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RESEARCH ARTICLE

HPLC Method for Quantification of Berberine in Wild and Micropropagated *Tinospora cordifolia* – An Important Ayurvedic Medicinal Plant

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ABSTRACT

The present study was attempt to established for rapid clonal propagation of valuable medicinal plant, *Tinospora cordifolia,* through *in vitro* culture using nodal explants from selected plant. And a sensitive, simple and accurate HPLC method had been developed for quantification of berberine, a isoquinoline alkaloid in dry stem of wild and micropropagated *Tinospora cordifolia* (wild) Miers. ex Hook.f. & Thoms. The chromatographic analysis was performed using pet- ether, methanol, aqueous, chloroform extracts of wild and micropropagated plant stem methanol extract, on using a solvent system, comprising of Acetonitrile: water 60:40 (V/V) as mobile phase at a flow rate of 0.5ml/min. And 265nm gives good separation of berberine at Rt 5.15min. The maximum of berberine content was observed in methanol extract of micropropagated plant stem when compared to wild test samples. The proposed HPLC method is rapid and accurate for quantitative monitoring of berberine in *Tinospora cordifolia.*

KEYWORDS

Tinospora cordifolia, Micropropagation, Berberine, HPLC

INTRODUCTION

Nature has been a source of medicinal agents for thousands of years, and an impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine. The plant-based traditional medicine systems continue to play an essential role in health care, with about 80% of the world's inhabitants relying mainly on traditional medicines for their primary health care.[1] Plant products also have an important role in the health care systems of the remaining 20%, Who reside in developed countries. Many medicinal plants species are disappearing at an alarming rate, as a result of rapid agricultural and urban development, deforestation and indiscriminate collection. If this trend continues, mankind will, forever, loss some of the most important sources of drugs. The tissue culture technique has been proved very efficient in rapid mass propagation and conservation of these important multipurpose medicinal plants.[2]

Medicinal plants play a vital role for the development of new drugs. Interest in herbal drugs is growing due to their efficiency, low toxicity and absence of side effects. Unfortunately, the quantity and quality of the safety and efficacy data on herbal medicine are far from sufficient to meet the criteria needed to

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support their use worldwide. One of the reasons is lack of adequate or accepted research methodology for evaluating herbal medicine. The search for new pharmacologically active compounds for drugs development is an important issue but not the only one, as the trends toward using standardized plant extract of high quality, safety and efficacy will continue. Standardization is as essential measurement for ensuring the quality control of the herbal drugs.[3] one of the important methods of standardization of herbal drugs is marker based standardization. It helps in adjusting the herbal products to a defined content of a constituents/s which have therapeutic activity.

Tinospora cordifolia is one such plant which is widely used in indigenous system of medicine.[4] it is a large, glabrous, succulent, deciduous climbing shrub belonging to the family menispermaceae.[5]

It is distributed throughout tropical India subcontinent, Sri Lanka and china, ascending to an altitude of 1200m. The stem of *Tinospora cordifolia* is rather succulent with long filiform fleshy aerial roots from the branches. The bark is creamy white to grey, deeply left rosette like lenticels. The leaves are membranous and cordate. The flowers are small and yellow or greenish yellow.[6]

Tinospora cordifolia known as Amrita (Guduchi) in Sanskrit, Shindilkodi in Tamil, it is a widely used in folk and ayurvedic systems of medicine. The term Amrita is attributed to its ability to impart youthfulness, vitality and longevity to the consumer.[7]

The large numbers of compounds have been isolated from the aerial parts, stem and roots of *Tinospora cordifolia*. Guduchi is widely used in Ayurvedic system of medicine "Rasayanas" to the immune system and the body resistance against infections. In modern medicine *Tinospora cordifolia* used for the treatment of general weakness, fever, dyspepsia, dysentery, gonorrhoea, urinary diseases, viral hepatitis and anaemia more recently, the immunomodulatory properties, antineoplastic activities and hypoglycemic activity have been reported.[8,9]

The present comparative analysis was aims at quantification and standardization of wild and micropropagated *Tinospora cordifolia* stem material using berberine as marker, the proposed HPLC method is simple, precise, and sensitive can be used for detection, monitoring and quantification of berberine in *Tinospora cordifolia.*

MATERIALS AND METHOD

Plant Materials

The stem plant material of *Tinospora cordifolia* was collected fresh from Vellore District area in Tamilnadu. The plant stem was authenticated by the Herbarium of Botany Directorate in National Institute of Herbal Science, Plant Anatomy Research Center, Chennai. A voucher specimen (No: TC08) was deposited in the Center.

Micropropagation of *Tinospora cordifolia*

Tinospora cordifolia, micropropagate through *in vitro* culture using nodal explants from selected plants. Best shoot induction was observed on MS basal medium supplemented with 4.36 µM KIN (Kinetin) produces 2.32± 0.1 cm length with 1.8 ± 0.1 numbers of shoots with 70 % response when compared with other Cytokinins BA (N6 – benzyladenine) and 2iP (N6 -2-isopentenyl adenine). After a month of shoot induced culture, creates a phenolic exudation problem in shoot formation, media had discoloration and explants were browning and the buds were break so, further analysis protocols were found by treating with different controlling substances from that, silver nitrate (20%) with KIN (4.36 µM) gives 100% response with 3.01 ± 1.0 cm length of shoots with 2.01 ± 0.1 numbers of shoots within 16 days of culture.

Nodal explants of *Tinospora cordifolia* were cultured on MS medium with various concentrations of BA alone and in combination with NAA (α- napthyl acetic acid) or IAA (Indole -3- acetic acid) for multiple shoot proliferation. Among the different experiments, BA (8.82 µM) alone showed better growth response and produced 4.81 ± 0.2 numbers of shoots with an average length of 3.1 ± 0.1 cm

after 20 days of culture. Small shootlet were transferred to shoot elongation medium supplemented with 8.82 µM of BA alone. An average length of 4.82 ± 0.4 cm with 4.61 ± 0.2 numbers of shoots produced, 76% of response.

The elongated shootlets transferred to half strength MS medium and 6.43 µM of IBA (Indole -3- butyric acid) with 3% sucrose produce 5.2 ± 0.2 rootlets per plant with average root length of 3.2 ± 0.1 cm after 27 days. Rooted plantlets were transplanted *ex vitro* and raised in pots containing red soil, vermiculite and farmyard manure in 1:1:1 ratio, kept under green house conditions for one month followed by their field transfer. Approximately 80% of plantlets survived.

Preparation of Extract from Wild and Micropropagated *Tinospora cordifolia*

The dried powdered stems of wild and micropropagated *Tinospora cordifolia* were allowed to pass through ss sieve (20 mesh). It was defatted by treating with petroleum ether (60-80^{o}) and then extracted at separately and then to exhaustion (soxhlet) with various solvents like methanol, aqueous and chloroform. The solvents were removed under vacuum to get solid mass and use further analysis.

Preparation of Standard Solution

A stock solution of standard berberine was prepared in 5ml volumetric flask by dissolving 1.7mg of accurate weighed berberine standard in about 3ml of solvent (acetonitrile: water) 60:40 followed by sonication for 5min. and finally making the volume up to mark with solvent.

Preparation of Sample Solution

Stock solution of samples was prepared by transferring 3.8mg of accurately weighed; pet-ether extract in 1ml solvent (acetonitrile: water) 60:40, 2.8mg of methanol extract in 1ml solvent, 2.7mg of aqueous extract in 1ml solvent and 2.0mg of chloroform extract of *Tinospora cordifolia* stem power in the volumetric flasks and then sonicated for 15min. at room temperature. The content of the flask were filtered thought whattman filter paper No.41. The filtrate was collected and use further analysis.

Chemical Materials

Acetonitrile, methanol, chloroform, petroleum ether, were used of AR grade (S.D. Fine chemicals, Baroda) and standard berberine from sigma Alrich, Bangalore.

Chromatographic Conditions

Injection volume - 20µl

Flow rate – 0.5ml/min.

Mobile phase – Acetonitrile: water (60:40)

Detection wave length – 265 nm

Mode – isocratic

Retention time (Rt) – berberine 5.15min.

RESULTS AND DISCUSSION

In the present study, the auxiliary buds on the nodal cuttings showed visible growth after five days in culture and most of them were grow into shoots within 20 days. Shoots formation was affected by the concentration of hormones used in the medium. Among the different Cytokinins (BA, KIN, and 2iP) the better result was produced only in nodal explants with KIN 4.36µM, produces 2.23 ± 0.1 cm length with 1.8 ± 0.1 numbers of shoots with 70% response when compared with other Cytokinins (Table 1). The Primitive role of KIN for shoot initiation has been documented in many other medicinal plants too, such as *Saussurea obvallata*[10] and *Holarrhena antidysenterica.*[11] After, the determination of best treatments for shoots induction. The effect of different concentration of Cytokinins (BA, KIN, and 2iP) alone and combination of BA+ NAA and BA + IAA on shoot proliferation in nodal explants of *Tinospora cordifolia* in MS medium. Among the different experiment, BA (8.82 µM) alone showed better growth response and produced 4.81± 0.2 numbers of shoots with an average length of 3.9±0.1cm after a 20 days of culture with 80% of response (Table 2). Similar observations have been reported for *Bauhinia variegata*[12] and *Holarrhena antidysenterica.*[13,14]

Table 1: Influence of Cytokinins on Shoots Induction from Nodal Explants of *Tinospora cordifolia* in 20 days Culture

BA (µM)	KIN(µM)	2iP(µM)	No. of Shoots	Shoot length (cm)	% of Response
00.44	0	0	Callus	0	0
03.21	0	0	0	0	0
06.42	0	0	0	0	0
13.31	0	0	1.0 ± 0.0^{d}	0.34 ± 0.1^{e}	30^{d}
0	00.46	0	1.2 ± 0.3^{bc}	1.34 ± 1.1^{c}	40^{bc}
0	04.36	0	1.8 ± 0.1^{a}	2.23 ± 0.1^{a}	70^{a}
0	08.46	0	1.4 ± 0.1^{b}	2.01 ± 1.1^{b}	43^{b}
0	13.94	0	1.0 ± 0.1^{d}	0.62 ± 0.3^{cd}	32^{d}
0	0	00.49	Callus	0	0
0	0	04.26	1.2 ± 0.0^{bc}	0.21 ± 0.1^{f}	20^{e}
0	0	08.42	1.4 ± 0.1^{b}	0.23 ± 0.0^{f}	21^{e}
0	0	14.76	0	0	0

Explants were cultured on MS basal media supplemented with BA, KIN and 2iP. Data were recorded after 20 days of culture. Results represent mean ± SD of six cultures, ten replicated experiments. Values denoted by different letters in each column differ significantly at $p < 0.05$.

Table 2: Effect of Different Concentration of Cytokinins on Shoot Proliferation in Nodal Explants of *Tinospora cordifolia* on MS Medium

BA (µM)	KIN(µM)	2iP(µM)	No. of shoots	Shoot length (cm)	% of Response
04.22	0	0	2.42 ± 0.1^{c}	3.6 ± 0.3^{b}	60^{b}
08.82	0	0	4.81 ± 0.2^{a}	3.9 ± 0.1^{a}	80^{a}
12.64	0	0	3.21 ± 0.0^{b}	3.4 ± 0.1^{c}	52^{b}
0	06.23	0	2.11 ± 0.1^{c}	2.5 ± 0.4^{e}	55^{cd}
0	09.46	0	2.32 ± 0.2^{c}	2.2 ± 1.0^{fg}	63^{b}
0	12.44	0	2.0 ± 0.1^{d}	2.1 ± 1.1^{g}	60^{b}
0	0	03.34	1.26 ± 0.3^{e}	2.5 ± 0.5^{e}	55^{cd}
0	0	08.68	1.32 ± 0.5^{e}	2.8 ± 0.3^{d}	54^{cd}
0	0	13.32	2.0 ± 0.1^{d}	2.1 ± 0.2^{g}	50^{e}

Explants were cultured on MS basal media supplemented with BA, KIN and 2iP. Data were recorded after 20 days of culture. Results represent mean ± SD of six cultures, ten replicated experiments. Values denoted by different letters in each column differ significantly at $p < 0.05$.

Table 3: Effect of BA and KIN in Different Concentrations on Elongation of Shoot Regenerates from Nodal Explants of *Tinospora cordifolia* on MS Medium

BA (µM)	KIN(µM)	No. of shoots	Shoot length (cm)	% of Response
2.44	4.66	3.4 ± 1.1^{b}	5.2 ± 0.0^{b}	80^{c}
6.88	8.22	4.8 ± 0.4^{a}	6.8 ± 0.1^{a}	86^{a}
8.82	12.44	3.6 ± 1.3^{b}	4.87 ± 1.1^{c}	72^{b}

Explants were cultured on MS basal media supplemented with BA and KIN. Data were recorded after 20 days of culture. Results represent mean ± SD of six cultures, ten replicated experiments. Values denoted by different letters in each column differ significantly at $p < 0.05$.

The multiple shoots obtained from our experiments were short (below 2cm) and had condensed nodes. Therefore, transfer of these shoots to shoot elongation medium contains Cytokinins (BA, KIN and 2iP) alone or combinations. The best result was produced (Table 3) with the combination treatment of BA (6.88 µM) + KIN (8.22 µM) produces 86% of response with 4.8 ± 1.4 numbers and 6.8 ± 3.1cm length of shoots produced within 20 days of cultured (Fig. 1 H, I & J). Higher concentration of KIN show increase in shoot length 4.8cm and better growth with enlargement of single leaves.

The regeneration of shoots were excised and cultured on half strength of MS supplemented with 3% sucrose and different concentration of Auxins in IAA (1.76 – 17/13 µM), IBA (1.32 – 14.70 µM) and NAA (1.34 – 16.61 µM) were used in alone. The results were provided in Table.4. Rootining was noticed in all the concentrations of Auxins used, however, maximum number of shoots rooted in 6.43 µM IBA at 85% of response (Fig. 1. K) followed by 10.21 µM which produces 80% of response when compared with other concentration of Auxins at 27 days on medium. Several authors reported that IBA was an effective Auxin in the induction of roots in different ornamental, medicinal and fruit plants like chrysanthemum[15], carnation [16], neem[17], apple.[18] After 30 days of growth, lateral roots were produced from main root. The rooted plants were transplanted *ex vitro* and raised in pots (Fig. 1. L and Fig. 1 M) containing red soil, vermiculite and farmyard manure in 1:1:1 ratio, kept under green house conditions for one month followed by their field transfer. Approximately 80% of plantlets survived (Fig. 1. N).

Figure 1: Micropropagation of *Tinospora cordifolia* in Various Stages

A. Shoot induction from nodal explants of *Tinospora cordifolia* on shoot induction medium containing silver nitrate allowing with supplementary of 4.36 µM KIN. B. Shoot induction with 4.36 µM KIN in M.S. media after 20 days. C. Second sub culturing of the growing initiation culture along with leaves. D. Elongation culture with supplementary of KIN (9.46 µM) to form single large leaf. E. Initial stage of multiplication in shoot culture medium with BA (8.82 µM). F. Multiple shoots proliferation from nodal explants on shoot induction medium. G. Multiplication of shoot in M.S. culture medium allowing with BA (8.82 µM) after 4 weeks. H. Shoot elongation on M.S. medium with BA (6.88µM) and KIN (8.22 µM). I & J. Elongated shoot supplementary with BA (6.88 µM) and KIN (8.22 µM) after 12 and 28 days respectively. K. *In vitro* rooting on Half strength M.S. medium with IBA (6.43 µM). L & M. Acclimatized plantlets in pots. N. Five month old tissue cultured *Tinospora cordifolia* plants in pot.

The current method was conducted to identifying and quantifying the berberine from wild *Tinospora cordifolia* plant stems at various different fractions and methanol extract of micropropagated plant stem. Berberine peaks from solutions of various extract of wild plant like methanol, aqueous, chloroform and methanol extract of micropropagated plant were identified by comparing their Rt values with these obtained by chromatography of the standard under the same conditions.

Table 4: Effect of Different Auxins on Rooting from *in vitro* Elongated Shoots of *Tinospora cordifolia* in Half Strength MS Medium

IAA (µM)	IBA (µM)	NAA (µM)	No. of roots	Root length (cm)	% of Response
01.76	0	0	4.3 ± 1.1^{d}	3.0 ± 0.4^{bc}	79^{c}
04.32	0	0	4.2 ± 1.1^{ef}	2.9 ± 0.3^{c}	78^{c}
08.56	0	0	4.3 ± 0.3^{d}	2.2 ± 0.1^{ef}	74^{d}
17.13	0	0	4.5 ± 0.4^{c}	3.2 ± 0.2^{a}	79^{c}
0	01.32	0	4.3 ± 1.1^{d}	2.1 ± 1.3^{g}	74^{d}
0	06.43	0	5.2 ± 0.2^{a}	3.2 ± 0.1^{a}	85^{a}
0	10.21	0	5.0 ± 1.1^{bc}	3.2 ± 0.4^{a}	80^{bc}
0	14.70	0	4.7 ± 1.0^{c}	2.3 ± 0.2^{ef}	78^{c}
0	0	01.34	3.2 ± 1.0^{g}	2.1 ± 1.0^{g}	60^{f}
0	0	05.43	4.1 ± 0.3^{f}	2.1 ± 0.1^{g}	60^{f}
0	0	10.22	4.6 ± 1.1^{c}	2.5 ± 0.0^{d}	62^{e}
0	0	16.11	3.5 ± 0.2^{g}	2.8 ± 1.0^{c}	61^{e}

Explants were cultured on half strength MS basal media supplemented with IAA, IBA, NAA and 3% of Sucrose. Data were recorded after 27 days of culture. Results represent mean ± SD of six cultures, ten replicated experiments. Values denoted by different letters in each column differ significantly at $p < 0.05$.

The peaks of Rt 5.15min. was observed in the chromatograms obtained from fractions like methanol, aqueous, chloroform and methanol extract of micropropagated plant, the chromatograms of standards and test samples are shown in Fig 2 to 6 respectively.

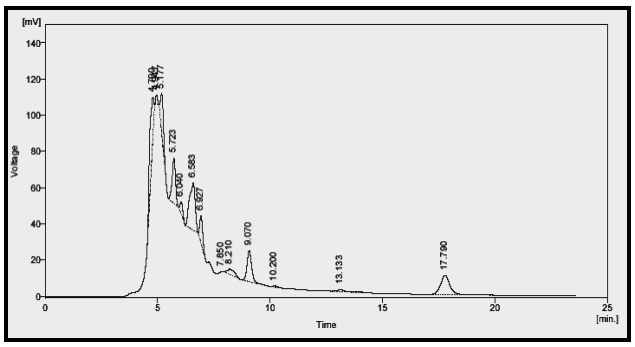

Figure 2: HPLC Chromatogram of methanol extract of wild *Tinospora cordifolia* peak at the Rt 5.15min. correspond to berberine detected at a wavelength of 265nm. (Sample -I)

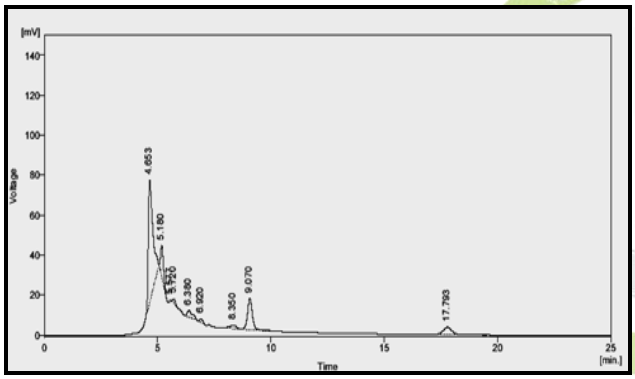

Figure 3: HPLC Chromatogram of Aqueous extract of wild *Tinospora cordifolia* peak at the Rt 5.15min. correspond to berberine detected at a wavelength of 265nm. (Sample -II)

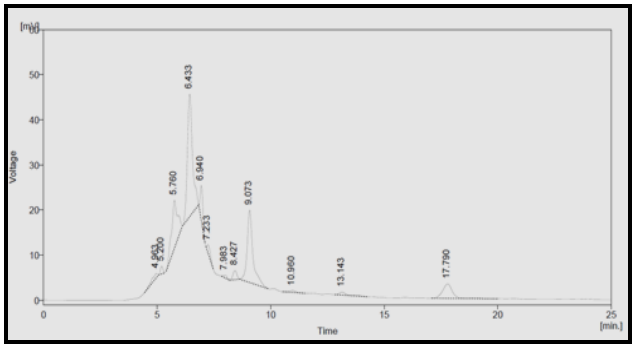

Figure 4: HPLC Chromatogram of Chloroform extract of wild *Tinospora cordifolia* Peak at the Rt 5.20 min. correspond to berberine detected at a wavelength of 265nm. (Sample -III)

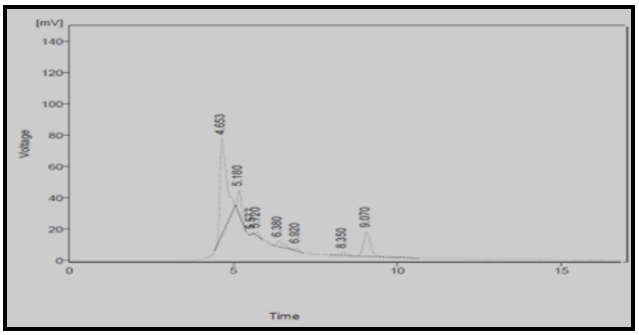

Figure 5: HPLC Chromatogram of methanol extract of micropropagated *Tinospora cordifolia peak at the* Rt 5.18min. correspond to berberine detected at a wavelength of 265nm. (Sample - IV)

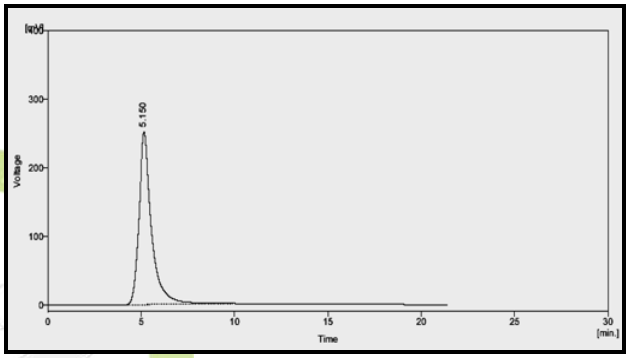

Figure 6: Standard berberine peak at the Rt 5.15min. detected at a wavelength of 265 nm

The berberine content of fractions of sample – I (methanol), sample –II (aqueous), sample –III (chloroform), sample –IV (methanol extract of micropropagated plant) was 0.223% (W/W), 0.150% (W/W), 0.020% (W/W), and 1.2 % (W/W) respectively. In wild plant, more amount of berberine was present in methanol extract when compared to other fractions, but when compared to wild the micropropagated plant had more amount of berberine present.

CONCLUSION

As a result of the over-exploitation of plant material from natural stands for traditional medicinal purposes, the standardization of the regeneration protocols for *Tinospora cordifolia* medicinal plant was becoming important. The protocols will facilitate conservation of the species and could also serve as an alternative source of materials for use. The regeneration protocol described herein would benefit the conservation of *Tinospora cordifolia*, which was

extensively used in traditional medicine. The HPLC technique is quite, simple, accurate, precise, reproducible and sensitive. This method can be used for routing analysis of berberine in cured drugs and prepared formulations and also it's used for standardization and quality control of herbal products of traditional medicine containing *Tinospora cordifolia* as an ingredient can be explored.

REFERENCES

1. Dubey, N. K,, Kumar, R., Tripathi, P. (2004). Global promotion of herbal medicine: India's opportunity. *Current science*, *86*, 37-41.
2. Fay, M. F. (1992). Conservation of rare and endangered plants using in vitro methods. *In Vitro Cellular & Developmental Biology-Plant*, *28(1)*, 1-4.
3. Liang, Y. Z., Xie, P., & Chan, K. (2004). Quality control of herbal medicines. *Journal of Chromatography B*, *812(1)*, 53-70.
4. Kirtikar, K. R., & Basu, B. D. (1933). Indian medicinal plants. *Ind. Med. Plants*. 77.
5. Anonymous. Wealth of India; Raw materials, Volume X. New Delhi: CSIR; 1976.
6. Nadkarni, K. M., Nadkarni, A. k. Materia Medica, Vol – I. 3rd ed. Mumbai: M/S. Popular Prakasan, Pvt. Ltd; 1976. P. 1221.
7. Sivarajan, V. V., Balachandean, I. *Ayurvedic Drugs and their plants sources*. New Delhi: Oxford and IBH Publishing Co. Pvt. Ltd; 1999.
8. Singh, S. S., Pandey, S. C., Srivastava, S., Gupta, V. S., Patro, B., & Ghosh, A. C. (2003). Chemistry and medicinal properties of Tinospora cordifolia (Guduchi). *Indian Journal of Pharmacology*, *35(2)*, 83-91.
9. Sivakumar, V., Dhana, Rajan M. S. (2011). Antioxidant activity of *Tinospora cordifolia* experimental diabetes. *IJPSR, 2(3)*, 608, 93-95.
10. Joshi, M., & Dhar, U. (2003). In vitro propagation of Saussurea obvallata (DC.) Edgew.—an endangered ethnoreligious medicinal herb of Himalaya. *Plant Cell Reports*, *21(10)*, 933-939.
11. Raha, S., & Roy, S. C. (2001). In vitro plant regeneration in Holarrhena antidysenterica wall, through high-frequency axillary shoot prolieferation. *In Vitro Cellular & Developmental Biology-Plant*, *37*(2), 232-236.
12. Mathur, J., & Mukunthakumar, S. (1992). Micropropagation of Bauhinia variegata and Parkinsonia aculeata from nodal explants of mature trees. *Plant cell, tissue and organ culture*, *28(1)*, 119-121.
13. Kumar, R., Sharma, K., & Agrawal, V. (2005). In vitro clonal propagation of Holarrhena antidysenterica (L.) Wall. through nodal explants from mature trees. *In Vitro Cellular & Developmental Biology-Plant*, *41*(2), 137-144.
14. Martin, G., Geetha, S. P., Raghu, A. V., Balachandran, I., & Ravindran, P. N. (2005). Micropropagation of Holarrhena pubescens. *Journal of Tropical Medicinal Plants*, *6(1)*, 111.
15. Hoque, M. I., Jahan, M. T., & Sarker, R. H. (1998). In vitro shoot regeneration and ex vitro rooting in Chrysanthemum morifolium Ramat. *Plant Tissue Cult*, *8(1)*, 157-164.
16. Shibli, A. R., Ajlouni, M. M., Shatnawi, A. M., & Abdullah, A. B. (1999). An effective method for in vitro production of disease-free carnation (Dianthus caryophyllus cv. Balady). *Plant Tissue Cult*, *9*, 159-166.
17. Sarker, R. H., Islam, M. R., & Hoque, M. I. (1997). *In vitro* propagation of neem (*Azadirachta indica* A. Juss.) Plant from seedling explants. *Plant tissue Cult.*, *7*, 125-133.
18. Caboni, E., and Tonelli, M. G. (1999). Effect of 1, 2- benzisoxazole -3-acetic acid on adventitious shoot regeneration and *in vitro* rooting in apple. *Plant Cell Rep.*, *18*, 985-988.

