (upto 2.5µM) were found to facilitate growth when NAA levels were low, but retard growth when NAA levels were high. Callus growth was more effectively supported on a MS medium with full strength, rather than at half strength MS medium.[16] Overall, a medium based on the combined use of NAA, BAP was suitable for both the initiation and sustained growth of *Podophyllum hexandrum* callus tissue.

HPLC Analysis[17]

The Qualitative tests for the identification of Podophyllotoxin and the methods for there quantitative estimation was carried out by HPLC. Roots parts and callus were extracted from *Podophyllum hexandrum* under different conditions and these extracts are analyzed using 1 mg ml^{-1} sample concentration and compared with that of the standard using same concentration (100µl). Callus under different condition contain Podophyllotoxin.

Extract percentage yield shown in **Table -2.**

Table 2: Showing HPLC data for ethanolic extracts and Standard Podophyllotoxin.

Sample	RT	Area (m.V/s)	Percentage of Podophyllotoxin%
Standard	6.940	2150.2213	-
Root extract	6.890	120.12	5.46
Callus extract	6.80	17.10	0.78

HPTLC Analysis[18]

The Qualitative tests for the identification of Podophyllotoxin and the methods for there quantitative estimation is carried out by HPTLC. Different parts were extracted from *Podophyllum hexandrum* under different conditions and these extracts are analyzed using 5 mg ml^{-1} sample concentration and compared with that of the standard (98%) using 1mg ml^{-1} concentration (10µl). Callus under different condition contain Podophyllotoxin.

Extract percentage yield shown in **Table 3.**

Table 3: Showing HPTLC data for ethanolic extract and Standard Podophyllotoxin.

Sample	R_f	Area (m.V/s)	Percentage of Podophyllotoxin%
Standard-5	0.51	8630.5	-
Root extract	0.51	2415.4	5.48
Callus extract	0.51	352.2	0.79

Quantitative estimation of Podophyllotoxin was carried out using HPLC and HPTLC which revealed the presence of Podophyllotoxin in root extract and callus extract. Callus extracts showed presence of Podophyllotoxin.

CONCLUSION

Podophyllum hexandrum has been considered a rare and threatened species, large scale removal of its underground parts still continues at rates well over natural regeneration. Therefore special Attention needs to be given for its propagation and conservation. The use of seeds/plant material from different population would help to ensure the high Podophyllotoxin content by tissue culture techniques. The combined and sustained effort with seed germination, *in vitro* culture and estimation is required to reduce the pressure on natural population.

Study was successful in standardizing the media for tissue culture, aseptic germination, callus Initiation, Extraction and estimation of Podophyllotoxin from Callus and cultivated *Podophyllum hexandrum* extract.

ACKNOWLEDGEMENTS

We acknowledge the facilities provided to us by the Department of Pharmacy, Vivek College of Technical Education Bijnor (UP) India.

REFERENCES

1. Airi S, Rawal RS, Dhar U Purohit AN; Population studies on *Podophyllum hexandrum* Royle-a dwindling, medicinal plant of the Himalaya. Plant Genet. Resour. Newsl, 1997; 110: 29-34.
2. Gupta R, Sethi KL. Conservation of medicinal plant resources in Himalayan region, *Conservation of tropical plant resources*: B.S.I. Howrah, India, 1983; 101-7.
3. Sharma SK, Sharma S, Sharma K, Tiwari S; *Ethnopharmacological review on* Althaea Officinalis. World Journal of Pharmacy and Pharmaceutical Sciences, 2016; 5(7).
4. Giri A, Narasu ML; Production of Podophyllotoxin from *Podophyllum hexandrum*: A potential natural product for clinically useful anticancer drugs. Cytotechnology, 2000; 34: 17–26.
5. Choudhary DK, Kaul BL, Khan S; Cultivation and conservation of *Podophyllum hexandrum*- an overview. J. Med. Aro. Plant Sci, 1998; 20: 1071–3.
6. Sharma SK, Seshasai SM, Vipin S, Mohapatra S; Evaluation of analgesic and Anti-Inflammatory activity of *Abuliton indicum.* International Journal of drug development, 2013; 5(1): 402-07.
7. Kamil MW, Dewick PM; Biosynthesis of lignans alfa and beta- Peltetin. Phytochemistry, 1986; 25(9): 2089-92.
8. Kamil MW, Dewick PM; Biosynthetic relationship of aryltetralin lactone lignans to dibenzylbutyrolactone lignans. Phytochemistry, 1986b; 25: 2093-102.
9. Jackson DE, Dewick PM; Aryltetralin lignans from *Podophyllum hexandrum* and *Podophyllum peltatum.* Phytochemistry, 1984; 23(5): 1147-52.
10. Canel C, Moraes RM, Dayan FE, Ferreira D; Molecules of interest 'Podophyllotoxin'. Phytochemistry, 2000; 54: 15–20.
11. Sharma SK, Deopa D, Singh L; Current updates on Anti-Diabetic therapy. Journal of drug discovery and therapeutics, 2013; 3(6): 121-126.
12. Jackson DE, Dewick PM; Biosynthesis of Podophyllum lignans-II. Interconversion Arytetralin lignans in *Podophyllum hexandrum.* Phytochemistry, 1984; 23(5): 1037-42.
13. Goel HC, Prasad J, Sharma A, Singh B; Anti tumour and radio protective action of *Podophyllum hexandrum.* Indian Journal of Experimental Biology, 1998; 36(6): 583-7.
14. Badhwar RL, Sharma BK; A note on germination of *Podophyllum* seeds. Ind. For, 1963; 89: 445-7.
15. Semwal JK, Purohit AN, Gaur RD; Seed germination in some Himalayan alpine plants. Seed Res, 1983; 11(1): 42-6.
16. Sharma SK, Pandey AK, Singh L, Singh T; Review on *vetiveria zizanioides*: A Medicinal Herb. Journal of drug discovery and therapeutics, 2013; 7(1): 80-83.
17. Nadeem M, Palni LMS, Purohit AN, Pandey H, Nandi SK; Propagation and conservation of *Podophyllum hexandrum* Royle: an important medicinal herb. Biol. Cons, 2000; 92: 121-9.
18. Kadkade PG. Formation of Podophyllotoxin in *Podophyllum peltatum* tissue cultures. Naturwissenschaften, 1981; 68: 481-2.
19. Sharma SK, Pandey AK, Singh L, Singh T; An overview on *desmostachya bippinata*. Journal of drug discovery and therapeutics, 2013; 7(1): 67-68.
20. Kadkade PG; Growth and Podophyllotoxin production in callus tissue of *Podophyllum peltatum.* Plant Sci. Lett, 1982; 25: 107-15.
21. Chattopadhyay S, Srivastava AK, Bhojwani SS, Bisaria VS; Development of suspension culture of *Podophyllum hexandrum* for the production of Podophyllotoxin. Biotechnology Lett, 2001; 23: 2063-206.

22. VanUden W, Pras N, Visser JF, Malingre Th M; Detection and identification of Podophyllotoxin produced by cell cultures derived from *Podophyllum hexandrum* Royle. Plant Cell Rep, 1989; 8: 165-8.
23. Mishra N, Gupta AP, Singh B, Vijay KK, Ahuja SP; A rapid determination of *Podophllum hexandrum* by reverse phase High Performance Thin Layer Chromatography. Journal of Liquid Chromatography & Realated Technologies, 2005; 28: 677-91.