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Phytochemical investigations of *in vitro* propagated plant *Taxus wallichiana* Zucc. An endangered anticancer medicinal plant of Indian origin

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Abstract

An *in vitro* protocol for propagation of *Taxus wallichiana* Zucc. (Family: Taxaceae) is useful for production of anticancerous drugs. This plant became endangered due to over exploitation and hence, conservation is most essential which is possible through biotechnological approaches. In the present study, *T. wallichiana* was cultured by *in vitro* on various media, supplemented with different combinations of plant hormones. Germination of seeds and callus development under different conditions were studied. 2, 4-D (2.0 µM) and kinetin (0.5 µM) gave satisfactory results for both initiation and sustained growth of callus in Gamborgs B5 medium. Woody plant (WP) medium was found to be effective in ascertaining totipotency of the callus by showing organogenesis. Furthermore, histochemical study of callus showed the presence of high amount of proteins, insoluble polysaccharides and RNA. Phytochemical studies of *T. wallichiana* ethanolic extract showed the presence of lignans, protein, carbohydrate, flavonoids and alkaloids. Thereafter, taxol was isolated from extract using column chromatography and identified by TLC and finally estimated by HPLC chromatography. The results revealed that percentage of taxol presence in the isolated compound was 1.22 which was more than that of callus and natural plant extract (0.85% and 0.55%, respectively).

Key words : *T. wallichiana* Zucc., media, histology, phytochemical study, isolation, TLC, HPLC, taxol

1. Introduction

Plants are only recent focus as a source for the discovery of new molecules of medicinal value for drug development because plant constituents have the potential to cure any diseases including serious cancer treatment. From past few decades, medicinal plants have been proved to be a prime natural source for cancer therapy with minimal side effects (Polu *et al*., 2015). Six million new cases are reported every year but these anticancer plants are becoming endangered in their natural habitats due to over-exploitation for the root extraction purposes. Plant cell culture systems represent a potential renewable source of valuable medicinal compounds and preparation of various pharmaceutical aids such as flavors, fragrances and colorants (Mulabagal and Tsay, 2004; Taha *et al*., 2012). Biotechnological applications of plant cell culture presently the most updated reviews on current techniques in herbal field because it enhance the content of phytoconstituents with manipulation of hormonal level which was reported earlier by the several researchers in different plants (Das *et al*., 2006; Rao *et al*., 2015).

Of late, taxol a novel diterpenoid, isolated first from the bark of *Taxus brevifolia* Nutt. (Taxaceae), has been the most promising anticancer agent isolated in recent years. Furthermore, it is also reported from all the known spp. of *Taxus* including *T. wallichiana* and *T. baccata* which have anticancer potentiality. These plants are also rich in a variety of other related molecules namely; taxanes with different degrees of cytotoxicity and antitumor activities. Among these, *T. wallichiana*, belongs to the family Taxaceae, has very low content of taxol (0.04-0.1% on dry weight basis) in mature organs. These trees are very slow growing and their seeds have a long dormancy period of two years. Recently, *in vitro* culture of Taxus has been reported through embryo culture and somatic embryogenesis (Mahdinejad *et al*., 2015). Furthermore, many bibliographic literatures revealed *in vitro* regeneration of Taxus species, *viz*., *T. cuspidata, T. baccata, T. media, T. canadensis* (Globa *et al*., 2009), *T. brevifolia* (Chee, 1995), *T. wallichiana* Zucc. (Hien *et al*., 2004; Das *et al*., 2008). Cell suspension culture for the production of Taxol and rapid propagation and conservation of *T. wallichiana* was also studied by Hussain *et al*. (2011). The content of main active phytoconstituent, *i.e*., paclitaxel is very negligible. Hence, to procure sufficient amount of paclitaxel from naturally growing Taxus trees is require more number of trees (Jaziri *et al*., 1996) which leads further loss of existences of the plant. Many researches resulted that the estimated need of purified taxol per year is 250 kg which is procured from nearly 750,000 trees (Mahdinejad *et al*., 2015) and, hence, alternate biotechnological approaches are required to fulfill the demand on the supply of taxol for new drug development. *In vitro* culture study starting from aseptic seed germination, callus initiation, regeneration aspects, followed by phytochemical investigations, *viz*., chemical tests,

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extraction, isolation, identification and estimation of taxol content and its variation in the isolated compound, callus cultures and cultivated plant extracts are very limited. No recent literature revealed histological and histochemical studies of *T. wallichiana* callus from Indian origin. Hence, the present investigation was focused to determine the production and estimation of high value added phytochemicals especially taxol by the alternate biotechnological methods.

Highlights

- Germination of *T. wallichiana* seeds under different conditions.
- Establishment of callus in suitable medium with various concentrations of plant growth regulators.
- Histological study of callus to revealed the presence of proteins, insoluble polysaccharides and RNA.
- Phytochemical study of the extracted callus and isolation of constituents.
- Estimation of the taxol by HPLC and comparison of amount in callus, plant extract and isolated compounds.

2. Materials and Methods

2.1 Plant materials

Cultivated plants and seeds were collected from Almora region, Ranikhet, Uttarakhand and were authenticated by Dr. M. Nadeem, Senior Scientist, G.B. Pant Institute of Himalayan Environment and Development, Uttarakhand, India.

2.2 Culture media

Murashigae and Skoog medium (MS), Gamborg's B5 (B5) medium and Woody plant medium (WP) were procured from Hi-media, India. B5 was used as the basal medium for initiation of callus growth and WP medium, Hi-media, India was used for regeneration of the plants. All the growth regulators were collected from Hi-media, India and used in different concentrations for this present study, *viz*., 2,4-Di chlorophenoxy acetic acid (2,4-D), Kinetin, Indole acetic acid (IAA), Indole butyric acid (IBA), Gibberellic acid (GA_3), 6-Benzylaminopurine (BAP) and Naphthalene Acetic acid (NAA).

2.3 Preparation of medium

B5 medium was used as basal medium. 31.37 g lt^{-1} of medium with 8% of agar was dissolved in 500 ml of double distilled water and the pH of the medium was adjusted to 5.6, using 0.1(N) sodium hydroxide (supplied by Qualigens, India). Different combinations of hormones were added and mixed. They were sterilized in an autoclave at 121^0 C for 20 min.

2.4 Aseptic germination of seeds

Seeds were sterilized and made to germinate under aseptic conditions without contamination. Two different methods were adopted to break the seed dormancy. In first method, seeds were cleaned with double distilled water and placed in wet sterile filter paper in sterilized petridishes. The petridishes were imbibed with thin layer of sterile absorbent cotton impregnated with different concentrations (10-100 µM) of GA_3 and finally incubated at 25^oC in dark. In second method, seeds were imbibed in sterile distilled water for 24 h. and washed with mild detergent solution. Further soaked in 70% ethanol for 1 min, followed by treatment with 4% sodium hypochlorite (from Ranbaxy, India) for 15 min., then rinsed with sterile distilled water to remove the seed coats. Then endosperms were treated with 2% (v/v) sodium hypochlorite for 5 min., followed by washing 3 times with sterile distilled water. Finally they are transferred into different culture media, *i.e.*, ½ MS with GA_3 and ½ WP media with BAP and incubated at 25 ± 2^0 C.

2.5 Callus initiation and development

Callus was induced using B5 medium supplemented with different combinations of growth hormones, *viz*., 2,4-D, Kinetin, IAA, IBA, GA_3, BAP and NAA. Needles and shoots from mother plant were used as explant. The explants were washed and sterilized with distilled water then immersed in 2% sodium hypochlorite for 20 min. and further rinsed with double distilled water. Explants were aseptically cut to a size of 5 mm length with sterile scalpel and transferred in the sterile B5 medium and incubated in BOD incubator at 25 ± 2^0 C and observed for the growth.

2.6 Histology and histochemical studies of callus

Microtome was used to determine the anatomy and chemical composition of the cells and tissues by histology and histochemical studies, respectively. 75 days old calli grown on B5 media were taken for the present study. Series of methods were followed, namely; fixation of calli was done by using Carney's B after the treatment of calli with alcohol: chloroform: glacial acetic acid (6:3:1) and calli were fixed for 4-5 h. to determine the nuclear elements such as DNA, RNA, *etc*., followed by dehydration with alcohol and butanol followed by infiltration and embedding in paraffin wax at $58\text{-}60^0$C by paper boat technique, followed by sectioning of calli at thickness of 8 µm, used by rotary microtome and placed in slides by using 0.2% gelatin as adhesive, and finally deparaffinising by xylol solution, cleaning by using butanol, alcohol and finally dipped in 0.1% cell iodine solution for one minute before staining. Staining was done by 0.5% per-iodic acid Schiff's (PAS) reagent for 10 min. to identify the intensity and distribution of insoluble polysaccharides. Further 0.5% mercuric bromophenol blue (MBB) was employed for one minute to determine the concentration of proteins and finally 0.5% methyl green pyrolene (MGP) was used for 2 min. to differentiate RNA and DNA sites in the calli after staining with 0.1% toludine blue (TB, which was used to determine the intensity of RNA).

After staining the slides, the excess stains was removed by immersing in distilled water and passed through xylol to remove complete moisture and finally mounted with digital picture exchange (DPX) mountant.

2.7 Preparation of extract for chemical analysis

Obtained calli and dried plant part (needles) were refluxed 4 h., with ethanol and analyzed for various phytochemicals present in the calli as per the standard method described by Harborne (1973) and Evans (2002).

2.8 Isolation of taxol

Taxol was isolated from the ethanolic extract of the calli, cultured plant extract and cultivated plant extract by column chromatography using chloroform, methanol and acetonitrile solvents.

2.9 TLC identification and HPLC estimation of taxol

Taxol extracted from callus and plant of *T. wallichiana* was identified by TLC method, using standard paclitexal (Taxol) (Kwak *et al*., 1995; Nguyen *et al*., 2001). Chloroform and acetonitrile (7:3) were used as mobile phase and silica gel GF_{254} was used as stationary phase. Sample was prepared by dissolving 50 mg in 10 ml of acetonitrile and diluted further from 1 ml to 10 ml with acetonitrile. Standard paclitexal was procured from Dr. M. Nadeem, Senior Scientist, G.B. Pant Institute of Himalayan Environment and Development, Uttarakhand, India as research sample and 10 mg of sample dissolved in 10 ml of acetonitrile and diluted 1 ml to 10 ml with same solvent. It was detected at 254 nm and derivatized with vanillin sulphuric acid spraying reagent.

All the samples were analyzed for taxol by HPLC (Shimadzu, India). The condition for HPLC as column: SS Wakosil II C-18 (250 X 4.6 mm), mobile phase: Acetonitrile, water and methanol (60:20:20), flow rate 1.0 ml per min. and compound detected at 227 nm.

2.10 Statistical analysis

Callus growth was analyzed by one way ANOVA study, followed by Tukey's multiple comparison post test and $p<0.05$ was considered as significant. Graph Pad Prism 5 software was used for this analysis.

3. Results

3.1 Aseptic germination of seeds

The results showed that seed germination (through excised embryo) was initiated in 30 d. by the second method where seeds were treated with mild detergent solution. The excised embryos showed proliferation and growth in ½ MS medium with 5 µM solution of GA_3 that showed in Figure 1.

½ MS medium with 5 µM solution of GA_3

Figure 1: Aseptically germinated seeds and callus from embryo of TB

3.2 Callus initiation and development

Different combination of growth regulators was prepared and growth of callus was observed. The results revealed that after 75 d. callus was initiated and developed in B5 medium supplemented with hormonal combination of 2,4-D and kinetin. Other combinations such as GA_3, IAA, IBA and BAP in B5 medium resulted no callus initiation even after 80 d. (Table 1). Results showed 4.0 µM 2,4-D and 1.0 µM of kinetin initiated callus in 75 d. and development started when sub cultured in 5.0 µM 2,4-D and 1.0 µM kinetin in B5 medium whereas 2.0 µM of 2,4-D in combination with 0.5 µM kinetin in B5 medium gave satisfactory development of callus after 80 d., determined by eyepiece micrometer (Tables 2 and 3; Figure 2). Statistically, all the columns were analyzed by Tukey's multiple comparison test. Further, correlation coefficient study revealed the diameter and length of calli grown in 80 d. was increased in comparison to 75 d. of calli (Table 4).

Further, regeneration of the shoots was observed on the 55th d. of incubation. Rooting started on the 65th d. and took about 80 d. for the plants to grow to a size of about 2 inches length with well-established root in WP medium (Figures 3 and 4).

2.0 µM of 2, 4-D and 0.5 µM kinetin

Figure 2: Callus of TB grown in B5 media

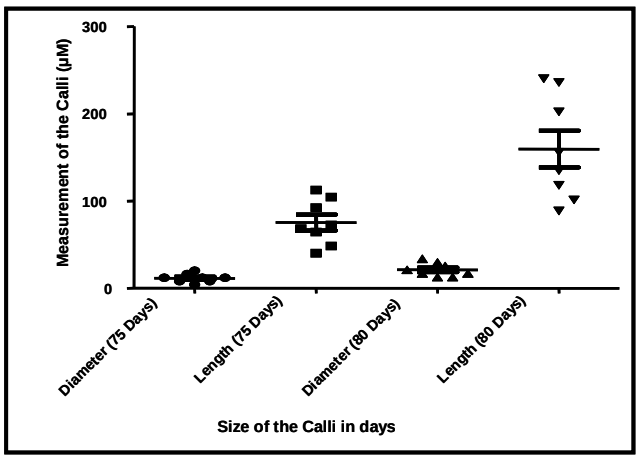


Figure 3 : Measurement of the calli of Tb in two different time period in different concentration of growth regulators in B5 medium

Table 1: Effect of growth hormones on growth of calli

Media composition	Conc. of 2,4-D (µM)	Duration of callus form	Growth	Media composition	Conc. of 2,4-D (µM)	Duration of callus form	Growth
B5 media	1.0		- -	B5 media	1.0		- -
31.37 g lt^{-1}	2.0		- -	31.37 g lt^{-1}	2.0		+ + +
+	3.0	75 days	- -	+	3.0	80 days	+
Agar 8.0%	4.0		+	Agar 8.0%	4.0		- -
+	5.0		+ + +	+	5.0		- -
Kinetin 1.0				Kinetin 0.5			
µM				µM			

- (+) Poor callus; (+++) Good callus growth; (- -) No callus.

Table 2: Showing diameter and length of calli (Multiplied by calibration factor, C_f) after 75 d. of growth (4.0 µM 2,4-D and 1.0 µM of kinetin in B5 medium)

Sample of calli	Divisions of eyepiece C_f = 4.018	Actual Diameter (µm)	Divisions of eyepiece C_f = 4.018	Actual length (µm)
1	3 × 4.018	12.054	12 × 4.018	48.216
2	2 × 4.018	8.036	10 × 4.018	40.18
3	5 × 4.018	20.09	23 × 4.018	92.414
4	4 × 4.018	16.072	26 × 4.018	104.468
5	3 × 4.018	12.054	17 × 4.018	68.306
6	2 × 4.018	8.036	16 × 4.018	64.288
7	1 × 4.018	4.018	18 × 4.018	72.324
8	3 × 4.018	12.054	28 × 4.018	112.504
Average		**11.551****		**75.338****

- **p< 0.05; significant when compared by one-way ANOVA study with Tukey's multiple comparison test.

Table 3: Showing diameter and length of calli (Multiplied by calibration factor, C_f) after 80 d. of growth (2.0 µM of 2,4-D and 0.5 µM kinetin in B5 medium)

Sample of calli	Divisions of eyepiece C_f = 4.212	Actual diameter (µm)	Divisions of eyepiece C_f = 4.212	Actual length (µm)
1	3 × 4.212	12.636	28 × 4.212	117.936
2	6 × 4.212	25.272	32 × 4.212	134.784
3	7 × 4.212	29.484	56 × 4.212	235.872
4	4 × 4.212	16.848	37 × 4.212	155.844
5	8 × 4.212	33.696	21 × 4.212	88.452
6	4 × 4.212	16.848	24 × 4.212	101.088
7	3 × 4.212	12.636	57 × 4.212	240.084
8	5 × 4.212	21.06	48 × 4.212	202.176
Average		**21.06*****		**159.530*****

- ***p< 0.001; significant when compared by one-way ANOVA study with Tukey's multiple comparison test.

Statistical data for the calli of Tw

Tukey's Multiple Comparison Test	q value	p <0.05
Diameter (75 d.) vs Length (75 d.)	5.490	**
Diameter (75 d.) vs Diameter (80 d.)	0.8184	ns
Diameter (75 d.) vs Length (80 d.)	12.74	***
Length (75 d.) vs Diameter (80 d.)	4.672	*
Length (75 d.) vs Length (80 d.)	7.247	***
Diameter (80 d.) vs Length (80 d.)	11.92	***

Table 4 : Correlation coefficient of calli measurement in two different time periods

Calli	Correlation coefficients			
	Diameter (75 d.)	Length (75 d.)	Diameter (80 d.)	Length (80 d.)
Diameter (75 d.)	1			
Length (75 d.)	0.521	1		
Diameter (80 d.)	0.433	0.060	1	
Length (80 d.)	0.136	0.526	-0.131	1

Table 5: HPLC data for ethanolic extracts of Tw and standard paclitaxel

Samples	R_t in minutes	Area (m.V/s)	Percentage of Paclitaxel
Standard (99% purity)	5.07	7644.9049	—
Plant extract	5.37	41.9841	0.55
Callus extract	6.51	64.9750	0.849
Column isolated sample	6.46	93.2736	1.220

3.3 Histology and histochemical studies of callus

0.1% MBB stain was used for detection of protein in the calli and resulted high concentration protein present in the calli that was identified with the intensity of the blue color in the section (Figure 5). Thereafter, 0.5 % TB showed the presence of high metabolic activity of the callus showcasing deep blue coloration (Figure 6). Furthermore, insoluble polysaccharides was identified with formation of deed magenta color, stained with PAS (Figure 7) and MGP stain was used to identify genetical materials like RAN and DNA which resulted minimum quantity of RNA with deep red coloration (Figure 8).

Figure 4: Regeneration of plantlet of Tb in WP media

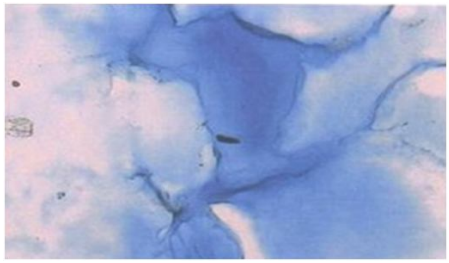


Figure 5: Section of callus stained with MBB for presence of total soluble protein

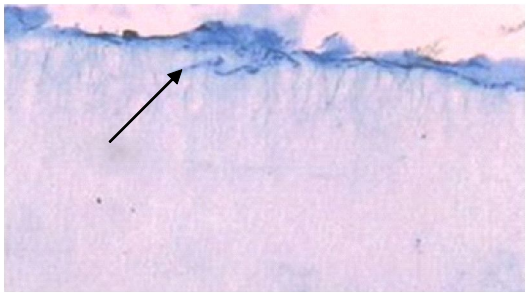


Figure 6: Section of callus stained with TB for presence of metabolic activity

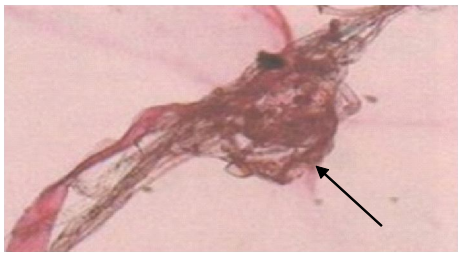


Figure 7: Section of callus stained with PAS for presence of insoluble polysaccharides

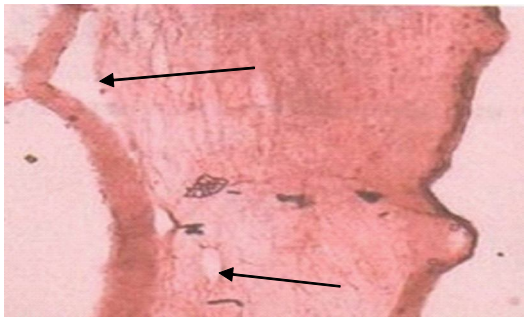


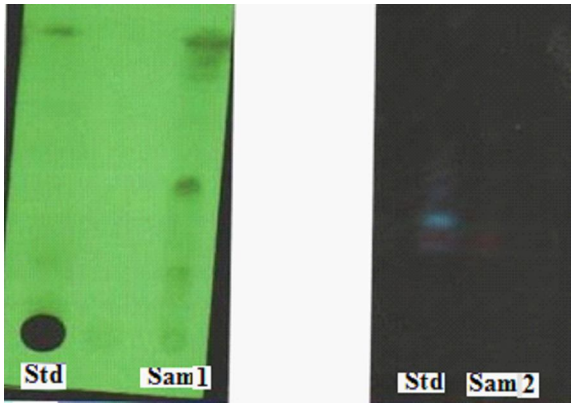
Figure 8: Section of callus stained with MGP for presence of RNA/ DNA

3.4 Chemical analysis of extracts

Ethanolic extracts of plant parts and calli of *T. wallichiana* were subjected to qualitative chemical analysis and revealed the presence of alkaloids, protein, carbohydrate, flavonoids and lignans.

3.5 Isolation and identification of taxol by TLC

Taxanes alkaloids were isolated from plant extract by column chromatography, using various solvents as per polarity and further identified by TLC method and the results revealed the presence of taxol in both plant (R_f = 0.59) and callus (R_f = 0.58) extracts when compared with the R_f of standard taxol at 0.61 (Figure 9).



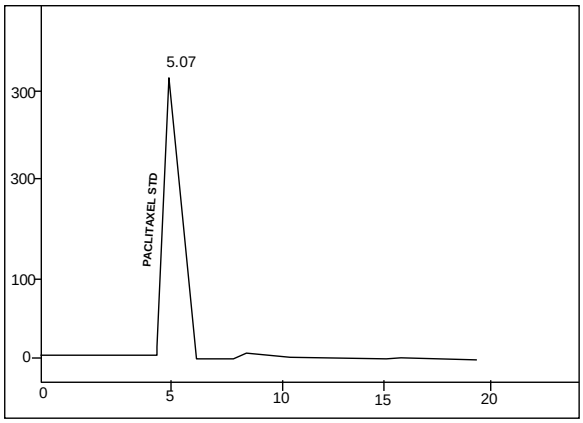
a) After derivatization b) Uv at 254 nm

Std= Standard taxol; Sam1= plant extract;
Sam 2= Callus extract

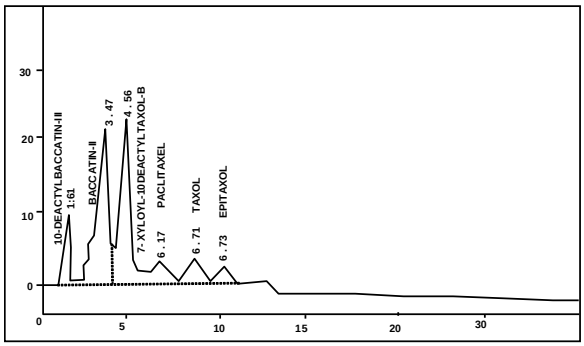
Figure 9: TLC of the extracted Tb samples

3.6 HPLC study

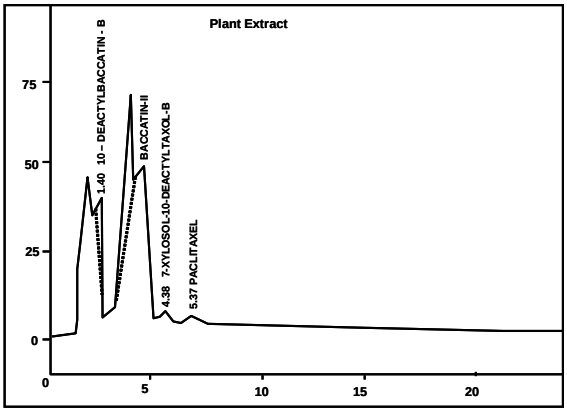
HPLC study was performed for all the samples and compared with the standard taxol and revealed the presence of taxol in all the samples of *T. wallichiana* which was confirmed by compared with the retention time (R_t). R_t of Standard taxol was 5.07 min. whereas taxol was eluted at R_t of 5.37 min., 6.51 min. and 6.46 min. for plant extract, callus extract and column isolated extract, respectively (Graphs 1, 2, 3 and 4) and the percentage content of taxol was relatively higher in column isolated extract (1.22 %) when calculated with the area of standard taxol (Table 5).



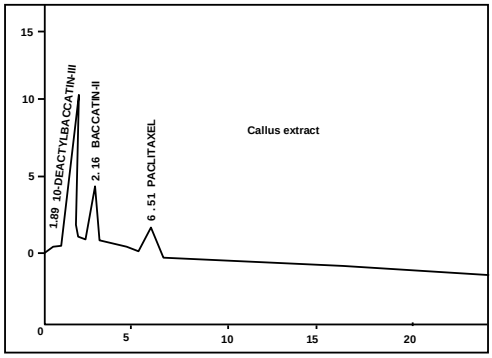
Graph 1: Standard paclitaxel in HPLC



Graph 2: HPLC for column isolated compounds from Tw



Graph 3: HPLC for ethanolic plant extract of Tw



Graph 4: HPLC for ethanolic callus extract of Tw

4. Discussion

The use of manipulated plant tissue culture for the production of chemicals and phytoconstituents has made great strides building on advances in plant sciences. The increased use of genetic tools and an emerging picture of the structure and regulation of pathways for secondary metabolism will provide the basis for the production of commercially acceptable levels of plant based product. The increased appeal of natural products for medicinal purposes coupled with the low product yields and supply concerns due to plant harvesting has gained interest in large scale plant cell culture technology. Hence, the present study has undertaken for establish suitable biotechnological methodology to increase availability of plant material for production of secondary metabolites for production of novel drugs.

Literature reveals the that the tissue culture of taxus species is difficult due to embryo dormancy (Flores *et al*., 1993), very slow germination (Flores and Sgrignoli, 1991; Anderson and Owens, 2001) and also seedling development in natural condition is very difficult (Hu *et al*., 1992). Hence, many treatments have been employed for improvement of seed germination of various taxus species (Flores *et al*., 1993; Zhiri *et al*., 1994; Chang and Yang, 1996). Looking at that, we have also used two different methods and finally germination started after one month in ½ MS medium supplemented with 5 µM of GA_3 when seed coats were removed aseptically. The results of the present study showed similar to the earlier report (Chang and Yang, 1996; Liao *et al*., 2006).

For callus initiation, full strength of B5 media provided satisfactory result than others basal media in combination of 2,4-D and kinetin which was in agreement with Jha *et al*. (1997) and Cusido *et al*. (2002). Further, regeneration of the plant was found on WP medium supplemented with varying concentration of plant hormones such as BAP and 2,4-D from needle explants which were correlated with the earlier reports (Wickremesinhe and Arteca, 1994; Chee, 1996). The satisfactory effect of 2,4-D on callus growth of *T. wallichiana* may be related to role of this plant hormone in blocking morphogenesis and favors differentiation and callus developments.

Callus was subjected to microtome by staining sections of callus with different dyes like MBB for protein, TB for metabolic activities, presence of proteins and polysaccharides by PAS and MGP for RNA and DNA study and revealed presence of protein, moderate metabolic activity and presence RNA. The study suggested that the cells were in high proliferation stage and contains either void of nuclei or the cells were not differentiated.

Identification of taxanes like baccatin-III, paclitaxel, epitaxol, 10-D acetylbaccatin-II, *etc.*, was carried out by using TLC. TLC pattern confirmed the presence of taxanes (taxol) when compared with standard taxol (Paclitaxel). Taxol and other taxanes fraction were isolated from methanolic plant extract using silica gel column chromatography using different solvents (chloroform, methanol and acetonitrile) as mobile phase. Quantitative estimation of taxol and other taxanes was carried out by HPLC using C-18 column and acetonitrile: methanol: water (60:20:20) as mobile phase. The method followed as reported earlier (Nadeem *et al*., 2002). The analysis revealed the presence of taxol in plant extract as well as in callus extract. The tissue culture method developed for callus production and isolated constituents from that, was found to be effective with taxane content comparable to that of the plant extract. The result also similar with the earlier studies reported by several researchers when estimated with HPLC data (Parc *et al*., 2002; Ashrafi *et al*., 2010).

5. Conclusion

T. wallichiana has been considered a rare, novel and endangered species with high demand anticancer agent. Therefore, special attention needs to be given for its propagation and conservation. It was reported that Taxus seeds have a long dormancy period of two years, hence, other methods of propagation have to be considered. Study was carried out successfully in standardizing media for tissue culture, aseptic embryo culture, callus initiation, regeneration, histochemical study of callus and estimation of callus as well as plant extract by HPLC analysis.

Further, Knowledge of biosynthetic pathways of desired compounds from this plant source as well as in cultures is often still in its infancy, and consequently, strategies are needed to develop an information based on a cellular and molecular level and preparation of semi synthetic derivatives from taxanes using baccatin III, as a lead antineoplastic molecule.

Conflict of interest

We declare that we have no conflict of interest.

Acknowledgement

The authors would like to thank to Dr. M. Nadeem, Senior Scientist, G B Pant Institute of Himalayan Environment and Development, Uttarakhand, India, for authentication and supplied of Taxus plant for the present study.

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