Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

IN VITRO TUBERIZATION AND QUANTITATIVE ANALYSIS OF COLCHICINE USING HPTLC IN *GLORIOSA SUPERBA*. L AN ENDANGERED MEDICINAL PLANT OF PACHAMALAI HILLS, A PART OF EASTERN GHATS, TAMIL NADU.

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ABSTRACT

Gloriosa superba. L has been a source of medicine right from ancient times. The tubers of this plant are sold in Indian herbal market as an important source of an alkaloid colchicine. Surface sterilized seeds of *Gloriosa superba* were soaked overnight in 1% GA_3 on the next day seeds were planted on germinating media containing MS basal salts with 0.5 mg/l GA_3 and 1.0 mg/l BA, 1% sucrose and 0.8% agar. 72.5% of seed germination was observed. The germinated seeds were transplanted on MS basal medium supplemented with 1.0 mg/l BAP, 0.05 mg/l GA_3, 9.5 mg/l NAA and 6% sucrose which led to 90% tuber induction within 6 weeks of culture. Since there is a great demand of colchicine in the market, we have made an attempt to estimate the colchicine content in different parts of the plant like leaf, seed, pericarp, tuber and *in vitro* produced tuber using High Performance Thin Layer Chromatography, using a mixture of Ethyl acetate:Methanol (10:1.3 v/v) as mobile phase and precoated silica gel F_{254} TLC aluminium sheets as the stationary phase. The detection of spot was carried out at 350nm. The calibration curve was found to be linear between 100 to 600 ng/spot for colchicines. The results revealed that *in vitro* tuber had highest amount (0.14249%) of cochicine, followed by *in vivo* seed (0.10900%), tuber (0.05761%), leaves (0.46470%) and pericarp (0.04574%). The proposed method can be used to determine the colchicine content in *Gloriosa superba*.

KEY WORDS: ***Gloriosa superba*. L, Colchicine, HPTLC, *In vitro* tuberisation.**

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INTRODUCTION

Gloriosa superba (Liliaceae) is an indigenous medicinal plant commonly known as glory lily, distributed throughout India from 68.7° to 97.25° E and 8.4° N to 37.6° N and 0 to 2043 m.a.s.l. The plants of this species have proved highly adapted to extreme conditions, including temperatures ranging from 22°C to 32°C (Seemanti Ghosh *et al*., 2008), studies reveal that all parts of the plant, especially the tubers are extremely toxic due to the presence of a highly active alkaloid, colchicine. Apart from its action on mitosis, colchicine reduces the inflammation and relieves the pain associated with acute gout (Insel, 1996). Colchicine therapy diminishes the metabolic activity of leukocytes, resulting in reduced phagocytosis of urate microcrystals, thereby interrupting the cycle of new crystal deposition (Seegmiller *et al*.,1962). In ayurveda and Yunani systems of medicine, the tuber is well known due to its pungent, bitter, acrid, heating, anthelmintic, laxative, alexiteric and abortifacient nature. It is widely used in the treatment of ulcers, leprosy, piles, inflammations, abdominal pains, intestinal worms, thirst, bruises, infertility and skin problem (Kirtkar and Basu, 1935). Glory lily is among some of the modern medicine's most important plant actually facing local extinction. The Pachamalai hills are part of Eastern Ghats, Tamil Nadu, known for the abundance of medicinal plant wealth. The tribal people of this place are called as 'Malayalis'. They depend only on medicinal plants for all ailments, and the over exploitation of medicinal plants has lead to endangeredness of the most valuable plants. Due to this reason *Gloriosa superba* is getting endangered in Pachamalai hills (Soosairaj *et al*., 2007). Various factors like excessive collection of the plant by people, susceptibility towards many insects and poor seed germination have lead this plant to become endangered (Sivakumar and Krishnamurthy, 2004). The use of *in vitro* cultures enables the selection of uniform individuals for studying the morphogenetic aspects of tuber formation process under controlled conditions (Ulloa *al*., 1997). The present study deals with the *in vitro* tuberization of *Gloriosa superba* through *in vitro* regeneration techniques. We have attempted to compare the colchicine content in different parts of the *in vivo* plant with the *in vitro* produced tuber through High Performance Thin Layer Chromatography (HPTLC), in order to establish the significance of tissue culture technique.

MATERIALS AND METHODS

Collection of plant material

The plant materials (*Gloriosa superb*) were collected from Pachamalai hills, a part of Eastern Ghats, Tamil Nadu, during harvest season of November (Fig 1A). The seeds were used as explants for induction of rhizome production through *in vitro* techniques. And other parts were shade dried, powdered and used for HPTLC analysis.

In vitro tuberization

The seeds of *Gloriosa superba* were washed with 5% Teepol (detergent) for 10 min and kept in running tap water for 30 min. they were surface sterilized by immersing in 70% for 2min, followed by 0.1% mercuric chloride for 3 min for the purpose of breaking the dormancy of the seeds. Seeds were rinsed with sterilized distilled water several times under asceptic conditions and immersed in 1% GA_3 for overnight in asceptic condition. On the next day, seeds were washed five times with sterile distilled water and planted on seed germinating medium containing MS basal medium (Murashige and Skoog, 1962) with GA_3, BA, 1% sucrose and 0.8% Agar. The germinated seeds were transplanted on corm-producing medium containing MS basal salts BAP, GA_3 and NAA.

Quantification of colchicines in G.superba samples by HPTLC technique

1.0g of the powdered plant material was dissolved in 10 ml of methanol individually and kept for about 15 minutes at a temperature of 30°C and filtered, made upto the volume with methanol. 10mg of standard colchicine extrapure (Sigma-Aldrich, St.Louis, USA) of purity (98% w/v) was dissolved in 10ml of methanol by sonication. Specific quantity of the sample and standard was applied with the help

of Linomat IV as bands on the Pre-coated silica gel F_{254} TLC aluminium sheets and developed with mobile phase- Ethyl acetate : Methanol (10:1.3), upto about 7.5cm. The developed chromoplate was dried by a hair-dryer. The plate was scanned at 350nm by using the Camag TLC plate Scanner III. The content of colchicine was calculated by comparing the peak areas of sample and standard spots winCATS Planar Chromatography Manager program (Ver. 1.4.3) (Wagner and Bladt, 1996).

RESULTS

The seeds of *Gloriosa superba* are very much prone to insect attack and having very less viability for germination. The seeds were collected from wild plants of Pachamalai hills (Fig. 1B & C), surface sterilized and planted on Seed germinating medium consisting of MS basal salts with GA_3, BA, 1% sucrose and 0.8% agar. Highest germination percentage (72.5%) was observed when the medium containing MS basal salts supplemented with 0.5 mg/l GA_3 and 1.0 mg/l BAP. (Fig. 1E) (Table-1).The germinated seeds were transferred in the corm-producing medium containing MS basal salts with BAP, GA_3 and NAA. Maximum percentage (90%) of *in vitro* tuberization (3.5cm length) and rooting were observed when the MS medium fortified with 1.0mg/l BAP, 0.05mg/l GA_3 and 9.3mg/l NAA with 6% of sucrose. 50% of *in vitro* tuberization with small rhizome (1.5cm) and 25 roots were observed when the MS medium supplemented with 1.25mg/l BAP, 0.05mg/l GA_3 and 9.4mg/l NAA was used in the culture medium. It was inferred that when BAP and NAA concentration was increased, tuberization was decreased and root induction was increased (Table-2).

In vitro tubers (Fig 1F&G), *in vivo* leaf, seed, tuber (Fig. 1D), and pericarp were thoroughly washed, shade-dried, and powdered to use in the estimation of colchicine content through HPTLC. Different compositions of mobile phase for HPTLC analysis were tested in order to obtain high resolution, symmetrical and reproducible peaks for colchicine. The desired resolution of compound was achieved by using Ethyl acetate: Methanol (10:1.3) as the mobile phase. On this system, separation was good and peaks of colchicines were well defined. The scanning wavelength of 350nm was found to be optimal for high sensitivity for colchicine spots. During the development of the HPTLC method, it was observed that a pre-saturation of TLC chamber with mobile phase for at least 1 hour was required to obtain a good separation. Estimation of the colchicine was carried out by comparing the peak area of sample with the standard. The samples were subjected to HPTLC quantification and the results obtained were tabulated (Fig 2 to 7). Colchicine content was quantified in raw material of *G.superba* and the values in percentages were found to be AB1 (0.14249%), AB2 (0.05761%), AB3 (0.04574), AB4 (0.04647%) and AB5 (0.10900%). The maximum colchicine content was seen in the AB1 i.e, *in vitro* tuber of *G. superba* L. and followed by *in vivo* seeds, tuber, leaf and pericarp. An interesting feature was the presence of a reasonably high quantity of colchicine in the pericarp which is normally discarded after harvesting of seeds (Table-3).

Table 1
Effect of growth hormones on in vitro seed germination of Gloriosa superba.

Growth Hormone (mg/l)		No. of seeds inoculated/ tube	No. of seeds germinated (Mean ± S.D)	Percentage of seed germination
BA	GA_3			
0.5	0.5	4	1.1±0.95	27.5
1.0	0.5	4	2.9±0.94	72.5
1.5	0.5	4	1.3±1.15	32.0
2.0	0.5	4	0.9±0.7	22.0
2.5	0.5	4	No germination	-

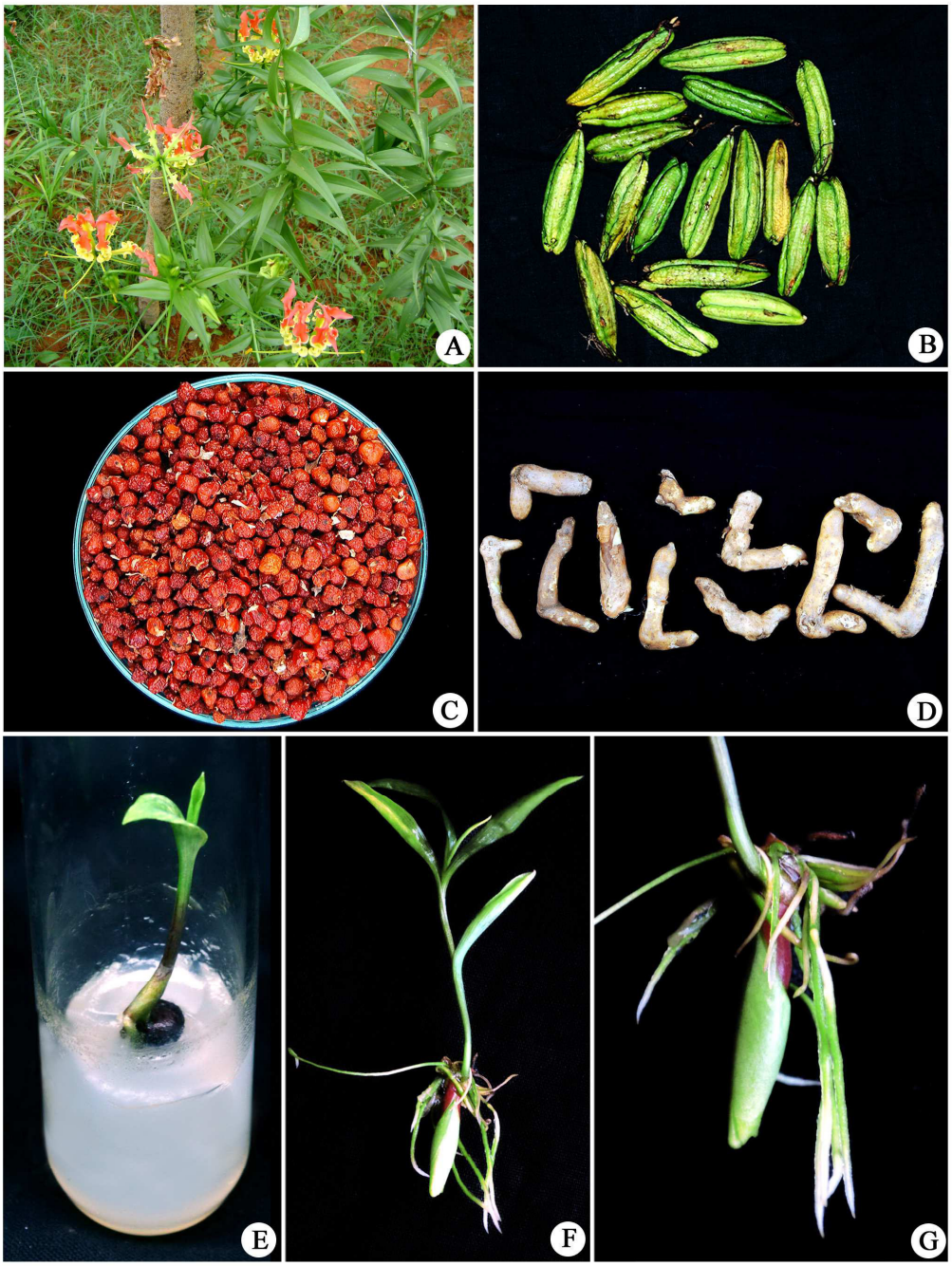
Table 2
Effect of growth regulators on in vitro tuberization when incorporated in MS medium supplemented with 6% sucrose

BAP (mg/l)	GA_3 (mg/l)	NAA (mg/l)	Percentage of tuberization	Length of rhizome (Mean± S.D)	No. of roots formed in rhizomes
0.50	0.05	9.1	25	1.2±0.1	3-4
0.75	0.05	9.2	65	2.5±0.7	9-11
1.00	0.05	9.3	90	3.5±0.3	10-15
1.25	0.05	9.4	50	1.5±0.5	20-25
1.50	0.05	9.5	No tuberization	-	-

Table 3
Quantification of colchicine from different samples

S.No.	Samples	Sample ID	Peak area	Amount detected in the sample (%)
1.	*In vitro* tuber	AB1	15127.0	0.14249
2.	*In vivo* tuber	AB2	9175.0	0.05761
3.	*In vivo* pericarp	AB3	7284.8	0.04574
4.	*In vivo* leaf	AB4	7400.5	0.04647
5.	*In vivo* seeds	AB5	17360.0	0.10900

Figure-1
Morphology and *in vitro* tuberization of *Gloriosa superba* L.

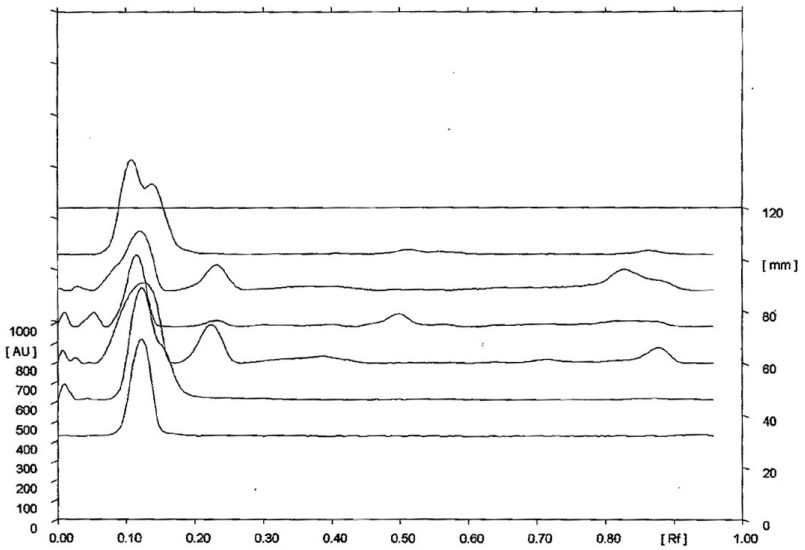


A. Habit
B. Fruits
C. Seeds
D. Tubers
E. *In vitro* seed germination
F. *In vitro* plantlet with tuber
G. *In vitro* tuberization with roots

Figure-2
Quantification of Colchicine by HPTLC

winCATS Planar Chromatography Manager

All tracks at Wavelength



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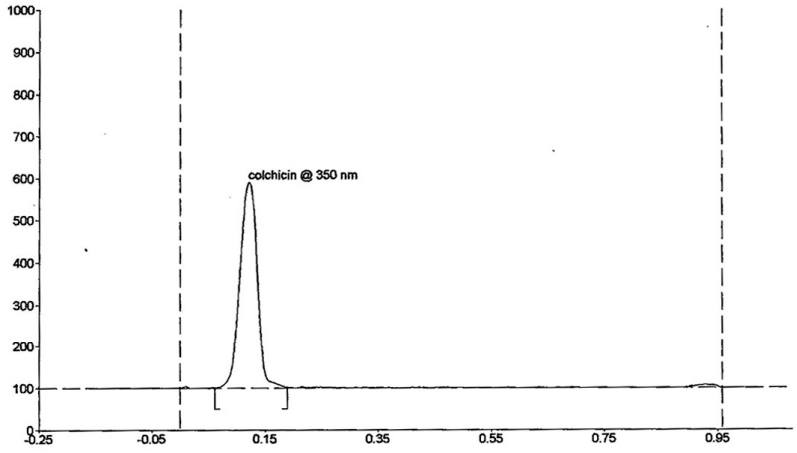
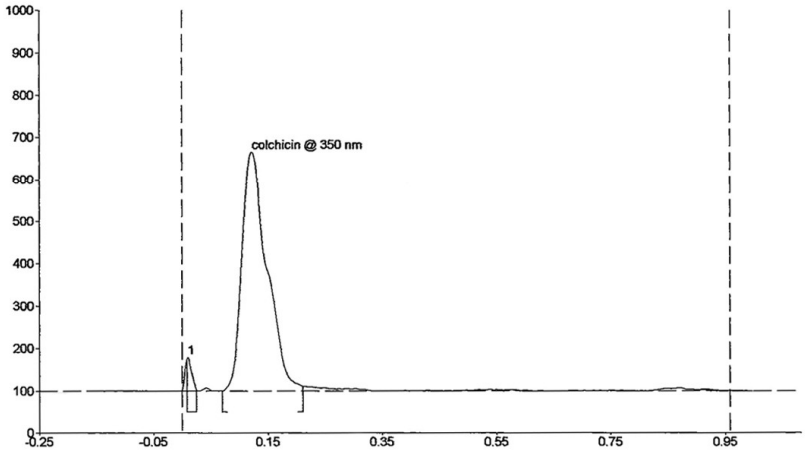


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.06	2.0	0.12	490.7	100.00	0.19	2.8	10615.9	100.00	colchicin

Figure-3
Quantification of Colchicine by HPTLC

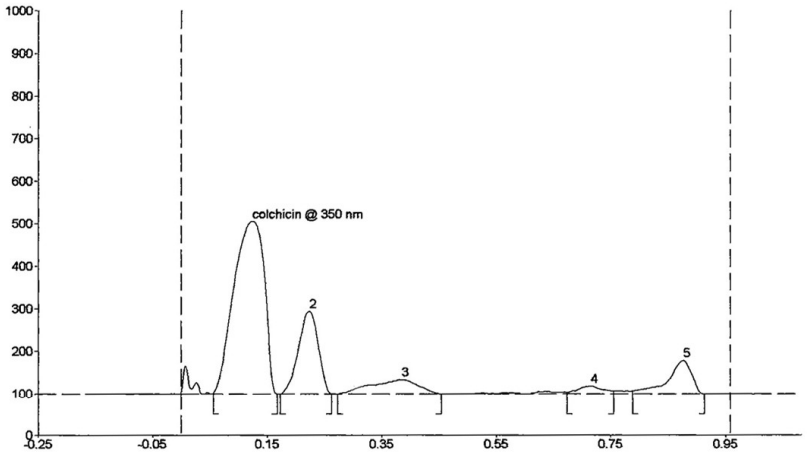
winCATS Planar Chromatography Manager

Track 3, ID: AB5



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.01	76.4	0.01	79.5	12.32	0.03	3.3	495.7	2.78	unknown *
2	0.07	0.2	0.12	566.3	87.68	0.21	11.6	17360.0	97.22	colchicin

Track 4, ID: AB1

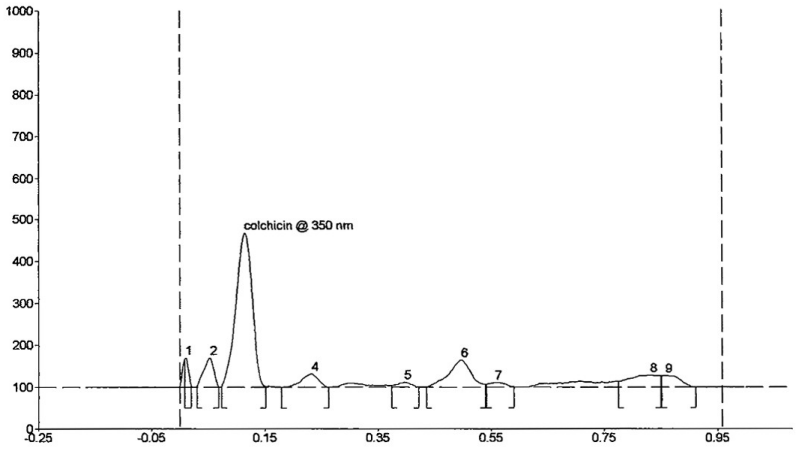


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.06	1.8	0.13	406.2	55.43	0.17	0.3	15127.0	61.28	colchicin
2	0.17	0.3	0.23	194.2	26.50	0.26	0.7	4674.3	18.94	unknown *
3	0.27	1.0	0.39	34.5	4.71	0.46	0.1	1993.0	8.07	unknown *
4	0.68	4.2	0.72	19.4	2.65	0.76	7.6	590.7	2.39	unknown *
5	0.79	7.1	0.88	78.5	10.72	0.92	0.1	2299.1	9.31	unknown *

Figure-4
Quantification of Colchicine by HPTLC

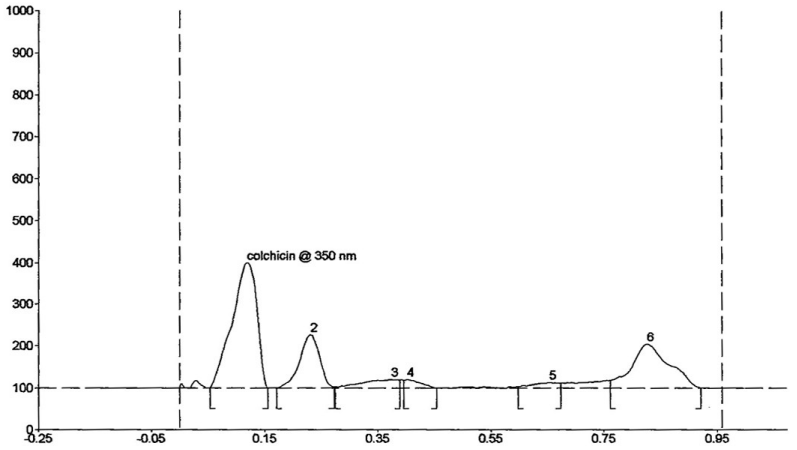
winCATS Planar Chromatography Manager

Track 5, ID: AB4



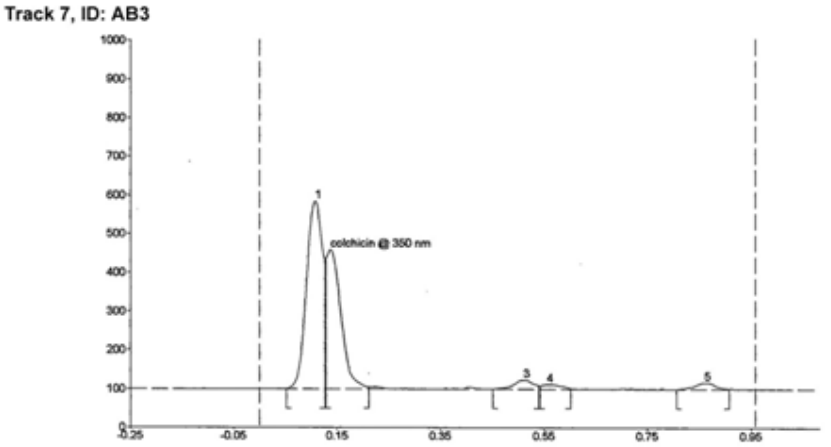
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.01	68.1	0.01	71.0	10.34	0.02	4.9	366.0	2.75	unknown *
2	0.03	2.2	0.05	70.9	10.32	0.07	2.0	888.2	6.68	unknown *
3	0.07	1.6	0.12	368.1	53.59	0.15	3.4	7400.5	55.67	colchicin
4	0.18	0.2	0.23	31.7	4.62	0.26	0.1	664.3	5.00	unknown *
5	0.38	4.7	0.40	12.1	1.76	0.42	0.9	214.4	1.61	unknown *
6	0.44	0.8	0.50	65.0	9.46	0.54	7.7	1796.0	13.51	unknown *
7	0.54	7.9	0.56	11.3	1.65	0.59	1.5	238.3	1.79	unknown *
8	0.78	14.1	0.83	29.1	4.23	0.85	26.9	1102.3	8.29	unknown *
9	0.85	26.8	0.86	27.7	4.04	0.91	0.1	623.2	4.69	unknown *

Track 6, ID: AB2



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.05	0.5	0.12	301.2	51.23	0.16	0.2	9175.0	48.25	colchicin
2	0.17	0.2	0.23	127.2	21.63	0.27	2.7	3124.6	16.43	unknown *
3	0.28	2.7	0.38	20.6	3.50	0.39	19.6	952.1	5.01	unknown *
4	0.40	19.1	0.40	20.3	3.46	0.46	0.6	428.1	2.25	unknown *
5	0.60	4.2	0.66	13.7	2.33	0.68	12.2	455.7	2.40	unknown *
6	0.76	19.3	0.83	105.0	17.85	0.92	0.2	4881.8	25.67	unknown *

Figure-5
Quantification of Colchicine by HPTLC
winCATS Planar Chromatography Manager



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.05	0.2	0.11	484.0	53.96	0.13	335.9	10524.8	54.46	unknown *
2	0.13	336.3	0.14	359.0	40.02	0.21	6.4	7284.8	37.70	colchicin
3	0.45	0.6	0.51	24.5	2.73	0.54	9.5	658.7	3.41	unknown *
4	0.54	9.5	0.56	12.3	1.37	0.60	2.8	344.2	1.78	unknown *
5	0.81	0.7	0.86	17.2	1.92	0.91	1.8	512.4	2.65	unknown *

DISCUSSION

The seeds of *Gloriosa superba* have poor seed germination and susceptibility towards pests. Hence, the standardization of *in vitro* germination of seeds is a boon for mass propagation of this plant. In the present study highest percentage of seed germination was observed when the MS medium fortified with 0.5 mg/l GA_3 and 1.0 mg/l BAP. Our results correlate with Sivakumar *et al.,* (2003) who achieved over 40% of seed germination in *Gloriosa superba* when MS medium fortified with 1.44µM GA_3 and 4.44µM BA. The germinated seeds were transferred to corm-producing media containing BAP, GA_3 and NAA. *In vitro* tuberization was achieved after four weeks. Higher concentrations of NAA induced rooting in the shootlets probably due to "carry over" effect of BAP in these shoots which were continuously subcultured on medium supplemented with BAP. 1.0mg/l BAP lead to the proliferation of multiple shoots and 9.3mg/l NAA induced prolific rooting of the shoots. When BAP and NAA at this concentration were used together, formation of tubers occurred. BAP alone suppressed rooting and NAA alone did not favour proliferation of shoots. Incorporation of GA_3 (0.05 mg/l) along with optimal concentration of BAP and NAA enhanced the percentage of cultures showing tuber formation. GA_3 acted in synergism with BAP and NAA in the process of *in vitro* tuberization. BAP and NAA have shown to influence *in vitro* rhizome formation which is evident by the work of Shimasaki and Uemoto (1991) in the orchid *Cymbidium goeringii*. While, *in vitro* corms were produced in *Gloriosa superba* L. using MS medium supplemented with 17.22µM 2iP, 9.52µM ADS and 10µM ANL from dormant corm buds as explants. For corm production from non dormant corm bud explants, MS medium supplemented with 9.28µM Kin, 2.72µM ADS, and 10µM ANL were used (Sivakumar *et al*., 2003).

Estimation of colchicine by HPTLC reveals that the *in vitro* tuber produced maximum amount of colchicine followed by the *in vivo* seeds, tuber, leaf and pericarp. The present work highlights the importance of *in vitro* culture of this plant for the quantitative analysis of the phytoconstituent. The study also suggests that the biochemical mechanisms used to produce the large array of compounds are not adversely affected by *in vitro* propagation. Our observations are supported by the work of Lakshmi *et al*., (1991) who has reported the maximum occurrence of colchicine in seeds of *Gloriosa superba*. Shah *et al*., (2006) reported that HPTLC can be used to determine the drug content of marketed formulations. The development and validation of a HPTLC method for the simultaneous estimation of Atorvastatin, Calcium and Ezetimibe was carried out by using a mixture of Chloroform: benzene: methanol: acetic acid (6.0:3.0:1.0:0.1 v/v/v/v) as the mobile phase (Chaudhari *et al*., 2006). Three different methods of extraction of colchicine was studied and the concentration was quantified using high performance liquid chromatography (HPLC) in six different species of *Gloriosa* (Bharathi *et al*., 2006). Therefore, the data derived from these experiments have provided the chemical basis for the wide use of this plant as therapeutic agent for treating various ailments. Furthermore, this data may be handy in probing biochemistry of this plant in the future. The significance of the study is to highlight the importance of *in vitro* tuberization in *Gloriosa superba*. *In vitro* tubers have several advantages, such as disease free after transplanting to soil, no dormancy period, year-round cultivation is possible. The colchicine content is also high comparing to the tubers grown in *in vivo* conditions. The production of *in vitro* tubers takes short period comparing with *in vivo* tubers. The study also suggests the industries to employ *in vitro* techniques for the mass propagation and cultivation of this highly medicinal and economically valuable plant for abundant colchicine production.

ABBREVIATIONS

HPTLC- High Performance Thin Layer Chromatography, MS-Murashige and Skoog (1962), BA- N^6-benzyladenine, NAA- α-Naphthalene acetic acid, GA_3- Gibberellic acid, BAP- Benzyl amino purine,

ACKNOWLEDGEMENT

Authors are thankful to the Department of Science and Technology, New Delhi, for providing the DST FIST-Sponsored program to our Department. Authors are grateful to the University Grants Commission, New Delhi, for providing financial support through UGC Major Research Project.

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