

EVALUATION OF METHANOLIC EXTRACTS OF *IN VITRO* GROWN *TINOSPORA CORDIFOLIA* (WILLD) FOR ANTIBACTERIAL ACTIVITIES

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ABSTRACT

Tinospora cordifolia commonly known as Guduchi, is an endangered, medicinal plants found throughout India, used in Ayurvedic rasayans to improve the immune system, memory and mental intelligence. It is also regarded as a liver projector. Due to ever growing demand, the tissue culture techniques have been employed for multiplication and conservation of the important medicinal plant like *Tinospora cordifolia*. Thus in order to obtain *in vitro* plants, a protocol was developed for rapid clonal propagation of *Tinospora Cordifolia* through nodal explants on Murashige and Skoog medium containing different concentration of growth regulators BAP (1- 2 mg/l) and Kinetin (1- 2 mg/l). The elongated shoots transferred on MS medium supplemented with NAA (0.5- 1.0 mg/l) in order to develop roots thus *in vitro* grown rooted plants were transferred to sandy soil. The methanolic extract of this plant was found to have antimicrobial activity against *Bacillus subtilis* (MTCC8), *E coli* (MTCC1), *Staphylococcus aureus* (MTCC98), *Salmonella typhi* (MTCC737). The largest zone of inhibition (18 mm) was found against *Staphylococcus aureus*. The preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, saponins, carbohydrates, and steroids in *in vitro* grown products like callus, leafyshoots and roots of *Tinospora cordifolia*.

Keywords: *Tinospora cordifolia* (Willd), *In vitro* multiplication, methanolic extracts, antibacterial activity.

INTRODUCTION

Medicinal plants have contributed immensely to health care. An impressive number of modern drugs have been isolated from natural sources, notably of plant origin¹.

Tinospora cordifolia (Willd.) Miers Hook.f.&Thoms.is a widely used in folklore and Ayurvedic systems of medicine, belongs to the family menispermaceae It is a glabrous climbing shrub with heart shaped leave and distributed throughout tropical Indian subcontinents². Plants have been a source of medicine in the past centuries and today scientists highlighted the potential health beneficial principles from phytal sources. The WHO estimates that up to 80% of people still rely mainly on traditional remedies such as herbs for their medicine³. The notable medicinal properties reported are anti-diabetic, anti-periodic, anti-spasmodic,

anti microbial, anti-inflammatory, anti-arthritic, antioxidant, anti-allergic, anti-stress, anti-leprotic, antimalarial, hepatoprotective, immunomodulatory and antineoplastic

activities^{4,5,6}. Its root, stem and leaves, are used for their medicinal properties. The root of this plant is known for its anti-stress, anti-leprotic and anti-malarial activities⁷. The anti-microbial activity of *T. cordifolia* was observed in root, stem and leaf extracts on pathogenic microorganisms^{8,9,10}.

The stem of *Tinospora cordifolia* is one of the constituents of several ayurvedic

preparations used in general debility, dyspepsia, fever and urinary diseases^{11,12}.

An impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine¹³.

Therefore, the micropropagation protocols for elite stocks of *T. cordifolia* are urgently needed. Some studies have reported *in vitro* plant regeneration and micropropagation of

T. cordifolia needed. Micropropagation through culture of explants having pre-existing meristem is powerful options which allow multiplying genetically stable and true-to-type progeny of the species that are rare, endangered and difficult to propagate^{14,15}. *In vitro* propagation in *T. cordifolia* was reported through nodal segments through axillary shoot proliferation^{16,17}. Micropropagation ensures not only continuous supply of plant throughout the year but also prevent the destruction of the natural population of medicinal plant^{18,19}. During past few years, there has been an increase interest for *in vitro* multiplications and germplasm

conservation of rare, endangered, aromatic and medicinal plants^{20,21}, to develop of efficient protocols for its regeneration through tissue culture methods. The available literature on the medicinal properties of this plant pertains mainly to the field grown plants. Although the plant was successfully raised under *in vitro* tissue culture condition, to study the antimicrobial activity of *in vitro* raised plant of *T. cordifolia*. In this report, we describe the procedure for proliferation, maintenance of *in vitro* grown plants and callus, both on MS medium supplemented with growth regulators and antimicrobial activity of methanolic extract of *T. cordifolia* as well as its preliminary phytochemicals sceneing.

MATERIALS & METHODS

Source of Explants

In order to obtain *in vitro* plants, a protocol was developed for rapid clonal propagation of *Tinospora cordifolia* through mature nodal explants. The explants were collected from the campus of the department of Botany, B R A Bihar University, Muzaffarpur. These nodal segments were cuts into 1.5cm to 2.0cm length with single node & internode intact. These nodal & internodal cutting were washed with 5% (v/v) detergent solution (tepol) for 10minutes followed by rinsed with running tap water for several times. In the laminar chamber the nodal segments were further treated with 70% alcohol for one minute followed by 0.1% (w/v) mercuric chloride treatment for 5 minutes, aseptically explants were washed with sterile distilled water for 3 to 4 times dried using sterile blotting paper which was ultimately used as explants for raising *in vitro* cultures.

Growth medium & culture condition

Murashige and Skoogs²² (MS) medium 1962 was used as nutrient source with or without growth hormones such as BAP, KN, and NAA either individually or in combination for culture explants. Sucrose 3% and agar 0.8% (Hi media) were used as carbon source and gelling agent respectively. The pH of the medium was adjusted to 5.8 and autoclaved at 121°C for 20 minutes. The cultures were maintained in the culture room at 25±C 2°C, under white fluorescent light.

Shoot organogenesis and elongation

For multiple shoot induction from nodal explants were culture on MS medium supplemented with BAP (1- 5 mg/l) alone or combination with Kinetin (1-2 mg/l) (Table 1). Data on percentage

of responding explants and number of shoots per explants were recorded after 25 days of initiation culture.

In vitro rooting plant establishment

Production of plantlets with rooting *in vitro* is important for successful establishments of regenerated plants in soil. The auxin NAA was used to induce rooting from *in vitro* raised shoot lets. A range of concentration was tested (0.5, 1.0, 1.5 mg/l). for rooting . The maximum rooting was obtained on MS with NAA (0.5mg/l). The well rooting plant lets were transferred to plastic cups containing sandy soil.Plants started producing fresh shots and roots after one week of transplanting. Later they were transported to the field condition and the survival rate was 70%.

Phytochemical Screening

The role of medicinal plants in disease control has been attributed to antioxidant properties of their constituents²³. The protective effect of plant products are due to the presence of several components such as enzymes, proteins, vitamins²⁴, carotenoids²⁵ flavonoids²⁶ and other phenolic compounds²⁷. The methanolic extract of *in vitro* grown plants of *Tinospora cordifolia* (Miers.) were subjected to different tests to identify the nature of chemical constituents present in the plant material. The crude extract were screened qualitatively for the phytochemical constituents utilizing standard methods of analysis^{28,29}.

Preliminary Phytochemical Screening

The aqueous extract of the plant samples were prepared by soaking 100gms of *in vitro* grown *Tinospora cordifolia* plant extract in 200ml of methanol solvent.

A. Test for alkaloids: Presence of alkaloids in *Tinospora cordifolia* extract was determined by using two reagents, Drangendroffs reagent and Mayer's reagent . One ml extrate was mixed with 2ml of Drangendroffs reagent yielded orange colour similarly one ml of extract mixed with few drops of Mayer's reagent resulted into buff coloured precipitate. Both of these test confirms the presence of alkaloids in the methanolic extract.

B. Test for Carbohydrates (i) One 1ml of extract was mixed with 5ml Benedict's reagent. The content was boiled for 5 minutes, yielded. The develop colour indicated the presence of carbohydrates in mehanolic extract.

(ii) In 1ml of extract mixed with few drops of Molisch's reagent followed by few drops of conc. H₂SO₄. A red or dull violet colour was precipitated indicate the presence of carbohydrates in methanolic extract of plants.

C. Test for Reducing sugar: One ml of the extract mixed with, few drops of Fehlings B. The brown precipitate was indicated the presence of reducing sugar in methanolic extract.

D. Test for Steroids: One 1ml of the extract was mixed with 10ml chloroform followed by addition of H₂SO₄. The red yielded, conforms the presence of steroids in methnolic extract.

E. Test for Terpenoids: One ml of the extract was mixed with 2ml CHCl₃ followed by added few drops of conc. H₂SO₄. A reddish brown colour appeared at interface, conforms the presence of terpenoids.

F. Test for Saponins: One 1ml of filtrare was diluted with 2ml distilled water, was shaken vigorously and allowed to stand for 10 minutes. Development of foam on surface of the mixture, lasting for 10 minutes indicated the presence of saponins.

G. Test for flavonoids: The 1ml of filtrate was mixed with 2ml of 10% lead acetate. Development of brown precipitate indicated the presence of flavonoids (Trease and Evans, 2002).

.Further work is necessary to isolate and purify the compounds of *in vitro* grown *Tinospora cordifolia* plants and callus extracts, which will allow the scientific community to recommend their utilization as an accessible alternative to synthetic antibiotic.

Antibacterial Assay

Preparation of Plant Extract

In Vitro grown plants, callus, were weighted and grinded with help of motor & pestle. About 50gm of power grinded material was extracted in soxhlet apparatus with 250ml of methanolic solvent³⁰. The extracted solvent was removed from the extract under reduced pressure with rotary vacuum evaporator. The sticky greenish-brown substance was obtained was stored in refrigerator condition till use.

Culture of microorganism

Both gram positive and gram negative bacteria were used as test organism for this study. The organisms like *Escherichia coli* MTCC 2845, *Salmonella typhi* MTCC 737, *Staphylococcus aureus* MTCC 98, *Bacillus subtilis* MTCC 441, were used for antibacterial assay. The bacteria were grown in nutrient broth media at 37°C, for antibacterial assays. The disc diffusion method³¹ was adopted for antimicrobial study of plant extract. Briefly, 20ml of media was transferred aseptically into each sterile petri dishes and allowed to solidify. An over night grown inoculums (100µl) suspension was spread uniformly over the agar medium using sterile glass rod for uniform distribution of bacteria. The readily prepared sterile discs were loaded with different concentrations of about 10-20 µg of plant extract of *Tinospora cordifolia*. The paper diffused discs were placed on the medium and plates were incubated at 37°C for 24 hours. The antibacterial activity was recorded by measuring the width of the clear inhibition zone around the disc in millimeter.

RESULTS AND DISCUSSION

Morphogenetic responses of the nodal explants of *T. cordifolia* cultured on MS medium containing BAP either alone or in combination with kinetin and NAA are summarized in (Table1). Explants cultured on MS medium without cytokinin failed to induce shoot proliferation. The stimulating effect of BAP on multiple shoot formation has been reported for several medicinal plant species^{32,33,34}. The frequency of axillary shoots initiation and development per explants were increased with an increased in concentration of BAP up to 2mg/l (Fig- A).

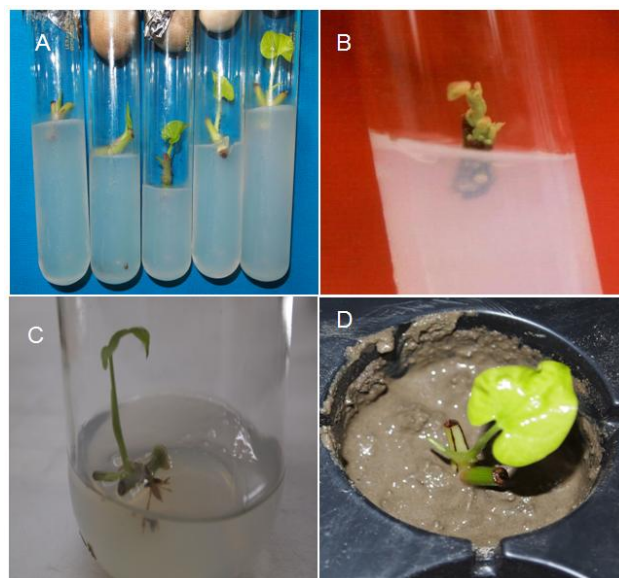


Figure 1: *In vitro* clonal propagation of *Tinospora cordifolia* (Miers.) from internodal explants.

- A. Shoot development and elongation from nodal explants on MS + BAP (1mg/l).**
B. Multiple shoot formation from nodal explant on MS + BAP (1mg/l) + KN (0.5mg/l).
C. *In vitro* root induction on MS + NAA (0.5mg/l).
D. Hardening of tissue culture raised plantlets.

Table 1: Effect of cytokinins and auxin and their interaction on shoot proliferation from Nodal explants of *Tinospora cordifolia*.

Growth regulator gm/l	concentrati on	% of response	Induction of shoots per explants	Induction of roots per explants
BAP	.5	50	1.0	0.0
	1	70	1-2	0.0
	2	60	0.0	0.0
KN	.5	40	1.0	0.0
	1	70	1.0	0.0
	2	60	0.0	0.0
BAP+ KN	.5 + .5	70	2-3	0.0
	1+ .5	74	3-4	0.0
	1+ 1	65	1	0.0
NAA	.5	0.0	0	1-2
	1	50	0	0.0
	2	40	0	0.0
BAP + NAA	.5 + .5	80	2-3	4-5
	1 + .5	90	3	5.0-7.0

The higher concentrations of BAP not only reduced the number of shoots proliferation on nodal explants but also resulted in stunted growth of the shoots. When the explants were cultured on MS medium with BAP 1mg/l and kinetin (0.5mg/l) the number of shoots proliferation was increased in each explants (fig-B). When BAP was used in combination with NAA the inductions of shoot as well as root both take place (Fig-C). The complete plantlets were carefully removed from the medium and washed gently with sterilized double distilled water to remove any traces of medium on roots. Then they were transferred to sterile soil. Plantlets were hardened by keeping inverted glass beakers onto them to provide high humidity, controlled temperature and the intensity of light could be assured by cool, white fluorescent tubes. After two weeks, plantlets were gradually transferred to partial sunlight for acclimatization. Finally, they were transplanted to earthen pots containing sterile garden soil (Fig- D). After 22-28 days, these plantlets were simultaneously exposed to natural environment, where 70% plantlets survived well in the field (Table- 2).

Table 2: Data on Survival of transferred Regenerated Plants

	No. of Plants	of Plants Survived	% Response
Transferred for Hardening	30	22	73.3
Transferred for Acclimatization	20	14	70

In this investigation, during the process of transfer to field the survival percentage was found to be decreased. There are various reasons for low survival of regenerated plants after transfer to natural soil. Gangopadhyay et al.³⁵ (2002) reported that *in vitro* derived roots are often physiological functioning when in contact with the soil. The efficient micro propagation technique described here may be highly useful for raising disease free quality planting material of *T. cordifolia* for commercial and off season cultivation which not only help in improving economic condition of the farmer but also fulfill the market demand of herbal industries.

The antibacterial activity of aqueous, ethanolic and chloroform stem extract of *T. cordifolia* against number of Gram negative and Gram positive bacteria was reported by suggesting significant antimicrobial activity of ethanolic extract³⁶. The plants and their extracts used against microbial infections due to the presence of secondary metabolites such as phenols, flavonoids, tannins, terpenoids etc³⁷ have documented the use of natural products as new antibacterial drugs. Phytochemical investigations of crude plant extracts shows the presence of active principles in the *in vitro* grown plant parts like leaves, stem, roots, and callus. Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. Plant produces these chemicals to protect itself but research works demonstrates that many phytochemicals can protect humans against diseases. Knowledge of the chemical constituents of plants is desirable because such information will be

of valuable for the synthesis of complex chemical substances. Preliminary phytochemical analysis of methanolic extract revealed the presence of alkaloids, carbohydrates, reducing sugar, glycosides, steroids, terpenes, saponins, and flavonoids (Table-3). The inhibitory effects of this medicinal plant on the test microorganisms may therefore, be due to the presence of the above phytochemical components.

Table 3: Phytochemical screening of methanolic extract of *Tinospora cordifolia*.

Sl. No.	Phytochemical Test	Methnol Extract
1	Alkaloids	+++ ++
2	Carbohydrates	+++
3	Reducing Sugar	+++
4	Glycosides	++
5	Steroids	+++ ++
6	Terpenes	+++ +
7	Saponins	+++
8	Flavonoids	++

+++ Prominently Present, ++ Moderately Present, + Slightly Present

The antibacterial activity of aqueous, ethanolic and chloroform stem extract of *T. cordifolia* against number of Gram negative and Gram positive bacteria was reported by suggesting significant antimicrobial activity of ethanolic extract (Jeychandran et

al., 2003). In the present work, the antimicrobial activity of methanolic extracts of *in vitro* grown products like callus, plantlets and roots of *T. cordifolia* was examined, the result shown in the Table- 4.

Table 4: Evaluation of antibacterial activity of the methanolic root extract of *in vitro* grown plants of *Tinospora cordifolia*.

S.No	Microorganisms	Zone of Inhibition(m m) 10mg	Zone of inhibition(m m) 20mg
1	<i>Escherichia coli</i> (-ve)	10mm	15mm
2	<i>Salmonella typhi</i> (-ve)	8mm	10mm
3	<i>Staphylococcus aureus</i> (+ve)	13mm	18mm
4	<i>Bacillus subtilis</i> (+ve)	9mm	16mm

(+ve) gram positive bacteria, (-ve) gram negative bacteria

The methanolic extracts of *in vitro* grown plants and callus showed a broad spectrum of activity against all the bacterial strains at the tested concentration of 10 - 20µg/disc. They exhibited greater zone of inhibition for *Staphylococcus aureus* (18mm). The least activity was observed for *S. typhi* (8mm). The plants and their extracts used against microbial infections due to the presence of secondary metabolites such as alkaloids,

phenols, flavonoids, terpenoids etc^{22,23} have documented the use of natural products as new antibacterial drugs. Preliminary phytochemical analysis of methanolic extract revealed the presence of alkaloids, carbohydrates, reducing sugar, glycosides, steroids, terpenes, lignin, saponins, tannins and flavonoids. The inhibitory effects of this medicinal plant on the test microorganisms may therefore, be due to the presence of the above phytochemical components. The isolation, and characterize, of the photochemical compounds of the *in vitro* grown plants as potent antibacterial agents.

CONCLUSION

In conclusion, an efficient protocol was developed for multiple plant regeneration of an important medicinal plant *Tinospora cordifolia* (will). The protocol reported in this study can be used for rapid and large scale propagation of *Tinospora cordifolia*. The methanolic extract of *in vitro* grown *Tinospora cordifolia* was found to be

sensitive to different microorganisms, thus this plant could be utilized as a natural source of antimicrobial drugs. Further studies are needed to isolate, characterize, and elucidation of the phytochemical compounds of the in vitro grown plants for formulation of antimicrobial drugs.

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