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CHROMATOGRAPHIC DETERMINATION OF TYLOPHORINE CONTENT IN *IN VITRO* RAISED PLANTS OF TYLOPHORA INDICA- AN ENDANGERED MEDICINAL PLANT

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ABSTRACT: Introduction: *Tylophora indica* member of family Asclepiadaceae contains therapeutically important alkaloids such as tylophorine ($C_{24}H_{27}O_4N$), tylophorinine ($C_{23}H_{25}O_4N$), and tylophrinidine ($C_{22}H_{22}O_4N$). The plant is valued for its medicinal importance and hence, it becomes essential to adopt methods to explore its potential for metabolite content. **Methodology**: *De novo* adventitious shoot formation from leaf explants occurred on Murashige and Skoog's medium with 8.8 µM 6- benzyladenine in combination with adenine sulphate (1.35 µM) where greatest number of 55 ± 2.88 shoots per culture were produced. For establishing callus cultures, leaf explants were cultured on 29.4 µM naphthalene acetic acid and 4.65 µM kinetin medium. Caulogenesis (40.0 ± 1.45 shoots per culture) from green compact callus occurred on 8.8 µM benzyladenine. The best *in vitro* rooting responsewas recorded when half strength of Murashige Skoog's media was used. Plants hardened under open field conditions recorded 90% success rate. Leaves from directly raised and callus regenerated plants were used for tylophorine estimation. High performance thin layer chromatography was employed for purification of tylophorine using solvent system comprising of toluene: chloroform: ethanol: ammonia (4: 3.5: 1.5: drop). **Results**: Quantitative analysis of all the samples showed that maximum amount of tylophorine (80µg/ml) was detected in leaf callus-regenerated plants of *T.indica* whereas, directly obtained plants contained 71 µg/ml of tylophorine. **Conclusion**: This is a first comparative report where plants raised directly from the explants and through callus cultures were analyzed for tylophorine content using an advanced approach of High performance thin layer chromatography.

KEYWORDS: *Tylophora indica,* tylophorine, high performance thin layer chromatography, de novo, *in vitro*

Abbreviations: BA: benzyladenine, NAA: naphthalene acetic acid, K: kinetin, HPTLC: high performance thin layer chromatography, MS: Murashige and Skoog's medium.

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1. INTRODUCTION

Medicinal plants are important source of bioactive compounds, which are used as pharmaceuticals, agrochemicals, fragrance ingredients and food additives, medicinal and other dietary supplements [1]. Out of nearly 50 members of the family Asclepiadaceae, which are well distributed over Africa, Asia, Australia and the Pacific Islands, only 23 have been reported to contain phenanthroindolizidine alkaloids and among these most of the alkaloids have been obtained from Tylophora indica [2]. These alkaloids like tylophorine (C24H27NO 4), tylophrinidine (C23H25O4N) and anticancerous tylophorinidine (C22H22O4N) exhibited various pharmacological functions like hepatoprotective, antiallergic, immunomodulatory, diuretic, anti-asthmatic and antitumor activity [3,4]. The plant has drawn immense attraction in traditional medicines all over the world since these are safe, cheap and with no side effects. So, there is an ever-increasing inclination towards the identification, isolation, purification and characterization of active ingredients in crude extracts of these medicinal plants by various analytical methods. In view of the growing world population, increasing anthropogenic activities and rapidly eroding natural ecosystems, the natural habitats for these medicinal plant species are dwindling. The rising demand of plant-based drugs is creating heavy pressure on selected, high valued medicinal plants populations due to over harvesting. Thus, mass propagation of disease-free planting material is the general problem. To cope up with this alarming situation, the recent advances in Biotechnology especially Plant Tissue Culture has come up as a boon. The above mentioned causes prompted us to find an alternate method of rapid micro propagation of this plant species along with metabolite extraction to explore its medicinal properties.

2. MATERIALS AND METHODS

In vitro culturing:

For micropropagation, Murashige and Skoog's (1962) [5] medium (MS) was used. The media stocks were prepared in distilled water and refrigerated at 4ºC. Stock solutions of various auxins (like NAA) and cytokinins (like K, BA) were also prepared and all these were stored at 4º C. The requisite amount of salts, vitamins and growth regulators from respective stock solutions were added into a conical flask. As a carbon source, 2% sucrose (Hi-Media) was used unless otherwise specified and the final volume was made up to the required level with distilled water. The pH was

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adjusted to 5.7 with either 0.1N HCl or 0.1 N NaOH and medium was gelled with 0.8 – 1.0% w/v agar Type-I (Hi-media). 75 ml and 25 ml medium were dispensed in culture bottles and test tubes respectively. Bottles were closed with autoclavable plastic caps whereas tubes were plugged with non-absorbent cotton wrapped in muslin cloth and were steam sterilized by autoclaving at 121°C and 1.1 kg/cm^2 for 20 minutes. Leaf explants were washed under running tap water for 30 minutes to remove all adhering dust particles and microbes on the surface, followed by their immersion in teepol solution 1% (v/v) for 5 minutes followed by repetitive washings with tap water. Thereafter, the explants were treated with bavistin (0.1% w/v) for 10-12 minutes followed by repeated washings with water. Final sterilization was carried out in laminar flow hood by treating explants with 0.05 -0.1% (w/v) aqueous solution of mercuric chloride for 3-6 minutes (depending upon the explant) followed by thorough washings with sterile distilled water. On both the ends, a few mm portions of explants exposed to the sterilant were removed with the help of a sharp sterile secateur. The explants were then cultured on variously supplemented MS medium. Leaf segments (4-5 mm) in size were cultured on variously supplemented MS medium for *de novo* adventitious shoot formation directly from the explants. MS medium supplemented with 6- benzyladenine (4.4- 8.8 µM) either alone or in combination with adenine sulphate (1.35 µM) was used for producing greatest number of shoots/explant. For establishing callus cultures, NAA and 2, 4-D in combination with K were used. A combination of NAA (9.0- 29.4 µM) along with different concentrations of K (2.2- 4.65 µM) were used for inducing callus from the cut ends or along the entire surface of the explant. To obtain high frequency shoot differentiation, calli were transferred to different concentrations of BA (8.8- 9.84 µM) or K (2.2- 4.65 µM). Microshoots were rooted on half strength basal MS medium for root induction. All the inoculated cultures were incubated in growth room under controlled conditions at a temperature of 25.0 ± 2° C with a photoperiod of 16-hours per day. Illumination was provided by cool white fluorescent tubes (Philips India Limited, Mumbai) at 50 µmol/m^2/s^1. Plantlets after acclimatization through various weaning stages were shifted to open field conditions.

Preparation and extraction of plant sample:

Leaf plant parts were collected from 2 yrs old healthy *in vitro* raised plants of *Tylophora indica.* They were washed thoroughly under running tap water to remove impurities and stubborn dust particles. Explants were shade dried at room temperature, Powderised to a particle size of 1mm using pestle and mortar, packed in airtight containers and stored at room temperature. Powderised explant samples were then processed thrice in cold with 100 ml of 1% acetic acid in methanol, following an extraction protocol of Rao and Brook, 1970[6]. The extracts were then fractionated thrice using ethyl acetate: HCl (1:1) and the final filtrate thus, obtained was analyzed on high performance thin layer chromatography (HPTLC).

3. RESULTS AND DISCUSSION

In Vitro Plant Regeneration

For *de novo* adventitious shoot formation, leaf explants were cultured on MS medium supplemented with 8.8 µM 6- benzyladenine either alone or in combination with adenine sulphate (1.35 µM). The caulogenic effect of BA as described in the present study is in consonance with other reports as well. Studies showed multiple shoot formation directly from leaf explants of *Tylophora indica* on MS medium supplemented with 22 µM BA with 0.65 µM adenine sulphate [7]. Effect of BA either alone or in combination with auxins has been demonstrated in many medicinal plants of family Asclepiadaceae. Nodular meristemoids differentiated from the cut ends and from the abaxial and adaxial surface of leaf lamina after 8 days (Fig 1a) and within 3 weeks the entire surface was covered with these nodular meristemoids that eventually grew into green leafy shoots. Figure 1b shows differentiation of a few shoots formed from nodular meristemoids after 4 weeks. Initially fewer shoots were formed from these meristemoids but in due course, these multiplied further forming many shoots (Fig. 1c). The highest regeneration frequency (85%) and maximum number of shoots (55 ± 2.88 per culture) were achieved on BA (8.8 µM) and adenine sulphate (1.35 µM). Repeated sub culturing accelerated the formation of shoots without any decline in their proliferation up to 3 subculture passages. Among different auxins (NAA, 2, 4-D, IBA and IAA) tested for establishing callus cultures, NAA and 2, 4-D in combination with K proved to be most effective. Good callus growth from the explants occurred on NAA (9.0-24.7 µM) supplemented medium, however, the addition of K enhanced the callus production many folds. Optimal callusing occurred on NAA (29.4 µM) and K (4.65 µM) medium where 98 % of the explants callused either at the cut ends or along the entire surface (Fig. 2 a). The callus grew further and within 30 days, the entire explant turned into a mass of green and compact callus. Callus when subcultured on the same medium proliferated further and showed sustained growth. To obtain high frequency shoot differentiation, calli were transferred to different concentrations of BA or K used either alone and in combination with each other. Best results were obtained on 8.8- 9.84 µM BA where 86 % of the cultures resulted in excellent shoot induction (40.0 ± 1.45 per culture) upto 3-4 subcultures. Initially 6-7 shoot initials differentiated from the callus after 2 weeks of culturing (Fig. 2 b) which grew further and developed many leaves. Figures 2 C show the formation of numerous healthy green leafy shoots after 4 weeks. There was a steady increase in the number of shoots per culture on subsequent subculturing. A perusal of literature reveals similar observations, where high shoot regeneration from leaf and stem calli in *T. indica* were observed when callus was subcultured on MS medium containing either K or BA, with best results in terms of number and growth of regenerants on 5 µM K[8]. High frequency shoot induction (26.8 ± 0.97 shoots/culture) were observed when calli were transferred to MS medium containing BA (5 µM) alone [9]. The

stimulatory role of cytokinins BA or K for satisfactory shoot bud organogenesis and plantlet production was also advocated in a number of other medicinal plants. For root induction, individual regenerated healthy shoots (5-6 cm) were carefully rescued from culture vessels and transferred onto to half strength MS medium, full strength MS medium and MS medium supplemented with different concentrations of IAA, NAA & IBA. Best root initiation, however, occurred on half strength MS medium where 4-6 healthy roots emerged in 90% of the cultures after 10 days (Fig. 3 a). The roots were long, white and branched. Beneficial effects of low salt MS medium for root initiation *in vitro* grown shoots have also been reported by other systems like *Adhatoda vasica* [10], *Plumbagorosea* [11] and *Grammatophyllum scriptum* [12]. Rooted plantlets were transferred successfully to the field conditions through successive hardening stages. Firstly, the plantlets were acclimatized on moist cotton for 12-15 days kept under growth room conditions (Fig. 3 b). Plantlets kept on moist cotton registered increase in shoot and root length, increase in the number of leaves and also exhibited better survival percentage in the subsequent weaning process. This step of hardening is well supported by observations from literature, where hardened rooted plantlets of *Saccharum officinarum*were hardened on moist cotton in open test tubes before their transfer to the soil [13]. Thereafter, the plantlets were shifted to poly bags containing the same potting mixture and were kept in growth room for another 2 weeks (Fig. 3c). Plantlets were watered periodically and monitored. The hardened plantlets in plastic bags were then transferred to green house for another 2 weeks before their final transfer to full sunlight outdoor (Fig. 3d). By this time, plants have become sturdy, developed an efficient root system, formed new leaves and became photosynthetically active.

Extraction and Estimation Of Tylophorine

Plants of *T.indica* were obtained directly from the leaf explants as well as from the calli obtained from them on variously supplemented Murashige and Skoog's medium and were successfully established in the field. Thereafter, the leaves were collected from twelve months old plants for the quantification of tylophorine. The excised leaves were powdered and processed using optimized extraction protocol and the final extract obtained was analyzed using HPTLC. A perusal of literature revealed that two alkaloids namely tylophorine and tylophorinine were also isolated from *Tylophora asthmatica* (syn. *T. indica*), followed by fractional crystallization of their mixed salts [14]. Similar studies for isolation of alkaloid tylocrebrine from *Tylophora crebriflora* was also carried out [15]. Chemical examination of *Tylophora mollissima* yielded caffeine as major alkaloid and tylophorine and tylophorinine as minor alkaloids using techniques like ultraviolet, infrared and mass spectroscopy [16]. Tylophorine, tylophorinine, tylophorinidine, septicine and isotylocrebrine were also isolated from *T. asthmatica* by chromatography on alumina [17]. Samples along with the standard tylophorine were loaded in separate tracks on pre coated silica gel fluorescent (5X5cm) plate. Track I contains standard tylophorine sample while track II was loaded with samples from

plants regenerated directly without any intervening callus stage whereas track III contains samples from plants regenerated from leaf callus (Fig. 4 A-C). The plate was developed in solvent system comprising of toluene: chloroform: ethanol: ammonia (4: 3.5: 1.5: drop) and scanned in scanner III at 258 nm. On comparison of Rf values of standard tylophorine (Rf 0.68) with sample peaks it was observed that tylophorine peak (Rf 0.68) was present in both the samples. Banding pattern of the plate was observed under UV scan at 254 and 366 nm, which clearly showed tylophorine bands in both the test samples (Fig. 4 D and E). Quantitative analysis of all the samples showed that maximum amount of tylophorine (80 µg/ml) was detected in plants regenerated from leaf callus whereas plants obtained directly without any intervening callus stage contained 71 µg/ml of tylophorine (Table 1).

Table 1. Estimation of tylophorine (µg/ml) in different leaf samples of *Tylophora indica.*

Track ID	Solvent System	Peak	End Position (Rf)	Area (AU)	Area %	Tylophorin(µg/ml)
Fig. 4 Track I (A) Standard	Toluene: chloroform: ethanol: ammonia (4:3.5:1.5: drop)	2	0.68	1246.6	49.00	------
Fig.4 Track II (B)	Toluene: chloroform: ethanol: ammonia (4:3.5:1.5: drop)	3	0.68	1099.6	69.00	**71**
Fig. 4 Track III (C)	Toluene: chloroform: ethanol: ammonia (4:3.5:1.5: drop)	7	0.68	9888.1	80.00	**80**

4. CONCLUSION

1. The present work is an attempt to investigate the dedifferentiation and redifferentiation responses of cells of various organs of*Tylophora indica* to varied and diverse chemical milieu.
2. *Tylophora indica* exhibited high degree of propensity for plant regeneration through different methods of micropropagation viz. forced axillary branching, *de novo* adventitious shoot formation.
3. Comparative study of secondary metabolites showed that plants regenerated from leaf callus contained higher amount of tylophorine.

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Different stages of *in vitro* plant regeneration in *Tylophora indica*

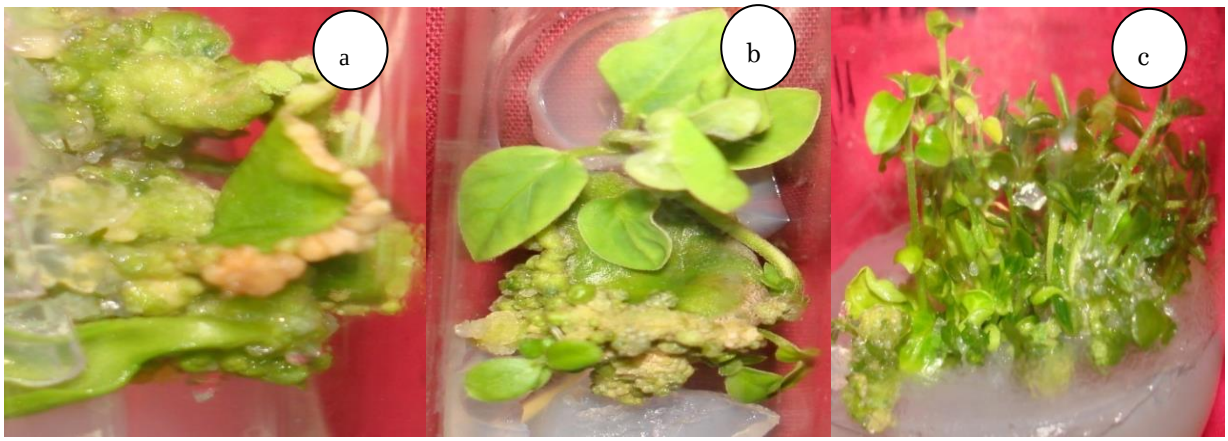

Fig.1: *De novo* adventitious shoot formation from leaf explants of *T.indica* on BAP (8.8 µM) + adenine sulphate (1.35 µM) supplemented medium. a) Formation of nodular meristemoids from leaf surface after 8 days of culturing. b) Adventitious shoot formation from nodular meristemoids after 4 weeks. c) Prolific shoot differentiation from nodular meristemoids on subsequent subculturing.

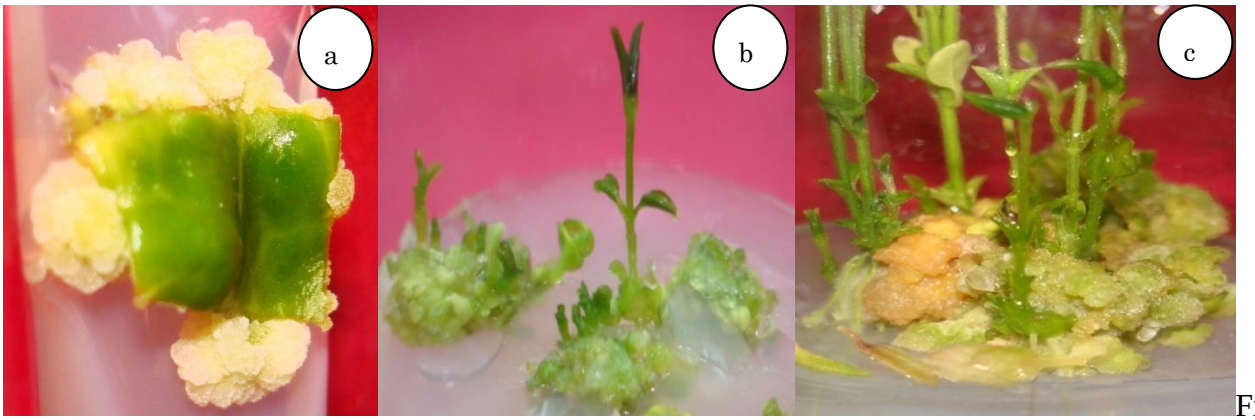

Fig. 2: Callus induction and organogenesis from leaf callus a) Actively growing callus on MS medium supplemented with NAA (29.4 µM) + K (4.65) b)Differentiation of a few shoots from leaf callus on 9.84 µM BA c) Formation of multiple shoots after 4 weeks.

Fig.3:Rooting of microshoots and acclimatization of plantlets. a) Healthy thick long roots formed on half strength basal MS medium. b) Plantlet transferred to moist cotton for initial acclimatization.

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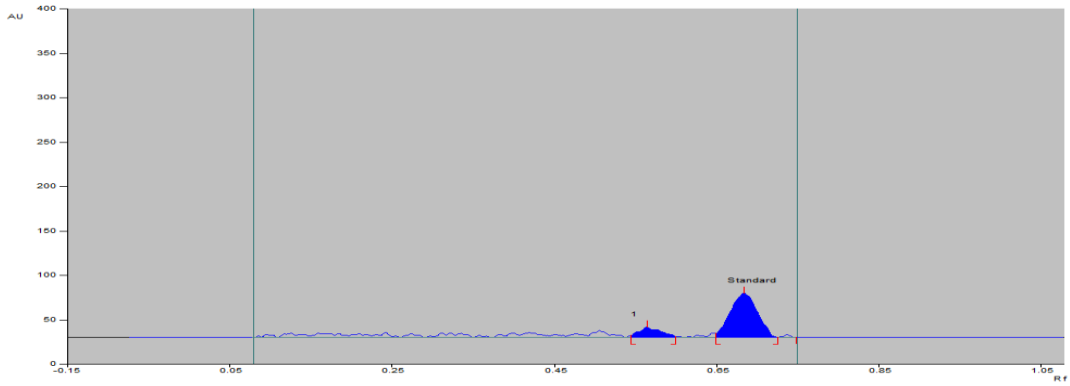
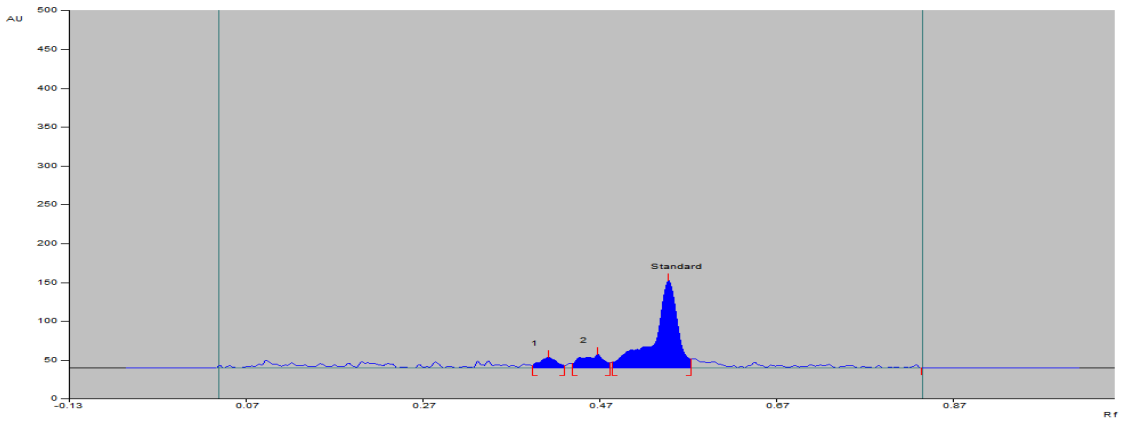
c) Plantlets in plastic cups containing potting mix d) Hardened plantlets in green house.

Fig. 4. HPTLC chromatogram of tylophorine extracted from leaf explants of *in vitro* raised plants of *T.indica*

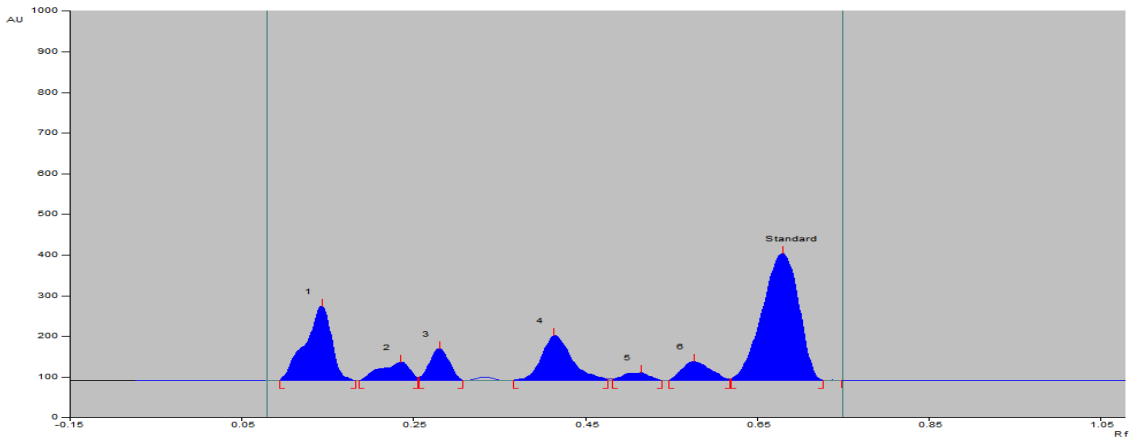
A.Track I loaded with standard tylophorine.



B.Track II loaded with leaf extract of plants regenerated directly without intervening callus stage.



C.Track III loaded with leaf extract of leaf callus-regenerated plants.



D.UV visuals at 254 nm showing bands of tylophorine (arrowhead) in both the samples.

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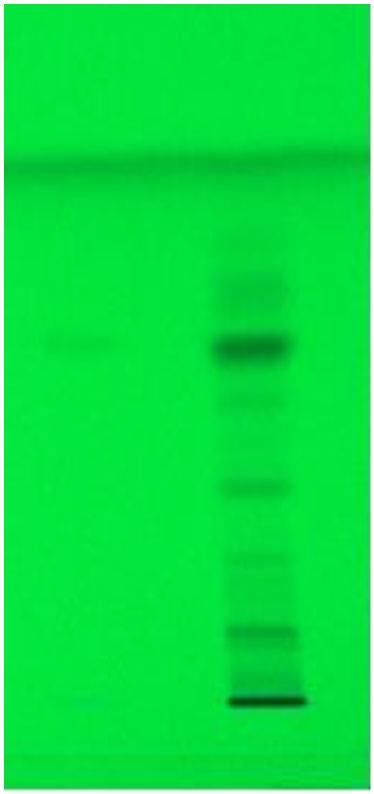
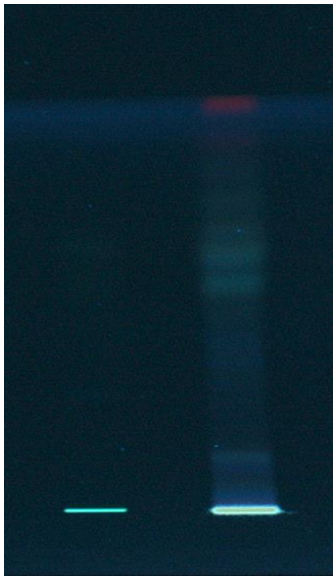


E.UV visuals at 366 nm showing bands of tylophorine in both the samples.



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CONFLICT OF INTEREST

The authors declare that no competing financial interests exist.

REFERENCES

1. Chaudhuri KN, Das S, Bandyopadhyay M, Zalar A, Kollmann A, Jha S, Tepfer D. Transgenic mimicry of pathogen attack stimulates growth and secondary metabolite accumulation. Transgenic Research. 2009; 18: 121–134.
2. Ali M, Ansari SH, Qadry JS. Rare phenathroindolozidine alkaloids and a substituted phenathreneTyloindane from *T. indica*. J. Natural Products. 1991; 54(5): 1271-1278.
3. Geller. E. The indolizidine alkaloids. J. Natural Products. 1982; 45:50–73.
4. Faisal M, Anis M. Rapid mass propagation of *Tylophora indica* Merrill via leaf callus culture. Plant Cell Tissue Organ Culture. 2003; 75:125-129.
5. Murashige T, Skoog F. A revised medium for rapid growth and bioassay with tobacco tissue cultures. PhysiologiaPlantarum. 1962; 15: 473-497.
6. Rao VK, Brook P. Alkaloids of *Tylophora indica* and *Tylophora dalzellii.* US Patent. 1970; 3:497-593.
7. Bera TK, Roy SC. Micropropagation of *Tylophora indica* by multiple shoot formation from mature leaf explant without callus intervention. Bot. Bull. Academia. 1993; 34: 83-87.
8. Faisal M, Anis M. *In vitro* regeneration and plant establishment of *Tylophora indica*: Petiole callus culture. *In vitro*Cellular and Development Biology- Plant. 2005; 41: 511-515.
9. Sahai A, Shahzad A, Anis M. High frequency plant production via shoot organogenesis and somatic embryogenesis from callus in *Tylophora indica*- An endangered plant species. Turkish Journal of Botany. 2010; 34: 11-20.
10. Nath S, Buragohain AK. Establishment of callus and cell suspension cultures of *Centellaasiatica.* BiologiaPlantarum. 2005; 3: 411-413.
11. Gopalakrishnan M, Janarthananm B, Lakshmi Sai G, Sekar T. Plant Regeneration from Leaf Explants of *Plumbagorosea*L. Plant Tissue Culture and Biotechnology.2009; 1: 79-87.
12. Abbas B, Listyorini FH, Amriati B. *In vitro* seeds germination and plantlets development of *Grammatophyllum scriptum*Lindl. (Orchidaceae). International Research Journal of Plant Science. 2011; 2, 5: 154-159.
13. Gill NK, Gill R, Gosal SS. Factorsenhancingsomaticembryogenesis and plantregeneration in sugarcane (*Saccharum offinarum*L.). Ind. J. Biotechnology. 2004; 3: 119-123.

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14. Rathnagiriswaran AN, Venkatachalam K. The chemical examination of *Tylophora asthmatica* and isolation of the alkaloids tylophorine and tylophorinine. Ind. J. Medical Research.1935; 22: 433.
15. Gellert ETR, Govindachari MV, Lakshmikantham IS, Ragade R, Rudzats N, Viswanathan J. The alkaloids of *Tylophora crebriflora*: structure and synthesis of tylocrebrine, a new phenanthroindolizidine alkaloid J. Chemical Society.1962; 1008-1014.
16. Vishwanathan N, Pai BR. Alkaloids of *Tylophora mollissima*J. Nat. Products. 1985; 48, 6: 997-998.
17. Govindachari TR. Five decades in the study of natural products. Proceedings of the Indian Academy of Science. 2012; 3: 114.

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