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Full Length Research Paper

Extraction of tylophorine from *in vitro* raised plants of *Tylophora indica*

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A method for mass propagation of *Tylophora indica* (Burm. f.) Merill. (Asclepiadaceae) from leaf explants and extraction of tylophorine was developed. Explants cultured on 9.0 µM α-naphthalene acetic acid (NAA) and 4.65 µM kinetin (K) resulted in callus formation under dark conditions after 7 to 8 days of culturing, whereas formation of nodular meristemoids was observed on 8.8 µM 6-benzyladenine (BA). Eventually, both the cultures developed green leafy shoots on their transfer to 9.84 µM 6-benzyladenine shoot inducing medium. Microshoots thus formed were cultured on basal MS root inducing medium, which resulted in the formation of long healthy roots within 10 to 12 days. Plantlets were successfully hardened and transferred to field conditions. Tylophorine was extracted from the leaves of regenerated plants using organic solvents such as like hexane, chloroform and dichloromethane and separated on high performance thin layer chromatography (HPTLC) using toluene: chloroform: ethanol: ammonia (4:3.5:1.5) as mobile phase. Amount of tylophorine obtained was 80 and 71 µg/ml from callus raised and directly cultured *in vitro* plants respectively.

Key words: *Tylophora indica*, high performance thin layer chromatography, tylophorine, 6-benzyladenine, α-naphthalene acetic acid.

INTRODUCTION

Tylophora indica (Burm. f.) Merill. (Asclepiadaceae) commonly known as “Antmool” is an important medicinal plant, traditionally used as a folk remedy in treatment of bronchial asthma, bronchitis, rheumatism, allergies and inflammation. The roots and leaves contain 0.2 to 0.46% therapeutically important alkaloids tylophorine, tylophorinine and tylophrinidine. Major alkaloid tylophorine has immunosuppressive, anti inflammatory (Gopalakrishnan et al., 1979), anti-tumor (Donaldson et al., 1968), stimulant of adrenal cortex (Udupa et al., 1991) and anti-amoebic (Bhutani et al., 1985) properties. Prior to this Gujrati et al. (2007) reported the hepatoprotective activity of alcoholic and aqueous extracts of leaves of *T. indica*. A process for extracting biologically active alkaloids from *T. indica* and *Tylophora dalzelli* comprising of acid extraction followed by solvent extraction using methanol, ethyl acetate, chloroform was provided by Rao and Brook, (1970). Similar studies for isolation of alkaloid tylocrebrine from *Tylophora crebriflora* were carried by Gellert et al. (1962). Viswanathan and Pai (1985) reported chemical examination of *Tylophora mollissima* and yielded caffeine as major alkaloid and tylophorine and tylophorinine as minor alkaloids using techniques like ultra violet, infrared and mass spectroscopy. Ratnagiriswaran and Venkatachalam (1935) had isolated two alkaloids from the plant *Tylophora asthmatica* (syn. *T. indica*), tylophorine and tylophorinine which were separated by fractional crystallization of the mixed salts. Similarly Govindachari (2002) isolated tylophorine, tylophorinine, tylophorinidine, septicine and isotylocrebrine by chromatography on alumina and reported the structure of tylophorinine and tylophorinidine with X- ray study. Due to its medicinal importance the plant is over exploited, therefore present invention embodies a method for the mass propagation of *T. indica* followed by extraction, separation and purification of alkaloid tylophorine.

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MATERIALS AND METHODS

Plant material and explant sterilization

Leaves were collected from mature field grown healthy plant of *T. indica*, maintained in green house at STEP, Thapar University Campus, Patiala and washed to remove all adhering dust particles and microbes with running tap water for half an hour followed by 1% (v/v) Teepol for 5 min. Surface sterilization was performed in 0.1% (w/v) aqueous solution of $HgCl_2$ for 2 to 3 min followed by 4 to 5 rinses in sterile double distilled H_2O.

Culture media and growth conditions

Culture media and growth conditions was performed as described by Murashige and Skoog's, 1962 (BMS) medium which was supplemented with 2% sucrose and 0.8 to 1% agar was used for all the experiments. The pH of the medium was adjusted to 5.6 ± 2.0 before the addition of agar. The media was autoclaved at 121°C for 15 min and was poured in tissue culture bottles. Cultures were maintained in growth room at a temperature of 25 ± 2°C, with intensity of illumination of 3500 lux for 16 h a day provided by cool white day light fluorescent tube lamps.

In vitro plant regeneration

Leaf explants were excised (3 to 5 mm in length) and planted on Basal Murashige and Skoog's medium, supplemented with different growth regulators such as NAA, K, BA and IBA (indole 3-butyric acid) either alone or in conjunction, with 20 replicates in each combination. Calli formed were subcultured after every 4 weeks. Synergistic action of NAA (7.35 to 29.4 µM) with K (4.65 to 18.6 µM) was studied for callus induction, whereas direct adventitious shoot formation was observed on BA (4.4 to 17.6 µM) either alone or in combination with adenine sulphate (2.71 to 10.84 µM). Microshoots thus formed were rooted on root inducing media and were hardened in soil and vermicompost potting mixes.

Extraction from dried leaves of *in vitro* raised plants

Leaves were collected from healthy mature, callus and directly raised in *vitro* plants cultured plants of *T. indica* grown and maintained in green house at STEP, Thapar University campus and washed under running tap water, dried and grounded to fine powder using mortar and pastle. Powder was washed twice with hexane to remove oil soluble components and was soaked in ethyl acetate overnight to extract components in the acetate layer. Extract was further filtered and pH was adjusted to 3 to 4 with HCl. Filtrate was diluted with 100 ml distilled water and concentrated in flash evaporator at 55 to 60°C to half of its volume. Filtrate was further washed thrice with dichloromethane and pH was adjusted in the range of 11 to13 with saturated NaOH. The extract was concentrated using flash evaporator and resuspended in chloroform for high performance thin layer chromatography (HPTLC) analysis.

Separation and recovery of tylophorine

Chromatographic separation of chloroform extract was realized using HPTLC purchased from CAMAG, Switzerland. Plant extract was applied with 100 µl syringe on pre coated silica gel 60F254 HPTLC plates (10 x 10 cm) with band length of 8 mm and track separation of 12 mm using Linomat V applicating device and nitrogen as spray agent. The chromatograph was developed in twin trough chamber using solvent system of toluene: chloroform: ethanol: ammonia (4:3.5:1.5: drop) and scanned in scanner III at 258 nm wavelength using lamp in absorption mode with spectrum scan speed of 100 nm/s. Apart from quantitative analysis, qualitative analysis was done to determine the concentration of tylophorine in the sample using standard tylophorine procured from Alexis Biochemicals. Stock solution for standard (tylophorine) was prepared by dissolving 0.5 mg standard in 0.5 ml of 100% ethanol. Concentration of tylophorine was calculated from the following formula:

Concentration (µg/ml) = Area of peak of std in test sample X Conc. of std / Area of std peak

RESULTS

Callus initiation and differentiation of shoots

Callus was induced from the abaxial, adaxial and cut surfaces of leaf lamina on BMS medium supplemented with different hormones. Callusing was observed on different concentrations of NAA (9.0 µM) with K (4.65 µM) where callusing initiated within 7 to 8 days in almost 100% cultures and the callus thus formed was light green, friable and capable of sustained growth (Figures 1a and b).

A comparative study conducted on the growth rate of leaf callus on above said combination under light and dark conditions (recorded in terms of percentage increase/day) clearly depicts that the rate of formation of callus was better under dark conditions where callusing started within 5 to 6 days which is otherwise 7 to 8 days under light conditions (Table 1).

Differentiation of shoots was observed at different concentrations of BAP (4.4 to 19.68 µM), however, best identified medium was BAP (9.84 µM) where callus showed shoot proliferation within 10 to 12 days after culturing (Figure 1c). Initially 7to 8 shoots proliferated but after frequent subculturing number of shoots increased to 35 to 40/explants.

Direct adventitious shoot induction

Differentiation of nodular meristemoids from cut ends and abaxial and adaxial surface of leaf lamina was also observed when cultured on BAP (4.4 to 17.6 µM) either alone or in combination with Adenine sulphate (2.71 to 10.84 µM), however best formation and growth of meristemoids was obtained on BAP (8.8 µM) after 10 to 12 days (Figure 1d). These nodular meristemoids cover the whole surface of explants within 3 weeks of inoculation. Eventually nodular meristemoids developed in about 70% of the cultures into green leafy shoots on same medium (Figure 1e). Initially fewer shoots were formed which latter developed into healthy 35 to 40 shoots per explant (Figure 1f).

Figure 1. (a) Initiation of callus from leaf terminals on NAA (9.0 µM) with K (4.65 µM) within 7-8 days of culturing (b) Growth of callus on same medium after further subculturing, callus formation was better under dark conditions where callusing started within 5-6 days (c) Shoot proliferation from leaf callus when cultured on 9.84 µM BAP after 10-12 days (d) Initiation of meristemoids from leaves on BAP (8.8 µM) after 10-12 days (e) Development of green leafy shoots from nodular meristemoids on same medium in about 70% of the cultures (f) Further proliferation of shoots on same medium after further culturing with formation of 35-40 shoots/explant (g) Rooting of Microshoots on half strength basal MS (h) Hardening of plantlets in moist cotton (i) Acclimatization of plantlets in soil: Vermicompost potting mix.

Rooting and acclimatization

Upon subsequent subculturing when sufficient number of shoots 3 to 4 cm in length were formed, they were separated from differentiating mass and cultured on MS medium supplemented with different concentrations of auxins. BMS supplemented with IBA (4.92 to 19.68 µM) and half strength BMS were used for root differentiation. Among these half strength basal MS medium produced long healthy roots within 10 to 12 days whereas on IBA (9.84 µM) roots were formed after 20 to 25 days hereby identifying the latter as better medium (Figure 1g). Microshoots along with roots were transferred to moist cotton jars covered with polybags to maintain high humidity and were kept in growth room for a week. The latter were then transferred to potting mixture of soil: vermicompost (1:1) and kept in green house under normal day length conditions. A survival rate of nearly 90% was obtained.

Extraction, separation and recovery of tylophorine

HPTLC of *T. indica* leaf extract was carried out. In callus raised *in vitro* plants densitometry evaluation at 258 nm yielded the presence of 7 different bands including the band for tylophorine at an Rf = 0.68 and 38.84% area which was revealed on comparison with standard track of tylophorine (Rao and Brook., 1970) with Rf = 0.68 and 81.42% area (Figure 2).

Table 1. Growth of callus on NAA (9.0 µM) and K (4.65 µM) medium under light and dark conditions.

Time (days)	Fresh weight (mg)		Average percentage increase/day (%)	
	Light	Dark	Light	Dark
0	-	-	-	-
10	178	200	-	-
20	327	486	5.90	8.33
30	508	728	4.33	3.98
40	612	1237	1.85	5.18

Average percentage increase/day was calculated as: (Final wt-Initial wt/10 / Final wt + Initial wt/2) X 100.

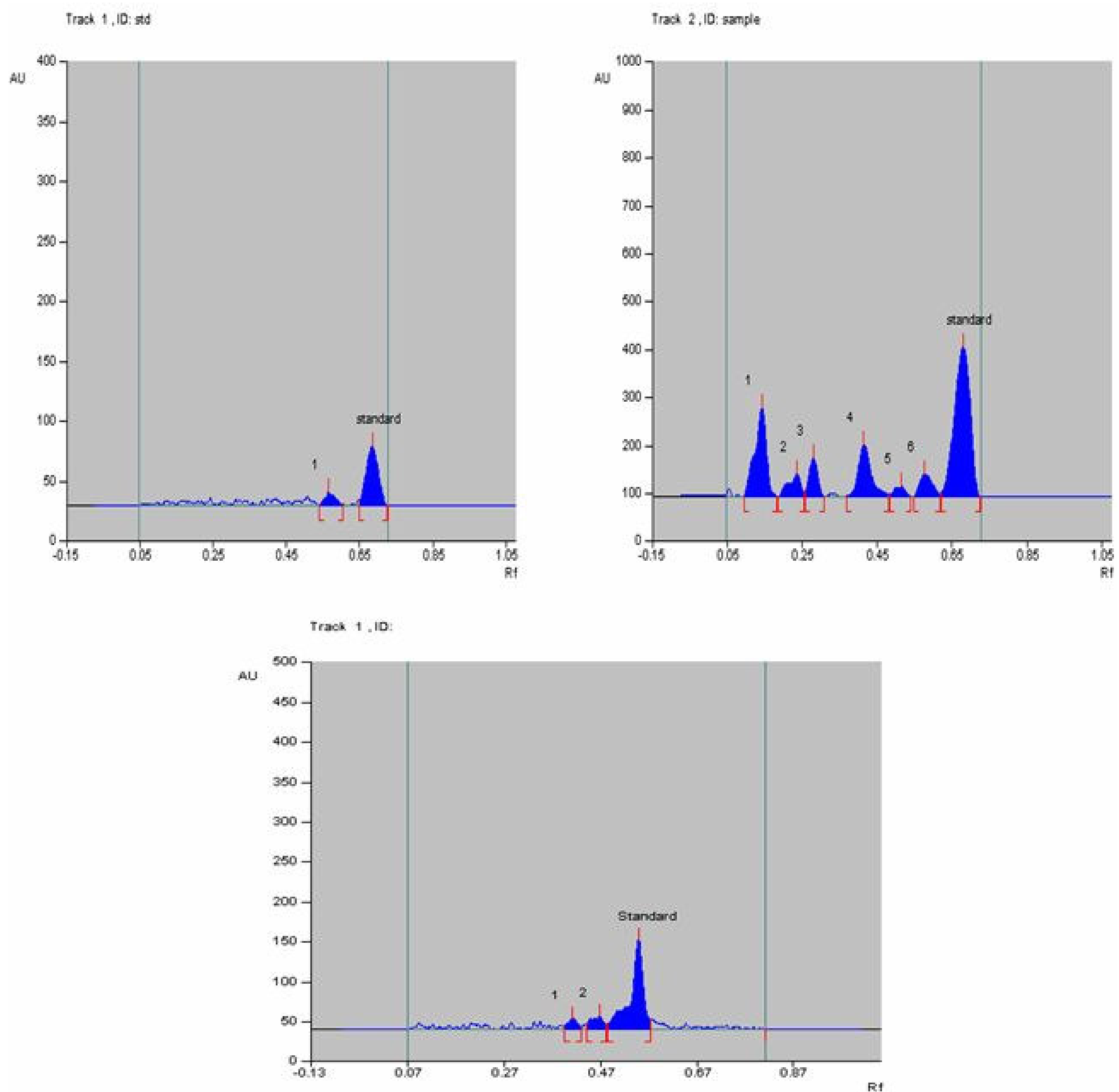

Figure 2. HPTLC of crude extract of *in vitro* callus raised and directly cultured *T. indica* showing seven different bands in track II and three bands in track III, whereas the band for standard (tylophorine) is in track I.

However, in directly cultured *in vitro* plants 3 bands were seen with tylophorine band at Rf = 0.68 and 80.93% area. Digital photo documentation at 254 and 366 nm was done to compare the position of standard band with the samples. The concentration of tylophorine in dried leaf extract of callus regenerated and directly cultured plants were found to be 80 and 71 µg/ml.

DISCUSSION

Our experiments on extraction of tylophorine from *in vitro* raised plants of *T. indica* confirm that nature and concentration of cytokinnin and auxin have a marked influence on callusing, growth and differentiation of the explant. Leaf segments of *T. indica* regenerated adventitious shoots directly from the cut ends and entire surface of the explant without the formation of intervening callus on BMS medium supplemented with BAP (8.8 µM). This is in accordance with observations by Chaudhari et al. (2004) for direct shoot bud formation from root explants of *T. indica* in the presence of BAP (10.7 to 26 µM). Callusing was induced on NAA and K combination, where callus formed was light green, friable and capable of sustained growth. However, as reported by Jayanti and Mandal (2001) induction of callus from leaf explants was reported on combinations of 2, 4-D (2 mg/l) and kinetin (1 mg/l). Faisal and Anis (2005) have achieved multiple shoot induction from the surface of leaf callus on BMS medium containing K (5 µM) whereas Thomas and Philip (2005) achieved plantlet regeneration from the callus on thidiazuron (8 µM) at which shoot regeneration was obtained from 100% of cultures with an average of 6.7 shoots per culture. For shoot proliferation, cytokinins are one of the most important factors affecting the response (Lane 1979; Stolz 1979; Bhojwani 1980; Garland and Stolz 1981). BA (9.84 µM) supplemented medium was used for shoot regeneration, wherein 35-40 shoots were formed per explant. As also reported by Bera and Roy (1992) induction of shoot was observed on BMS medium supplemented with BA (5 mg/l) and adenine sulphate (0.5 mg/l) from mature leaf explants of *T. indica.*

Induction and development of roots at the base of *in vitro* grown shoots is an essential and indispensable step to establish tissue culture derived plantlets in the soil. Shoots thus formed were excised and subjected to rooting on half strength BMS and basal MS supplemented with IBA. Half strength BMS produced healthy roots within 10-12, whereas 9.84 µM IBA produced roots within 20-25 days. All the rooted microshoots were successfully transferred to the pots through successive acclimatization process which showed 90% survival. HPTLC determination of dried leaf extract of extract of callus regenerated and directly cultured plants showed the presence of tylophorine at a concentration of 80 and 71 µg/ml, respectively. Rao et al. (1970) reported extraction of alkaloids from *T. indica* and *Tylphora dalzeii* using methanol, ethyl acetate and chloroform. Analysis of crude extract extracted using hexane, chloroform and dichloromethane was performed on high performance thin layer chromatography and densitometry evaluation at 258 nm showed the presence of tylophorine in crude plant extracts. In earlier reports different plant alkaloids have been estimated using techniques such as Infrared spectroscopy (Vishwanathan and Pai, 1985), direct-injection electrospray ionization mass spectrometry (Verma et al., 2007) and high performance thin layer chromatography (Chaudhari et al., 2004). We report HPTLC of crude extract from *in vitro* raised leaf explant of *T. indica* for identification and estimation of tylophorine and micropropagation protocol developed in the present study is an alternative method for propagating *T. indica* which has been reported to be declining very fast (Chandel and Pandey, 1991).

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REFERENCES

Bera TK, Roy SC (1989). Role of auxins in inducing embryogenic calli and other changes in *Tylophora indica*. In G. K Manna and U. Sinha (eds).Perspective in cytol. Genet., 6: 307-310.

Bhojwani SS (1980). *In vitro* propagation of garlic by shoot proliferation. Sci. Hortic., 13: 47-52.

Bhutani KK, Sharma, G L Sarin AN, Kaur R, Kumar V, Atal CK (1985). *In vitro* amoebicidal and bactericidal activities in medicinal plants. Indian Pharm. Sci., 47: 65-67.

Chandel KPS, Pandey R (1991) Plant genetic resources conservation0 a recent approach. In : Paroda RS and Arora RK (Eds) Plant genetic Resources- Conservation and Management. IBPGR Publ- New Delhi, India, pp. 247-272.

Chaudhari KN, Ghosh B, Jha S (2004). The root: a potential new source of competent cells for high-frequency regeneration in *Tylophora indica*. Plant Cell Rep., pp. 731-740.

Donaldson GR, Atkinson MR, Murray AW (1968). Inhibition of protein synthesis in *Ehrlich ascetes*- tumor cells by the pheanthrene alkaloids tylophorine, tylocrebrine and cryptopleurine. Biochem. Biophys. Res. Commu., 31: 104-109.

Faisal M, Anis M (2005). *In vitro* regeneration and plant establishment of Tylophora indica: Petiole callus culture. *In vitro* Cell. Dev. Biol. Plant, 41: 511-515.

Gujrati V, Patel N, Rao VN, Nandakumar K, Gouda TS, Shalam MD, Kumar SM (2007). Hepatoprotective activity of alcoholic and aqueous extract of leaves of *Tylophora indica* in rats. Indian J. Pharmacol., 39: 43-47.

Garland P, Stolz LP (1981). Micropropagation of Pissrdi plum, Ann. Bot. vol., 48: 387-389.

Gellert ETR, Govindachari MV, Lakshmikantham IS, Ragade R, Rudzats N, Viswanathan J (1962). The alkaloids of *Tylophora crebriflora*: structure and synthesis of tylocrebrine, a new phenanthroindolizidine alkaloid. J. Chem. Soc., pp. 1008-1014.

Gopalakrishnan C, Shankaranarayan D, Kameswaran L, Natarajan S (1979). Pharmacological investigations of tylophorine, the major alkaloid of *Tylophora indica*. Indian J. Med. Res., 69: 513-520.

Govindachari TR (2002). Five decades in the study of natural products.

Proc. Indian Acad. Sci., 3: 114.
Jayanthi M, Mandal PK (2001). *In Vitro* Cell. Dev. Biol. Plant, 37: 576-580.
Lane WD (1979). *In vitro* propagation of Spirea bumalda and Prunus cistena from shoot apices, Can. J. Plant Sci., 59: 1025-1029.
Viswanathan N, Pai BR (1985). Alkaloids of *Tylophora mollissima* J. Nat. Prod., 48(6): 997-998.
Ratnagiriswaran AN, Venkatachalam K (1935). The chemical examination of *Tylophora asthmatica* and isolation of the alkaloids tylophorine and tylophorinine. Indian J. Med. Res., 22(3): 433-441.
Rao VK, Brook P (1970). Alkaloids of *Tylophora indica* and *Tylophora dalzellii.* US Patent, 3: 497-593.
Stolz LP (1979). *In vitro* propagation of Acalypha wilkesiana. HortSci., 80: 290-292.
Thomas and Philip (2005). Thidiazuron induced high-frequency shoot organogenesis from leaf derived callus of a medicinal climber, Tylophora indica. *In vitro* cellular and Dev. Biol. Plant, 41: 124-128.
Upupa AL, Upupa SL, Guruswamy MN (1991). The possible site of anti-asthmatic action of *Tylophora asthmatica* on pituitary adrenal axis in albino rats, Planta Med., 57: 409-413.