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Phyto-pharmacological and plant tissue culture overview of *Tylophora indica* (burm f.) Merill.

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Abstract:
Tylophora indica is a climbing perennial plant indigenous to India, where it is grows wild in the southern and eastern region and has long-standing reputation as a remedy for asthma (hence the *Tylophora asthmatica).* The leaves of *Tylophora indica* are included in Bengal pharmacopeia since 1884. It is said to have laxative, expectorant, diaphoretic (Sweating), and purgative (Vomiting) properties. It has been used for the treatment of various respiratory problems besides asthma including allergies, bronchitis and colds, as well as dysentery and osteoarthritis pain. Tylophora has increasingly popular for treatment in asthma, based on its traditional use for this purpose, and several studies performed in 1970s. *Tylophora indica* is also still recommended for some of its other traditional uses, including hay fever, bronchitis and the common cold.
Keywords: *Tylophora indica,* Tylophorine, Anti-asthmatic, *Tylophora asthmatica.*

Introduction:

Tylophora indica (Burm f.) Merill. (Family: Asclepidaceae) commonly known as Antmul is a twining perennial plant distributed throughout southern and eastern part of India in plains, forests, and hilly places [1]. The plant is found growing normally in Uttar Pradesh, Bengal, Assam, Orissa, Himalayas and sub Himalayas in India [2]. It is a branching climber or shrub that grows up to 1.5 meters, leaves are obvate-oblong to elliptic-oblong, 3-10cm long and 1.5-7cm wide[3]. Roots Long fleshy with longitudinally fissured light brown, corky bark. Flowers minute, 1-1.5 cm across, in 2-3 flowered fascicles in axillary umbellate cymes. Calyx divided nearly to the base , densely hairy outside; segments lanceolate, acute. Corolla greenish yellow or greenish purple; lobes oblong, acute. Fruit a follicle, up to 7 × 1cm, ovoid lanceolate, tapering at apex forming fine mucro, finally striate, glabrous, Seeds 0.6-0.8 × 0.3-0.4cm long [4].

The plant has been reported to contain 0.2-0.46% alkaloids viz. Tylophorine, tylophorinine, tylophorinidine, (+)septicine, isotylocrebrine, tylophorinicine, sterols, flavanoids, wax, resins, and tannins[5]. The plant has been traditionally used for the treatment of bronchial asthma, jaundice and inflammation [3, 6]. Its antitumor, immunomodulatory, antioxidant, antiasthmatic, smooth muscle relaxant, antihistaminic, hypotensive, antirehumatic activities are scientifically proven. In Ayurveda , the plant has been used in treatment of asthma , dermatitis and rheumatism [1, 6]. Although the leaf and root of this plant are widely used for treating jaundice in Northern Karnataka, there is a paucity of scientific evidence regarding its usage in liver disorder [3]. The other reported activities include immunomodulatory activity, anti-inflammatory activity, anticancer activity and antiamoebic activity [7,8,9,10].

Fig 1: *Tylophora indica* (Burm f.) Merill.

Botanical name: *Tylophora indica* (Burm f.) Merrill.
Synonym: *Tylophora asthmatica* (Linn. F.).
Common Name: Antmul.
Other names:
Beng.- Antomul.
Bomb.- Pitmari, Kharaki-raena, Anthamul, Pitakari.
Guj.- Antamul.

Hindi- Antamuli.
Kan.- Kirumanji.
Mal.- Valli-pali.
Mar.- Pitakari, Khodki-Rasna.
Ori.- Mendi, Mulini.
Tam.- Naye-pallai.
Tel.- Veripala, Kukka-pala, Vettipala [4].

Geographical Source

It is Indigenous to India. The plant inhabits up to an elevation of 1,260m in the sub Himalayan tract and in central and in peninsular India. It also met within Eastern, North- East and Central India, Bengal and parts of South India[4].

Habitat

The plant is a perennial branching climber with long fleshy roots. It grows in planes and hilly places of India up to an attitude of 1,000 m in Bengal, Assam, Cachar, Orissa, and southern India [11].

Botanical Description

a) Macroscopy

Leaf 5-10 cm long, 2.5-5.7 cm broad, ovate or epileptic-oblong, acute or acuminate, often apiculate, glabrous, more or less pubescent especially when young, petioles 6-13mm long. The colour of leaves is green, odour is pleasant characteristic, and surface is smooth [4].

b) Microscopy

Leaf is composed of an outermost layer of thin walled, single layered epidermal cells covered by thin cuticle. Mesophyll differentiated into 2-3 layered palisade and 6-8 layered spongy parenchyma, the latter containing rosettes of calcium oxalate (druses). Epidermal peeling exhibits characteristics covering trichomes, multicellular (3 to 8 celled), uniseriate, bent and tapering at the end. Paracytic stomata are seen only on the abaxial surface. In the midrib region, collenchyma is present below the upper epidermis and above the lower epidermis. In the centre, xylem elements are arranged in an arc and phloem occurs on both sides of it. Many idioblasts with crystals are found in the ground tissue [4].

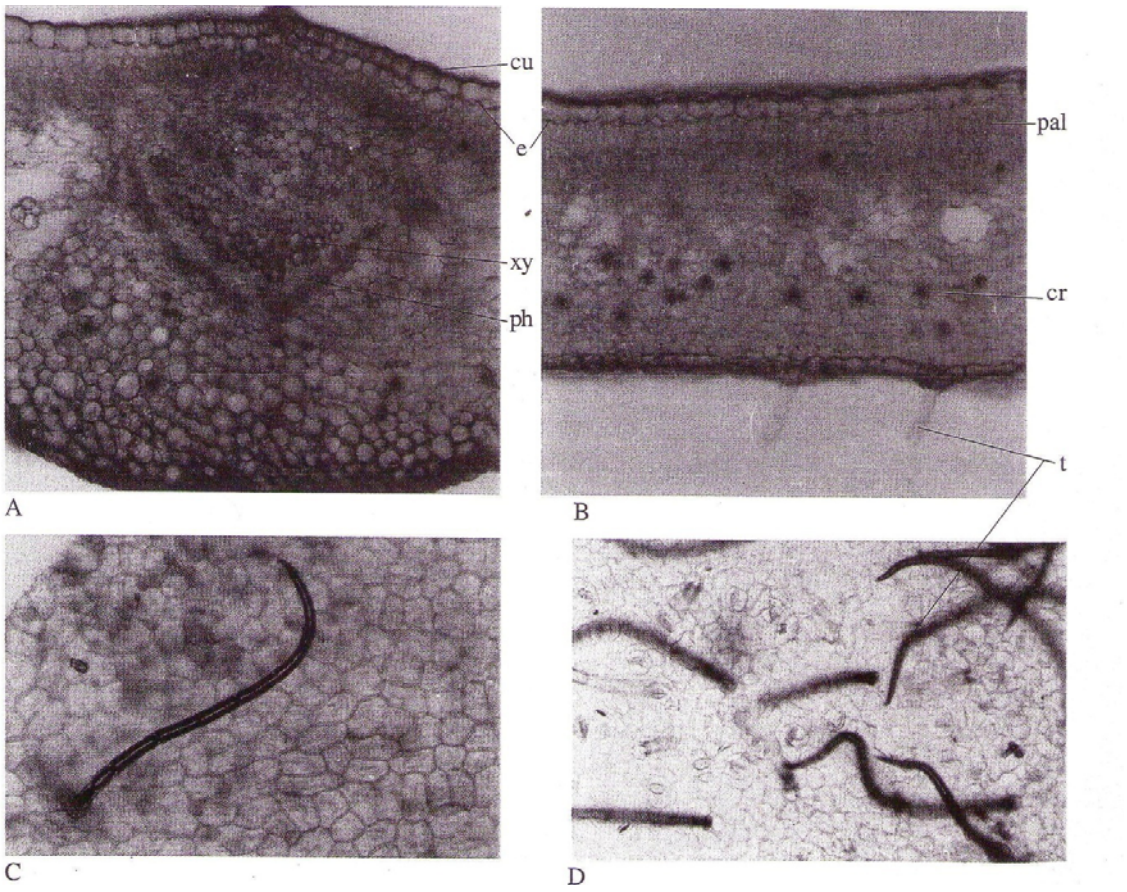


Fig 2: Microscopy of leaf of *Tylophora indica.* A. TS of Mid-rib portion, B. Section through Lamina region, C. Upper epidermal peel showing trichome, D. Lower epidermal peel showing multicellular trichomes.

Chemical constituents

The active constituents of *Tylophora indica* are phenanthroindolizidine alkaloids like tylophorine, tylophorinine, tylophorinidine and septidine. Recently some rare alkaloids namely tyloindicines A, B, C, D, E, F, G, H, I, and J, desmethyl-tylophorine, desmethyl tylophorinine, isotylocrebrine, anhydroustylophorinine, anhydrous-dehydrotylophorinine, γ-fagarine, skimmianine, 14-hydroxyisotylocrebrine, 4,6-desmethylisodroxy-o-Methyltylophorinindine have been reported. The non-alkaloidal compounds isolated from *Tylophora indica* are kaempferol, quercetin, α- and β- amyrins, tetratriacontanol, octaosanyl octacosanoate, sigmasterol, β-sitosetrol, tyloindane, cetyl-alcohol, wax, resin, coutchone, pigments, tannins, glucose, calcium salts, potassium chloride, quercetin and kaempferol. Steam distillation of an alcoholic extract of the air-dried root powder gave *p*-methoxy-salicyaldehyde and a small amount of oily matter [11].

Phytochemical Studies

The drug *Tylophora indica* contains major chemical constituents Tylophorine, kaempferol, α-amyrin and quercetin, other major alkaloids like tylophorindine, desmethyltylophorine, desmethyltylophorinine, desmethyltylophoridine, dehydrotylophorine, anhydrous-dehydrotylophorinine. Other alkaloids (+)-Septicine and (+)-isotylocrebrine from fresh leaf [4]. The new alkaloids include tyloindicines A-E, (+)-14-hydroxyisotylocrebrine and 4-6-desmethylisotylocrebrine. Tylophorine, 6-desmethyltylophorine, tylophorindine and 5-hydroxy-*o*-methyltylophorindine were the known alkaloids. Structural studies indicate that apart from tylophorindicne B all alkaloids possess the dibenzo-{f,h} pyrrolo {1,2b} isoquinoline skeleton but differ in the number, nature and distribution of the oxygen bearing substituents, in the presence or absence of C-13a or benzylic hydroxyls and an angular methyl function. Tylophorine B possess a cleaved substituted phenanthrene moiety nucleus and an angular methyl group on the indolizidine portion [12]. Although tylophora alkaloids are structural analogs, their potency of cytotoxicity, selectivity against NF-kB signaling pathway, and their inhibitory effects against protein and nucleic acid synthesis are different. Because they don't have an identical spectrum of targets, the studied are structural but may not be functional analogs [13].

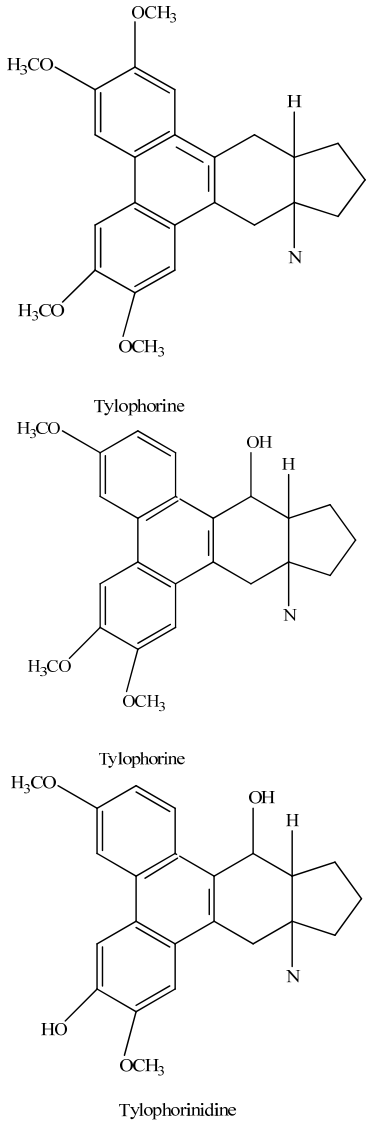


Figure 3: Chemical structures of chemical constituents found in *Tylophora indica*.

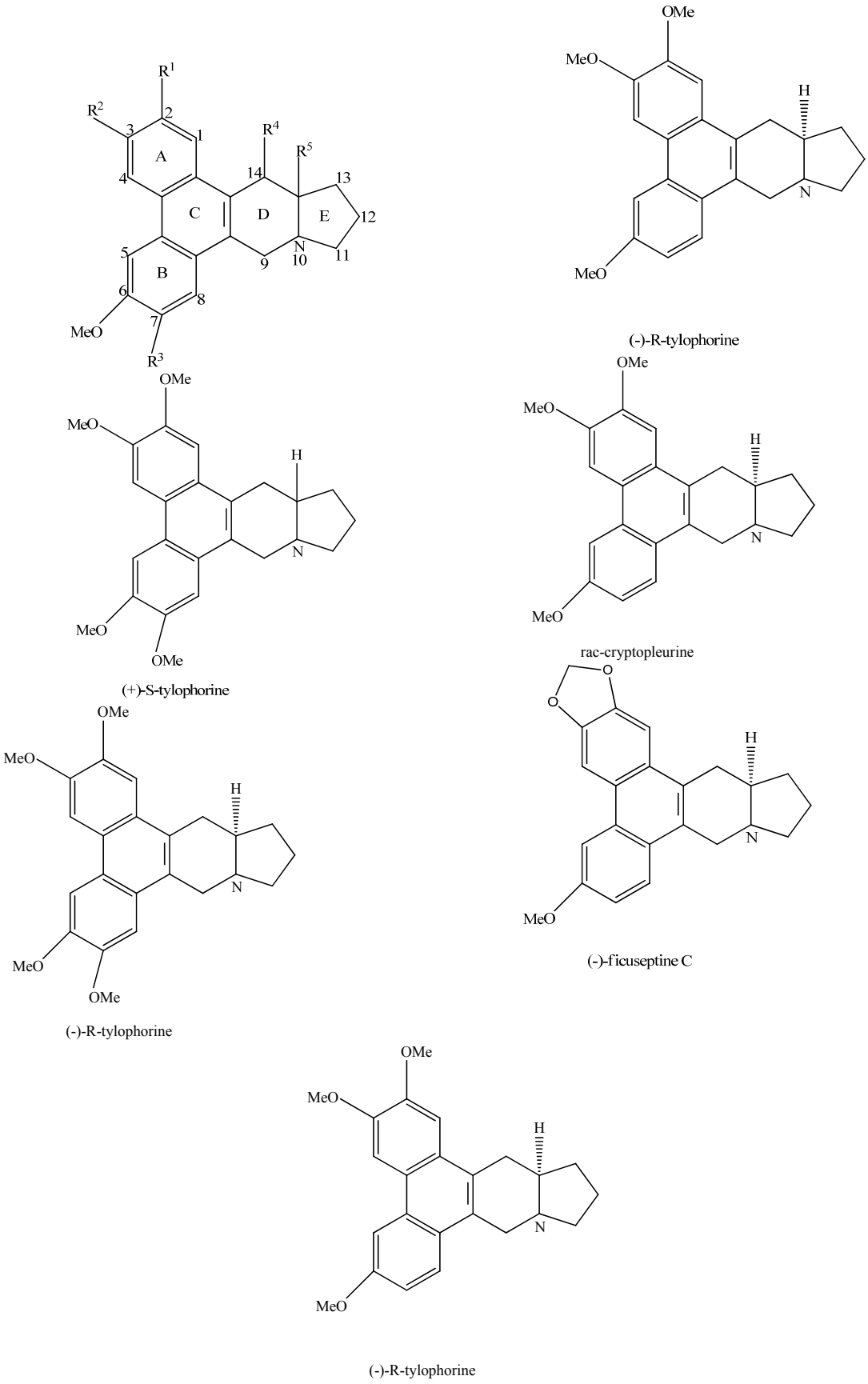


Figure 4: Chemical structures of tylophora alkaloids and phenanthrene-based tylophorine derivatives.

Pharmacological studies

Hepatoprotective activity:

The methanolic extracts of *Tylophora indica* leaves was screened for hepatoprotective activity in carbon tetrachloride induced hepatotoxicity in albino rats. *Tylophora indica* leaves exhibited significant reduction in serum hepatic enzyme when compared to rats treated with carbon tetrachloride alone [14]. The hepatoprotective activity of alcoholic (ALLT) and aqueous (AQLT) extracts of leaves of *Tylophora indica* against ethanol-induced hepatotoxicity. Ethanol induced significant changes in physical, biochemical, histologcal, and functional liver parameters. Pretreatment with ALLT and AQLT extract significantly prevented the physical, biochemical, hostological and functional changes induced by ethanol in the liver [15].

Lysosomal enzyme inhibiting activity:

The flavone fraction from *Tylophora indica* leaves showed significant dose dependent lysosomal enzyme inhibiting activity against adjuvant-induced arthritis at 20-50 mg/kg. Flavone fraction showed statistically significant inhibition of arthritis lesions ($p<0.05$) from day 18, ($p<0.025$) from day 20 and ($p<0.001$) from day 21 onwards in the adjuvant-induced arthritis studies which was compared to response of standard drug indomethacin[16].

Antiallergic activity:

The anti-allergic effect of *Tylophora indica* was compound with that of disodium cromoglycolate on perfused rat lung in sensitized rats by observing the changes in the volume of the perfusate per minute. Administration of aqueous extract of *Tylophora indica* and disodium chromoglycolate during perfusion of sensitized rat lung significantly increased the rate of flow. The action of *Tylophora indica* may be due to direct bronchodilator property and membrane stabilising and immune-suppressive effects [17].

Diuretic activity:

Aqueous and alcoholic extracts of *Tylophora indica* leaves were tested for diuretic activity in rats. The aqueous and alcoholic extracts of *Tylophora indica* leaves possess good diuretic activity. It is investigated that ethanol is most effective in increasing urinary electrolyte concentration of all the ions i.e sodium, potassium and chloride followed by chloroform and aqueous extracts while other extracts did not show significant increase in urinary electrolyte concentration[18].

Immunomodulatory activity:

Studies with tylophora alkaloids had shown that they inhibit cellular immune response like contact sensitivity to dinitro-flurobenzene and delayed hypersentivity to sheep red blood cells, in vivo. The alkaloids mixture suppressed IL-2 production at the lower concentrations. IL-1 production by activated macrophages on the contrary was doubled in the presence of inhibitory concentration dependent biphasic effect on con A induced mitogenesis. At lower concentrations they agument con A induced lymphoprolifration by enhancing IL-2 production. Inhibitory of proliferation at higher concentration of the alkaloids is due to inhibition of IL-2 production and activation of macrophages, which a cytostatic effect[19]. Crude extract of the leaves of Tylophora indica inhibited delayed hypersensitivity reaction to sheep red blood cells in rats when the alkaloid mixture was administered before and after immunization with these cells. The alkaloid mixture also inhibited contact sensitivity to dinitro-fluorobenzene in mice when given prior to or after contact sensitization. Lymphocytes taken from contact sensitized mice, when treated with tylophora alkaloid in vitro and transferred into naive syngeneic hosts, could suppress the transfer of delayed type hypersensitivity (DTH) response. However, the tylophora alkaloids could not suppress primary humoral (IgM) immune

response to SRBC in mice at the same dose. These studies suggest that tylophora alkaloids suppress cellular immune responses when administered at any stage during the immune response [20].

Mast cell stabilisation activity:

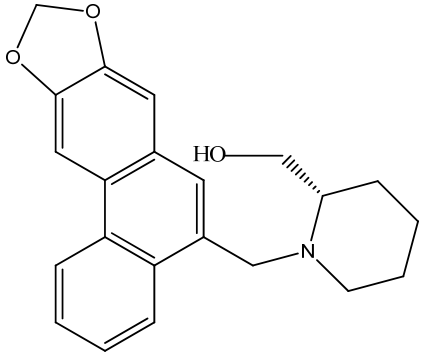
The total alkaloids of *Tylophora indica* were tested for mast cell stabilising effect in comparision with disodium cromoglycolate by challenging against three different mast cell degranulators, diazoxide, carbachol and polymixin B, in-vitro. The results suggest that tylophora alkaloids may have similar mechanism of action disodium cromoglyacte through cylic AMP [21].

Anti-Cancer Activity

Tylophorine not only retards the S-phase progression but also dominantly arrests the cells at G1 phase in HepG2, HONE-1, and NUGC-3 carcinoma cells. Moreover, tylophorine treatment results in down regulated cyclin A2 expression and overexpressed cyclin A2 rescues the G1 arrest by tylophorine. Thus, we are the first to report that the downregulated cyclin A2 plays a vital role in G1 arrest by tylophorine in carcinoma cells [22].

Anti-Tumor Activity

Tylophorine analogs had an inhibitory effect on cyclic AMP response elements, activator protein-1 sites, or nuclear factor-kappaB binding site-mediated transcriptions. In summary, these tylophorine analogs are a unique class of antitumor compounds that have a mode of action different from known antitumor drugs [23]. Polar phenanthrene-based tylophorine derivatives (PBTs) were designed, synthesized and evaluated as potential antitumor agents. The newly synthesized PBTs were evaluated for cytotoxic activity against the A549 human cancer cell line. Among them, *N*-(2,3-methylenedioxy-6-methoxy-phenanthr-9-ylmethyl)-l-2-piperidinemethanol (**34**) and *N*-(2,3-methylenedioxy-6-methoxy-phenanthr-9-ylmethyl)-5-aminopentanol (**28**) showed the highest potency with IC_{50} values of 0.16 and 0.27μM, respectively, which are comparable to those of currently used antitumor drugs [24].



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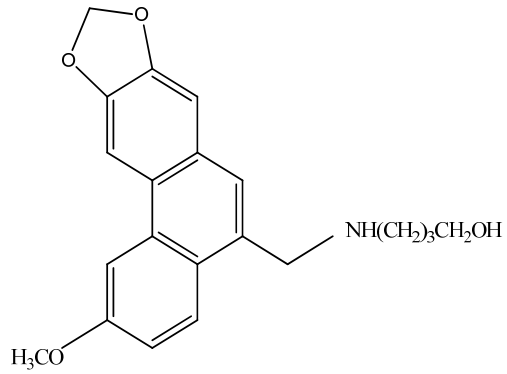

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Figure 5: Phenanthrene based tylophorine derivatives.

Antifeedant and antimicrobial activity:

Crude and pure extracts of *Tylophora indica* were investigated in view of antifeedant and antimicrobial activity. Pure compounds displayed strong antibacterial activity at lower concentrations in all tested bacterial starins except *E.coli.* while all the crude and pure compounds showed antifungal activity against *Aspergillus niger, Aspergillus fumigates* and *Trichoderma virdae,* the pure compounds had strong antifungal activity compared to crude extracts[25].

Anti-Asthmatic

A brief exposure of human peripheral leukocytes from asthmatic children to tylophorine (an alkaloid occuring in *Tylophora asthematica*) caused the stimulation of adenyl cyclase. This effect was not observed in the leukocytes from the nonasthmatic children or adults [26].

Plant tissue culture studies

An efficient procedure has been developed for inducing somatic embryogenesis from mature leaves of *Tylophora indica* (Burm f.) Merill., and important medicinal plant. Leaf sections were initially cultured on Murashige and Skoog's (MS) medium supplemented with thidiazuron (TDZ) in addition with 2,4-dicholorophenoxy acetic (2,4-D), particularly 0.5µm TDZ, along with 1.5µm 2,4 D was very effective in inducing somatic embryos. Plant were regenerated from in-vitro somatic embryos plated on semisolid medium devoid of growth regulators. Plantlets were obtained in 65% of the cultures with 2% sodium alginate coated embryos and control embryos showed 90% germination [27].

Organogensis callus formulation from immature leaf peices was obtained by using Murashige and Skoog (MS) medium supplemented with 7µ*M* 2,4-dichlorophenoxyacetic acid and 1.5µM 6-benzyladenine. On the medium 92% explants produced callus. The optimal hormone combination for plantlet regeneration was 8µM thidiazaron, at which shoot buds were originated from 100% of the cultures, with an average of 66.7 shots per culture. For roots formation half-strength MS-medium supplemented with 3µM indole-3butyric acid was used. Plants were transferred to soil, where 92% survived after 3mol of acclimatization [28].

Another protocol has been developed for high-frequency shoot regeneration and plant establishment of *Tylophora indica* from petiole-derived callus. Optimal callus was developed from explants on Murashige and Skoog basal medium supplemented with 10µM 2,4-dichlorophenoxyacetic acid and +2.5µM thidiazuron (TDZ). The in-vitro raised plantlets with well-developed shoot and roots were successfully established in earthen pots containing garden and soil and grown in a greenhouse with 100% survival. Four months after transfer to pots, the performance of in vitro propagated plants of *Tylophora indica* was evaluated on the basis of selected physiological and compared with ex vitro plants of the same age [29].

The effect of sugars, gibberellic acid (GA3) and abscisic acid (ABA) on somatic embryogenesis from internodal explant-derived callus of Tylophora indica (Burm. f.) Merrill has been investigated. Embryogenic calli were produced from internodal explants and the best result was achieved by using MS medium supplemented with 4micromol/L 2, 4-Dichlorophenoxyacetic acid (2, 4-D). Up to 69% of such embryogenic calli differentiated into somatic embryos with an average of 25 embryos per explant (per gram of the calli) on Murashige and Skoog (MS) medium containing 6micromol/L kinetin (Kn). The study reported here indicates that 200mmol/L sucrose with 6micromol/L Kn, 200mmol/L sucrose with 10micromol/L GA3 and 200mmol/L sucrose with 2micromol/L ABA significantly improved somatic embryogenesis in T. indica whereas glucose alone or in combination with sucrose had an inhibitory role. The embryos obtained developed normally and were easily converted into plants [30].

New and efficient transformation system for *Tylophora indica* using Agrobacterium rhizogenes strains LBA9402 and A4 to infect excised leaf and stem explants and intact shoots at different sites. The induction of callus and transformed roots was dependent on the bacterial strain, explant type and inoculation site used. Transformed roots were induced only in explants infected with A. rhizogenes strain A4, while an optimal transformation frequency of up to 60% was obtained with intact shoots inoculated at the nodes. Root growth and the production of tylophorine, the major alkaloid of the plant, varied substantially among the nine root clones studied. Both parameters increased over time in liquid cultures, with maximum biomass and tylophorine accumulation occurring within 4-6 weeks of growth in fresh medium. Interestingly, in liquid

culture, the culture medium also accumulated tylophorine up to concentrations of 9.78+/-0.21 mg l(-1) [31].

To develop a new micropropagation system for Tylophora indica, an important medicinal plant in India, using root explants as starting material. Root explants cultured on MS medium supplemented with 6-benzyladenine (BA) or 2-isopentyladenine (2iP) produced organogenic nodular meristemoids (NMs) within 4 weeks. Plantlets derived via somatic embryogenesis and shoot organogenesis were successfully hardened (88-96%) and transferred to the field [32].

Mature leaf explant derived callus of Tylophora indica (Burm. f.) Merrill yielded somatic embryos on MS medium supplied with BA(1-2 mg/L) or kinetin(1-5 mg/L) or kinetin/BA (1-2 mg/L) used along with IAA(0.1-1 mg/L). Maximum somatic embryos (30) could be recovered from 100 mg of embryogenic callus within 60 days at an optimum concentration of 2 mg/L of BA which was also best suited for providing the maximum conversion rate (90%) of embryoids to plantlets. Embryoids from all cultures germinated in the initiation medium and were transplanted to sterile vermiculite for hardening. After two weeks of hardening, the plantlets were transferred to the green house where they grew and established well showing a high rate of survival (90%) [33].

Protoplast culture and plant regeneration of an important medicinal plant *Tylophora indica* were achieved through callus regeneration. Protoplast were isolated fromleaf mesophyll cells and cultured at a density of 5×10^5 protoplast per gram fresh weight, which is required for the highest frequency of protoplast division (33.7%) and plating efficiency (9.3%). The calli developed shoot buds after 3-4 wk, and the frequencies of calli forming shoots varied from 5% to 44%. Optimum shoot regeneration occurred on MS medium supplemented with 5μM TDZ and 0.4 μM NAA. On this medium 44% cultures responded with an average number of 12 shoots per callus. Whole plants were recovered following rooting of shoots in ½ MS medium supplemented with 3μM indole 3-butyric acid [34].

Other Reported Work

Tylophorine B exhibits pronounced cytotoxicity and antitumor activity. In order to survey the structure selectivity to DNA afforded by tylophorine B, we have synthesized a variety of duplex, bulge- and hairpin-containing oligodeoxyribonucleotides. Their binding to tylophorine B has been assayed by fluorescence spectroscopy and thermal melting experiments. The results indicate that oligonucleotides interact with tylophorine B at submicromolar concentration, and the affinity for DNA bulge is optimal (with K_d of 0.018 μM). In addition, the bulged hairpin oligonucleotides are stabilized by binding to tylophorine B. These findings may shed some light on tylophorine B's mode of action in biological systems and result in the rational design of sequence-specific DNA binding molecules [35].

Tylophorine B exhibits 60% inhibition against tobacco mosaic virus (TMV) at a concentration of 1.0×10^{-6} g/ml. In our study, high affinity for TMV RNA and assembly origin of TMV RNA (oriRNA) was revealed, accompanied by the conformational change of RNA. Considering that TMV assembly begins with the specific recognition by the coat protein aggregate of oriRNA, and that tylophorine B has favorable interaction with oriRNA, we speculate that tylophorine B likely exerts its virus inhibition by binding to oriRNA and interfering with virus assembly initiation. This work may shed light on the possible molecular inhibition mechanism against TMV by tylophorine B, and provide clues in rational design of sequence-specific RNA binding antivirus drugs [36].

Tylophora indica plants have been shown to contain phenanthroindolizidine alkaloids of the tylophorine type.

Cinnamic acid-[2-^{14}C]was incorporated efficiently into these alkaloids supporting the hypothesis that ring A and C-10 and C-6 of tylophorine are derived from phenylalanine [37].

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