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Research Article

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TLC Based Analysis of Allelopathic Effects on Tinosporoside Contents in *Tinospora cordifolia*

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ABSTRACT

Plants may favorably or adversely affect other plants through allelochemicals. The objective of this study was to examine the allelopathic effects of P. pinnata, A. indica and Z. jujuba on phytochemical profile of Tinospora cordifolia. In current study auxiliary buds of plant were cultured on Murasinge and Skoog medium, proved to be the good for shoot as well as root induction. To ensure the quality of plants grown by culture the juice of the plants collected from various supports from the same garden was analyzed by thin layer chromatography (TLC). A total number of 11 spots were observed. Out of the 11 spots three of Rf value 0.192 (Tinosporaside), 0.551 (berberine) and 0.717 were found to be universally present in the plants used in the study, which can be used as chemical markers of the plant. Densitometric profiles of the chromatograms showed variable amounts of constituents in the juice of plants supported by various trees. Tinosporaside contents were found to be significantly high in the plant supported by A. indica tree as comparative the plant supported by wall. Berberine contents were found to be almost same in all the plants, a bit higher levels were observed in the plant supported by P. pinnata. Tinosporaside and berberine showed to have antioxidant properties. However, overall antioxidant activity was observed to be high because of highest levels of tinosporaside in the plant supported by A. indica. The TLC profiles of explants grown with different concentration of aqueous extract of A. indica were found to be highly affected when observed after 8 days of culture. The significant difference was observed in the tinosporaside contents after 15 days growth of tissue cultured plants in presence of A. indica root extracts.

Key words: Allelopathy, *T. cordifolia, A. indica, Z. jujube, P. pinnata*, phytochemical profile.

INTRODUCTION

The interaction of plants through chemical signals (allelopathy) has many possible agricultural applications. Decline in crop yields in cropping and agro-forestry system in recent years has been attributed to allelopathic effects. Allelopathy associated problems have been observed both in monocultures and multiples cropping system, continuous monoculture causes the accumulation of phytotoxins and harmful microbes in soil, which give rise to phytoxicity and soil thickness.

Guduchi (*Tinospora cordifolia* (willd). is a large, glabrous, deciduous climbing shrub belonging to the family Menispermacea and an important plant according to Ayurveda and traditional system of medicine. It is distributed through tropical Indian subcontinent and China, ascending to an altitude of 300 m. Guduchi is widely used in veterinary folk medicine/ayurvedic system of medicine for its general tonic, ant-periodic, anti-spasmodic, anti-

inflammatory, anti-arthritic, anti-allergic, anti-diabetic, aphrodisiac, anticarcinogenic and antimutagenic properties [1-4]. The plant is used in ayurvedic, "Rasayanas" to improve the immune system and the body resistance against infections [5]. The root of this plant is known for its anti–stress, anti-leprotic and anti-malarial activities [1, 6]. The extract of stem is reported to be useful in skin diseases.

T. cordifolia is used in Indian system of medicine to treat diabetes mellitus [7, 8]. It is reported that the daily administration of either alcoholic or aqueous extract of *T. cordifolia* decreases the blood glucose level and increases glucose tolerance in rodent. *T. Cordifolia* is reported to benefit the immune system in a variety of ways [5, 9]. Intrinsic factor such as genetics and extrinsic factors such as cultivation, harvesting, drying and storage conditions are the important factors that effects quantity of plant based drug material. Use of microscopy and chromatographic technique are widely used method for the identification of plant-based drugs. Therefore we standardized the phytochemical profiles of *T. cordifolia* supported by various tree and grown by tissue culture (in media only). We observed that chemo profile of plant changes rapidly as per the support due to allelopathic effects of supporting plants. These studies will help in selection of right chemo type having therapeutic efficacy and to multiply plant without affecting genotype.

EXPERIMENTAL SECTION

Collection of Plant Material: The fresh stems of T. cordifolia (Willd.) Miers ex Hook F & Thoms (Family: Menispermaceae) and root bark of *Azdirachta indica* were collected. Three different sample of T. cordifolia were used in the study along with tissue culture grown plants. The plants supported by *A. indica*, *Pongamia pinnata*, *Zizuphus jujuba* and supported by wall were collected.

Preparation of *Azadirachta indica* aqueous Extract: Collected root bark of *A. indica* was washed with distilled water, cut into fine small pieces and extracted over night with distilled water. Extract was filtered through Whatman filter paper No.1. The water was removed from the extract by keeping the extract at 37°C. The dried extract was stored in Refrigerator for further use.

***In- vitro* propagation of *T. cordifolia* (Wild.) Miers:** For *ex situ* cultures of *T. cordifolia* (Willd.) Miers., the Murashige and Skoog (1962) medium was used [10]. *T. cordiflia* explants were collected from fresh, healthy growing plants at RRI (Ay), Pune. Long nodal segments 2-3 cm with health axillary buds were selected for inoculation. Selected explants were washed with surfactant and by keeping under running water for half an hour. Thoroughly washed explants were surface sterilized with 0.1% $HgCl_2$ solution (100 ml), for 3 minutes and rinsed three times with distilled water to remove the traces of sterilent. Explants were trimmed at both ends to discard the dead tissues and inoculated onto the MS medium with or without root extract (*A. indica*). Medium was solidified with 0.7% agar. Cultures were maintained at 25± 2 ^{0} C under cool fluorescent light, with illumination of 3000 lux for 10 hours in light and 14 hours in dark for 3 weeks.

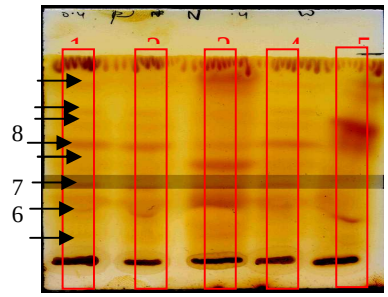
Preparation of Plant Juice: The collected plant materials were washed with distilled water and chopped into small fine pieces and juice is extracted from the material (2.0 ml), by using conventional method.

TLC Analysis: The 2µl juice of *T. cordifolia* was applied to thin layer chromatography plates (Merk, Silicagel 60 F_{254}), and developed in two solvent systems (i)-Butnaol: acetic acid: water (BAW) (50:10:40) and n-Butnaol: acetic acid: water (BAW) (70:20:10). Freshly prepared vanillin, Molybdophosphoric acid and Iodine spray reagents were used to detect the bands on the TLC plates.

RESULTS

Tissue Culture of *T. cordifolia*: An efficient protocol for *In vitro* micropropagtion of *T. cordifolia* (Linn.), an important shrub (Fig1) was developed. The simple MS media without any growth regulator was found to be effective for shoot and root formation in 80% plants. Shoot formation starts from day 5, root formation start after two weeks in the same media.

A



B

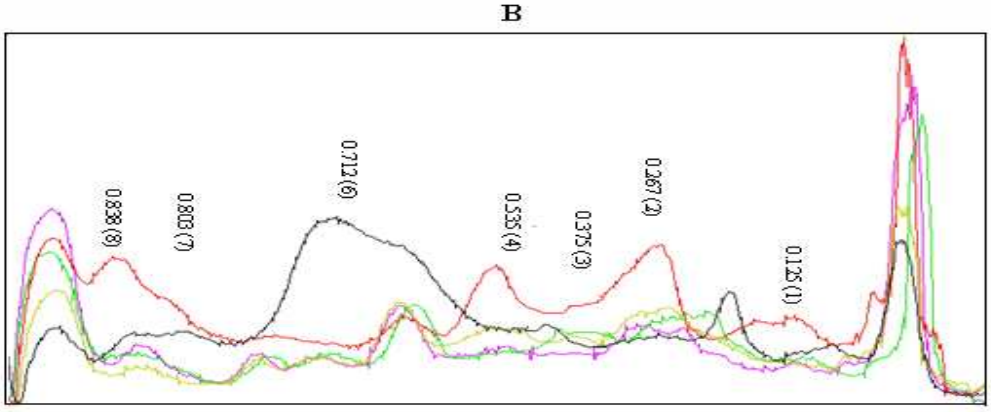


Figure 1: Comparative profile of *T. cordifolia* from various origins and grown in tissue culture in presence or absence of *A. indica* extract. The plates were developed with Iodine fumes. Fig A: Lane 1: plant grown in presence of A. *indica* extract after 15 days (8.0 mg/ml), Lane 2: *T. cordifolia* grown on *A. indica* (4.0 mg/ml), lane 3: *T. cordifolia* grown on *A. indica* (2.0 mg/ml), Lane 4: with *A. indica* extract after 8 days (1.0 mg/ml) and Lane 5: *T. cordifolia* grown on wall. Fig B: Densitometeric profiles of the same Lane 1: Pink, Lane 2: Green, Lane 3: Red, Lane 4: yellow, Lane 5: Black.

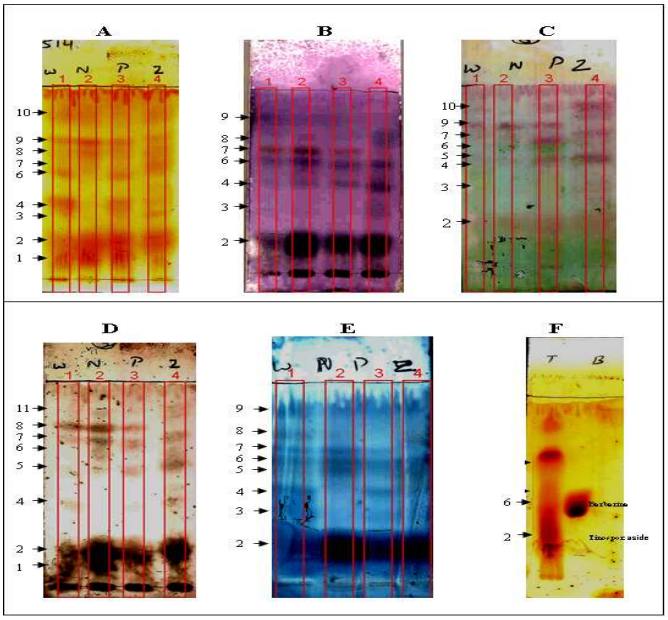


Figure-2 TLC Chromatograms developed in solvent system n-butanol: acetic acid: water (5:1:4). Fig (A) shows Iodine, Fig B: Vanillin, Fig C; Benzaldehyde, Fig D; Cerric sulphate and Fig E: Molybdenic acids spray. Fig F shows the standards of Tinosporaside (T) and berberine (B), the constituents of *T. cordifolia*.

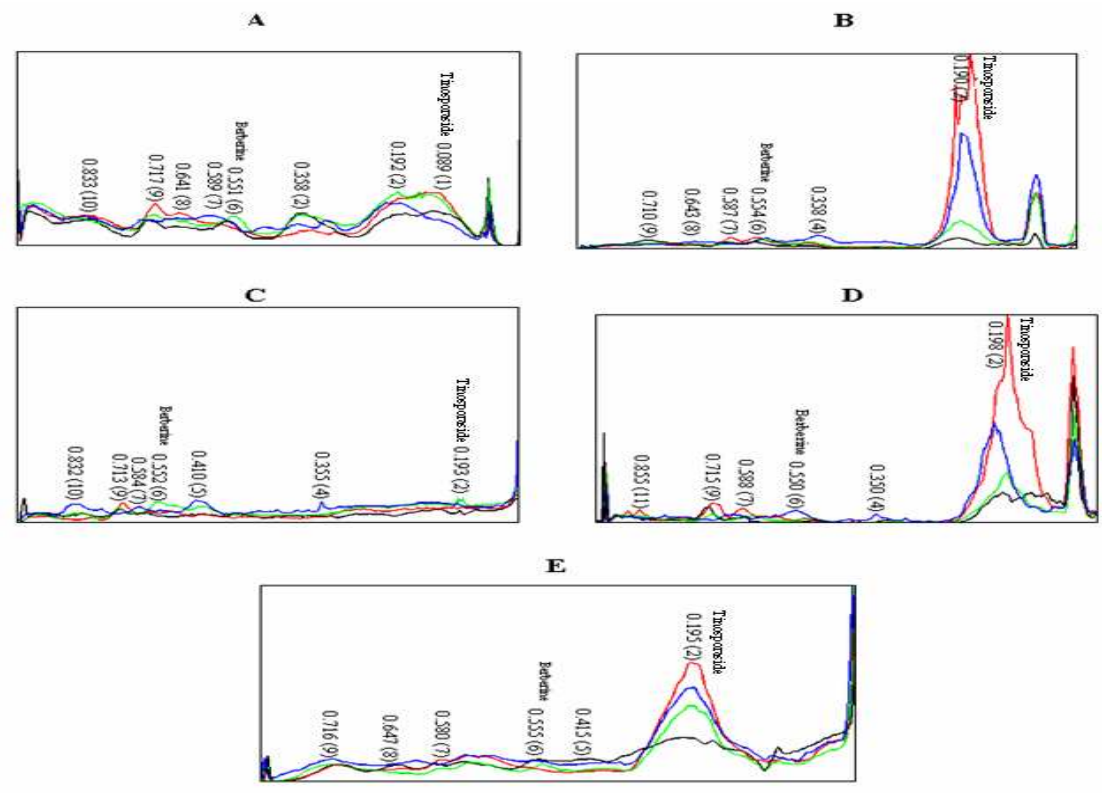


Figure 3: Comparative densitometric profile of TLC Chromatograms developed in solvent system n-butanol: acetic acid: water (5:1:4). Fig A: shows Iodine, Fig B: Vanillin, Fig C: Benzaldehyde, Fig D: Cerric sulphate and Fig E: Molybdenic acid spray. Lane 1: Black (Control, Plant from wall) Lane 2: Red (*A. indica*), Lane 3: Green (*P. pinnata*) and Lane 4: Blue (*Z. jujuba*).

TLC Profile of Juice: Juice of the plants collected form various source of the same garden was analyzed by developing the TLC plates in solvent system n- Butanol: Acetic acid: Water (50:10:40). To visualize the bands TLC plates were spayed with various reagents. Vanillin and Iodine spray was found to show maximum number of bands (Fig 2). All most 11 bands having Rf values 0.0.89, 0.192,0.253, 0.356, 0.410, 0.551, 0.587, 0.641, 0.717,0.883 and 0.858 were separated with the above solvent system (Table 1). Out of the 11 spots three of Rf value 0.192, 0.551 and 0.717 were found to be universally present in the plants used in the study. The spot of Rf 0.192 was found to be Tinosporaside, one of the major constituents of the plant. Tinosporaside having Rf 0.192 was found to be present in higher quantity in the plants supported by *A. indica* and *Z. jujuba* (Fig 3).

Densitometric profiles of Juice: Densitometric profiles of the TLC Plates showed variable amounts of constituents in the juice of plants supported by various trees (Fig 3). Tinosporaside contents were found to be highest in the plant supported by *A. indica* tree as comparative the plant supported by wall. Berberine contents were found to be almost same in all the plants, a bit higher levels were observed in the plant supported by *P. pinnata*. The levels of another unknown metabolite of Rf value 0.356 were found to high in the plant supported by *P. pinnata*. Profile for antioxidants showed the presence of highest quantity of antioxidants in the plant supported by *A. indica* (Table 1). Tinosporaside, berberine showed to have antioxidant activity. However, overall antioxidant activity was observed to be high in the plant supported by *A. indica* may be because of highest quantity of tinosporaside.

Table 1: Rf values of TLC Chromatograms developed in solvent system n.butanol: acetic acid: water (5:1:4).

Reagent used		Rf value										
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Iodine	**A**	0.089	0.192	-	0.358	-	0.551	-	-	0.717	0.833	-
	B	0.089	0.192	-	-	-	0.551	-	0.641	0.717	0.833	-
	C	0.089	0.192	-	0.358	-	0.551	-	0.641	0.717	0.833	-
	D	0.89	0.192	0.253	0.358	-	0.551	0.589	-	0.717	0.833	-
Vanillin	**A**	-	0.190	-	0.355	-	0.554	0.587	0.643	0.710.	-	-
	B	-	0.190	-	0.355	-	0.554	0.587	0.643	0.710.	-	-
	C	-	0.190	-	0.355	-	0.554	0.587	0.643	0.710.	-	-
	D	-	0.190	0.253	0.355	-	0.554	0.587	0.643	0.710.	-	-
Ceric sulphate	**A**	0.085	0.193	-	0.356	0.410	0.552	0.584	-	0.713	-	0.858
	B	0.085	0.193	-	-	0.410	0.552	0.584	-	0.713	-	0.858
	C	0.085	0.193	-	-	0.410	0.552	0.584	-	0.713	-	-
	D	--	0.193	-	0.356	0.410	0.552	0.584	-	0.713	-	0.858
Benzaldehyde	**A**	-	0.198	-	-	-	-	-	-	0.715	-	-
	B	-	0.198	-	-	-	-	-	-	0.715	-	-
	C	-	0.198	-	0.350	-	0.550	0.589	0.647	0.715	0.832	-
	D	-	0.198	-	0.350	-	0.550	0.588	0.647	-	0.832	0.855
Molybdophosphoric acid	**A**	-	0.195	0.253	-	0.415	-	0.580	0.645	0.716	-	-
	B	-	0.195	0.253	-	-	0.555	0.580	-	0.716	-	-
	C	-	0.195	0.253	-	-	0.555	0.580	-	0.716	-	-
	D	-	0.195	0.253	-	0.415	0555	-	-	0.716	-	-
Standard		-	-	-	-	-	-	-	-	-	-	-
Berberine		-	-	-	-	-	0.553	-	-	-	-	-
Tinosporaside		-	0.196	-	-	-	-	-	-	-	-	-

Supportive plants: A: wall; B: A. indica; C: P. pinnata; D: Z. jujuba

TLC Profile of Tissue culture grown plants: The grown plants in tissue culture were studied for comparative TLC profile of *T. cordifolia* with normal growing plant along tissue culture grown plant. Explants grown with different concentration aqueous extract of *A. indica* and TLC profile of explants juice was developed in solvent system n-Butanol: Acetic acid: Water (50:10:40). Fig 1 shows the comparative profile of *T. cordifolia* grown in tissue culture with different concentration of *A. indica* root extract and with normal plant. TLC Profiles were found to be highly affected when observed after 8 days of culture. But significant difference was observed among the TLC profile and tinosporoside contents of plants after 15 days growth of tissue culture plants in presence of *A. indica* root extracts (Fig 1).

DISCUSSION

T. cordifolia is very important plant according to Ayurveda as well as for the traditional healer. The stem of *T. cordifolia* is used as a therapeutic agent and because of this whole plant need to be destroyed, which disturbed its natural population leading to unavailability of good quality plant material for therapeutic use. Because of its heavy demand and indiscriminate use the plant is reported to be rare and endangered in several states of India by various authors. The plant species, which are supposed to be rare and endangered, should be conserved through developing efficient protocols for their multiplication.

An efficient protocol for *in vitro* micro propagation of *T. cordifolia* from auxiliary bud explants was developed. *In vitro* germination of the auxiliary buds was standardized on hormone free MS Medium. The maximum multiple shoot formation was achieved on simple MS from the decapitated auxiliary bud explants. It was observed that *In vitro* conditions provide an improved micro propagation environment and thus increased growth rates for the *T. cordifolia* plants. The improved plantlet quality and survival rates increased during the acclimatization period after deflasking. However, the trials undertaken in the current project did not include other conditions i.e. light CO_2 levels etc. It was found with *T. cordifolia* that optimum germination occurs with low light and ambient carbon dioxide levels.

In order to get maximum efficacy there is need to select correct chemotype of the plant. Even when there are many known chemotypes of a plant species, selection of the right chemotype to which clinical effects are attributed is difficult [11]. Therefore, by keeping in view above discussed limitations, in the present study we standardize the

TLC profile of *T. cordifolia* supported by various trees. The chemical constituent of the plants varies according to their allelopathic interactions, environment and genetic makeup. *T. cordifolia* is an herb and need support to climb up and grow efficiently. Every plant is of different nature and provides a microenvironment in its surrounding. The studies conducted to find out the chemical constituents variations in the *T. cordifolia* supported by various plants. Juice of the plants collected form various source of the same garden were analyzed by developing the TLC plates in solvent system n- Butanol: Acetic acid: Water (50:10:40). Vanillin and Iodine sprays showed all most 11 bands of Rf values 0.0.89, 0.192, 0.253, 0.356, 0.410, 0.551, 0.587, 0.641, 0.717, 0.883 and 0.858. Out of the 11 spots three of Rf value 0.192, 0.551 and 0.717 were found to be universally present in the plants used in the study. The spot of Rf 0.192 was found to be Tinosporaside, one of the major constituents of the plant. Tinosporaside having Rf 0.192 was found to be present in higher quantity in the plants supported by *A. indica* and *Z. jujuba* (Fig 3). Berberine levels were also found to be varied according to the support or environment at Rf 0.553 (Fig 3). Therefore, all the Tinosporaside, berberine and one unknown metabolite of Rf 0.717 can be used as chemical marker for the identification of plant or plant extracts.

Densitometric profiles of the TLC Plates showed variable amounts of chemical constituents in the juice of plants supported by various trees (Fig 3) Tinosporaside contents were found to be highest in the plant supported by *A. indica* tree as comparative the plant supported by wall. Berberine contents were found to be almost same in all the plants, a bit higher levels were observed in the plant supported by *P. pinnata*. Tinosporaside and berberine are reported to be active principles of plant [12, 13]. The levels of another unknown metabolite of Rf value 0.356 were found to high in the plant supported by *P. pinnata*.

The antioxidant, anti-inflammatory and immuno-modulatory properties of *T. cordifolia* has also been well documented [5, 14, 15]. Although, reports regarding the use of *T. cordifolia* as a neuroprotective were not available [14]. TLC profile for antioxidants showed the presence of highest quantity of antioxidants in the plant supported by *A. indica* (Table 1). Tinosporaside and berberine showed to have antioxidant activity. However, overall antioxidant activity was observed to be high in the plant supported by *A. indica*. Tinosporaside also showed to have maximum antioxidant activity. Therefore, the plants grown along with *A. indica* have maximum antioxidant activity should be used to get maximum benefit of therapy.

The tissue culture grown plants were studied for comparative TLC profile with normal growing plant. TLC profile of explants grown in presence of different concentrations of aqueous extract of *A. indica* and of others was developed in solvent system n-Butanol: Acetic acid: Water (50:10:40). Fig 1 shows the comparative profile of *T. cordifolia* grown in tissue culture with different concentration of *A. indica* root extract and with normal plant. At initial stages variations in TLC profiles were observed but minimum differences were noticed after 15 days culture (Figure 1). However, the profiles were found to have variations due to support differences as discussed above.

The observation leads towards the hypothesis that the rooting as well as aerial part of trees creates a microenvironment around the tree, which affects the chemo profile of *T. cordifolia*. The volatile compounds of *A. indica* that are present in environment probably affect the chemo profile of *T. cordifolia* through specific gene induction. However, how *A. indica* affects the growth of shrub is not known.

It is very important to standardize protocol for multiplication of endangered plants to preserve the nature and to standardize the TLC profile of plant for quality control purpose. A number of laboratories have standardized the TLC and HPTLC profiles of T. *cordifolia* without proper care toward the facts. Our studies clearly shows the importance of microenvironment provide by the trees in surroundings. Maximum antioxidant compounds were observed in the TLC profile of *T. cordifolia* grown along with *A. indica*. The present studies establish an efficient protocol for *ex-situ* conservation of *T. cordifolia*. We also standardized the TLC profiles of *T. cordifolia* from various sources. We observed the differences in TLC profiles of *T. cordifolia* supported by various trees. It was observed that all major bands observed in both cultured and field-grown plant of similar genetic makeup may have difference in chemo profiles due to environment. It was interested to observe that TLC profile of *T. cordifolia* changed along with its supporting tree. However, it is not known how the TLC profile of plant gets affected by the supporting trees contacts.

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