

Comparative Microbicidal Efficacy of Fractionated Extracts from *In Vitro* and *In Vivo* Raised Cells of *Tinosporacordifolia* Against MDR Pathogens

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ABSTRACT

The present study was conducted to explore the hidden potential of natural products synthesized in the medicinal plant *Tinosporacordifolia*. This plant is prioritized by National Medicinal Plant Board, New Delhi. Leaf and inter nodal segments were inoculated on MS Medium fortified with IBA (1.0 mg/L) produced callus after four weeks. The calli were brown due to phenolic substance secreted by the explant. This problem was overcome by using adjuvant PVP (0.1%). Further, secondary metabolites were isolated from callus and field leaf through Soxhlet extractor and fractionated by using column chromatography. The antibacterial activity of these fractionated extracts from *Tinosporacordifolia* callus and leaf were seen against multi drug resistance bacteria viz., *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa*, (ATCC 27853) & *Staphylococcus aureus* (ATCC 29213) and against plant pathogenic fungus *Fusicladium oxysporum* (MTCC 8608) and *Sclerotinia sclerotiorum* (MTCC 8785). All fractionated extracts showed antimicrobial activity but callus extracts were proved to be best in compare to leaf extracts. Furthermore, we are trying to analyze different bio active compounds through GCMS.

Key words: Medicinal plants, callus, natural products, PVP, Antimicrobial activity

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INTRODUCTION

Plant cells are factories of natural compounds which are used to produce drug to cure a number of ailments from prehistoric time as ethno medicines. These natural products have also been using in pharmaceuticals, agrochemicals, ingredients of flavors and fragrances, food additives and pesticides (Sharma et al. 2010). The natural habitats for medicinal plants are vanishing at a faster rate due to developmental activities of nation. Hence, it has become difficult to acquire natural compounds from the phytodiversity. Even though, pharmacological industries have produced a number of new synthetic antibiotics in the last few decades, but resistance to these drugs has increased in microorganisms (Wright 2010). This has encouraged industries, as well as scientists to consider the possibilities of investigation of cell cultures as an alternative supply for the production of plant pharmaceuticals (Yue et al. 2014).

In the search for alternative to produce desirable medicinal compounds from plants, biotechnological approaches especially plant tissue culture technology is found to have potential applications over traditional agriculture practices for the industrial production of bioactive plant metabolites (Barbulova et al. 2014).

Some of these natural products have been produced through callus culture using *Phyllanthusamarus* (Sen et al. 2009), *Verbesinaencelioides* (Karnawat et al. 2010) and *Polygonummultiflorum* (Zhou et al. 2011). The possible applications of plant cell cultures for the specific biotransformation of natural compounds have been reported (Sharma et al. 2012; Barbulova et al. 2014). Due to these advances, research in the area of tissue culture technology for the production of bioactive compounds has gained popularity beyond expectations.

Keeping in view the high demand of these bioactive compounds for commercial production of drugs, efforts have been made to utilize a medicinally potent plant *Tinosporacordifolia*(Willd.) Miersex Hook. F. and Thoms (family Menispermaceae) to analyze its therapeutic values against the microbes which have high resistance against the existing drugs. It is a large, deciduous,

climbing shrub found throughout India, especially in the tropical parts ascending to an altitude of 300 m. and also in certain parts of China (Anonymous 1976). It is rich with a variety of natural chemical constituents (Berberine, Tinosporin, Cordifolioside) (Patel et al. 2011; Sangeetha et al. 2013). These natural products have been used to cure a number of ailments like viral infections, cancer, diabetes, inflammation, immunomodulatory activity, neurological, psychiatric conditions, vasorelaxant, inflammation, microbial infection, hypertension, HIV etc. (Patel et al. 2009; Upadhyay et al. 2011; Nagarkar et al. 2013; Joladarash et al. 2014; Mittal et al. 2014).

This plant species has a huge variety of bioactive compounds; hence, it requires scientific attention for its conservation to continuous supply of these bioactive compounds. To fulfill the present day needs, callus culture is the alternative method to conserve and enhance the production of secondary metabolites under laboratory conditions. Therefore, the present investigation was carried out with the objectives to provide new vistas on the cell culture to elucidate the effect of plant growth regulators on callus cultures and role of different adjuvants to overcome leaching of phenolic substances. Isolation and fractionation of secondary metabolites of *in vivo* plant leaf and callus has been done. Antimicrobial efficacy of both the purified extracts in their fractions has evaluated against different pathogenic bacteria and plant pathogenic fungus by disc diffusion method and food poisoning method respectively. Serial dilution turbidity measurement assay has been also carried out to calculate minimum inhibitory concentration (MIC).

MATERIAL AND METHODS

Explant preparation:

Fresh twigs were collected from one year old plant and cut into 1.0-1.5 cm long internodal segments and leaf segments (1.0 cm²) for callus production. These explants were surface sterilized under running tap water to remove soil particles and other surface debris on plants followed by washing with 0.5% Tween 20 (1-2 minutes) for removal of surface contaminants. Subsequently, explants were subjected to

surface sterilization in laminar air flow by successively dipping in 70% ethanol for 1 min and 0.1% mercuric chloride for 1 min. Finally, the sterilized explants were rinsed with autoclaved distilled water for three times under aseptic conditions and used for inoculation.

Nutrient Medium and Hormone:

Murashige and Skoog (MS) Medium with different concentrations of auxin such as IBA, IAA, NAA, 2,4-D (0.5-5.0 mg/L) and cytokinin viz., BAP, Kinetin, TDZ, 2iP (0.5-5.0 mg/L) were used for callus production. The MS medium consisted of 95% water, macro and micronutrients, vitamins, amino acids, sugars. Besides, MS medium contained sucrose (3.0%) as a carbon source and agar powder (0.8%) as a gelling agent to make this medium semisolid.

Inoculation and culture of explants:

Surface sterilized explants were inoculated on MS medium fortified with various auxin viz. IBA, IAA, NAA, 2,4-D (0.5-5.0 mg/L) and cytokinin such as BAP, Kn, TDZ, 2iP (0.5-5.0 mg/l) for callus production. The culture vials were incubated at $25^{\circ} \pm 2^{\circ}$ C temperature under 16 h photoperiod with cool, white fluorescent lights (Philips India Ltd, Mumbai) of $25 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity and 8 hrs. dark cycle with $55 \pm 5\%$ relative humidity. All experiments were set with eight replicates and repeated at least thrice. All cultures were observed daily for the observation of gradual changes. Morphological changes were documented with the photographs and recorded. All data were subjected to statistical methods.

Experiment to overcome leaching:

After 15-18 days of inoculation green callus was produced but after 3-4 days it turns brown to black due to secretion of phenolic substances and stop further growth of callus. To overcome these problem explants were soaked in DI water for 2 hrs. Subsequently these explants were soaked in a mixture of activated charcoal, PVP, ascorbic acid and citric acid (1%: 0.1%: 0.05%: 0.05%) for 30, 45, 60 min. Finally these explants were washed with autoclaved distilled water and go through surface sterilization under laminar air

flow. For reduction of leaching MS medium was also fortified with different adjuvants viz., activated charcoal (0.1-5.0%), PVP (0.1-1.0%), Ascorbic Acid (0.01-0.5%), Citric Acid (0.01-0.5%) alone and in combination also.

Isolation of secondary metabolites:

Fresh leaves were collected and washed under running tap water to remove adhering soil particles. The leaves and 4 weeks old callus induced from inter nodal explants were dried at room temperature for one week to make it coarse powdered and stored in an air tight container. 20g of powdered samples of leaves and callus was extracted with methanol at 50°C temperature in soxhlet extractor apparatus (Borosil, India) of 72 h. The collected extracts were concentrated using a rotary evaporator to get a viscous mass at 45°C . The viscous masses were fractionated with different solvents using column chromatography. 2 gram dry extract was subjected to a silica gel-60 column chromatography and eluted with petroleum ether (100%), petroleum ether: chloroform (80:20, 60:40, 40:60, 20:80), chloroform (100%), chloroform: ethyl acetate (80:20, 60:40, 40:60, 20:80), ethyl acetate (100%), ethyl acetate: methanol (80:20, 60:40, 40:60, 20:80) and methanol (100%). All fractions were filtered using syringe filter ($0.22 \mu\text{m}$) and concentrated to dryness under reduced pressure below 60° using a rotary flash evaporator (Heidolph, India) for further analysis.

Antimicrobial assay:

All fractionated extracts were dissolved in DMSO (Dimethyl Sulphoxide) to give a final concentration of 100 mg/mL each. Three different bacterial strains *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 were used for antibacterial activity and two plant pathogenic fungus *Fusarium oxisporum* MTCC 8608 and *Sclerotinia sclerotiorum* MTCC 8785 were used for antifungal activity.

Minimum inhibitory concentration (MIC) of fractionated extracts were evaluated against bacteria and fungus by serial dilution turbidity measurement assay. For serial dilution turbidity measurement assay, nutrients broths

were prepared and distributed in several culture tubes. Each tube contains 10 mL of nutrient broth. The stock solution of isolated fractions was serially diluted at 10^{-1} , 10^{-2} and 10^{-3} level. The tubes were inoculated with 50 μ L of 24 h old bacterial suspension. Each tube was mixed with 0.1 mL of plant extract fractions of different concentration. The tubes were incubated for 24 h at 37°C and OD was taken at 600 nm by using spectrophotometer (Systronics, India) to measure the turbidity against a sterile blank. A negative control without bacterium and a positive control without plant extract were incubated for comparative purpose. All the experiments were done in triplicate.

Antibacterial activities were carried out by agar disc diffusion method. The nutrient agar plates were used for culture of all bacterial strains and for evaluating antibacterial activity. The bacterial suspension was spread equally on the nutrient agar plates using sterilized spreader. The sterile Whatman No 1 paper disc at 6 mm diameter was soaked in different fractionated extracts at minimum inhibitory concentrations evaluated by previous experiment. The disc without any solution, placed on the plates was act as control. These NA plates were incubated in for 24 h at 37°C. However, the susceptibility of the test organism was determined by measuring the diameter of the zone of inhibition (ZOI) and all data were statistically analyzed.

Antifungal efficacy was also analyzed by the standard food poisoning method. Different fractions of secondary metabolites were mixed in Potato Dextrose Agar (PDA) medium. The fungal strain was inoculated by point inoculation method with the help of sterile needle. PDA plate without plant extract was maintained as control. These plates are incubated in inverted position for 72 h. at 27°C. Each experiment was carried out in triplicate and diameter of the zone of inhibition was measured and all data were statistically analyzed.

RESULT AND DISCUSSION

Callus initiation was observed on inter nodal segments inoculated on basal MS medium supplemented with various auxins either alone or in combination such as IBA, IAA, NAA, 2,4-D. Optimum results were observed when

IBA alone was used with MS medium in different concentration (0.5, 1, 1.5, 2.5 and 5.0 mg/L). The highest biomass of callus was obtained on IBA (1.0 mg/L). The proliferation efficiency of through inter nodal segments was significantly higher when compared with leaf explants within three to four weeks. Swelling of inter nodal segments was followed by callus initiation within one week of inoculation (Fig.1A). Further, cells simultaneously absorb nutrients from the medium and callus proliferation was obtained on MS medium augmented with IBA (1.0 mg/L). Callus was pale green, spongy, and fragile in nature (Fig. 1B and 1C). In consonance to our results of callus production, Gururaj et al. (2007) also reported callus production on MS medium supplemented with IBA (1.0 mg/L) and 2ip (0.5-1.0 mg/L) via nodal segments explant of *T. cordifolia*. However, in contrast to our findings, Rao et al. (2008) shoed initiation of callus on MS medium fortified with NAA (4.0 mg/L) using leaf explant of *T. cordifolia*.

The callus changed its colour from brown to black after three weeks (Fig. 1D). This is mainly due to the secretion of phenolic compounds in the medium, which prohibited further growth of callus. Different adjuvants were tested to overcome this leaching issue, out of them PVP (0.1%) proved to be the best in leaching control (Fig. 1E and F).

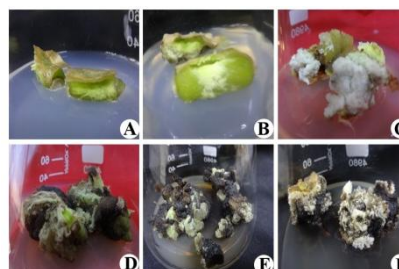


Figure 1-Different stages of callus production via inter nodal segments cultured on MS medium supplemented with IBA (1.0mg/L)

A: In vitro callus initiation after one week

B: Further growth of callus after two weeks

C: Growth of callus after sub culturing on the same medium

D: Browning of callus duet secretion of phenolic substances

E: Brown callus treated with PVP (0.1%)

F: Greening of callus treated with PVP (0.1%)

Subsequently, the callus was sub cultured after every two weeks on MS medium supplemented with IBA (1.0 mg/L) and PVP (0.1%).

Similarly, browning of medium and explants has been observed in *Dioscorea hispida* and *Dioscorea pentaphylla* (Shukla and Shukla, 2014). Sivakumar et al. (2014) also studied effect of four types of antioxidants and adjuvants on the control of explant and culture medium oxidation during *in vitro* cultivation of *T. cordifolia*.

Furthermore, secondary metabolites were also isolated from *in vivo* leaf and *in vitro* callus and purified by column chromatography for testing its antimicrobial efficacy. Total six fractions FC1, FC2, FC3, FC4, FC5 and FC6 were finally obtained from callus while six fractions FL1, FL2, FL3, FL4, FL5 and FL6 were obtained from leaf sample. All the fractions were assayed for testing its antimicrobial efficacy against three pathogenic bacterial strains and two plant pathogenic fungi.

In order to determine MIC, serial dilution turbidity measurement assay was carried out with a series of different concentration of extracted fractions of callus and leaf. The

results of serial dilution method are illustrated in Table 1. The fractionated extracts when added to the liquid medium, start their antimicrobial activities in terms of inhibition of the growth of microbes. This inhibition is measured in terms of optical density values (OD). The MIC value indicates that the fractionated extract of leaf and callus of *T. cordifolia* inhibit the growth of test organisms at different concentrations (30-50 ppm). The results of MIC assays showed that the antimicrobial activities of extracted fractions of callus (FC1-FC6) are as effective as extracted fractions of leaf (FL1-FL6) against different microbial strains tested. This is possibly due to the higher concentration of secondary metabolites in callus than leaf (Jain et al. 2012). The similar studies were also carried out using *Rhus coriaria* L. (Vahid-Dastjerdi et al. 2014), *Hydnora africana* (Wintola et al. 2015) and *Plantago lanceolata* (Ferrazzano et al. 2015) plant extracts.

Table 1: Minimum inhibitory concentration ($\mu\text{g/mL}$) of fractionated extract of callus and leaf against different pathogenic bacteria and plant pathogenic fungus

MIC Strain	MIC in $\mu\text{g/ml}$ (PPM)											
	FC1	FL1	FC2	FL2	FC3	FL3	FC4	FL4	FC5	FL5	FC6	FL6
<i>E. coli</i>	30	40	35	40	25	30	30	40	35	50	25	40
<i>S. aureus</i>	40	50	35	40	30	40	35	35	30	40	30	35
<i>P. aeruginosa</i>	45	45	40	40	35	40	40	50	35	50	30	40
<i>S. sclerotiorum</i>	50	50	50	50	40	45	45	50	40	50	40	50
<i>F. oxysporum</i>	45	50	45	50	40	45	45	45	40	45	40	50

All fractions of callus and leaf showed antibacterial as well as anti-fungal activity. The results of disc diffusion assays indicate that the fraction 3 of callus showed maximum ZOI against *E. coli*, while fraction 5 of callus showed maximum ZOI against *S. aureus* (Fig. 2A and B). For *P. aeruginosa* fraction 6 of callus proved to be the best (Fig. 2C). Plant pathogenic fungus *F. oxysporum* and *S. sclerotiorum* were analyzed by food poisoning method (Fig. 2D and E). Fraction 3 of callus showed maximum ZOI against *F.*

oxysporum and fraction 5 and 6 of callus were also showed significant ZOI against *F. oxysporum*, while fraction 6 showed maximum ZOI against *S. sclerotiorum*. Similarly to the MIC by serial dilution method, fractions of callus extracts showed better zone of inhibition (ZOI) by disc diffusion and food poisoning methods than fractions of leaf extracts. There are numerous reports in support to our findings (Srivastava et al. 2013; Castilho et al. 2014; Kumari et al. 2015; de Souza et al. 2015).

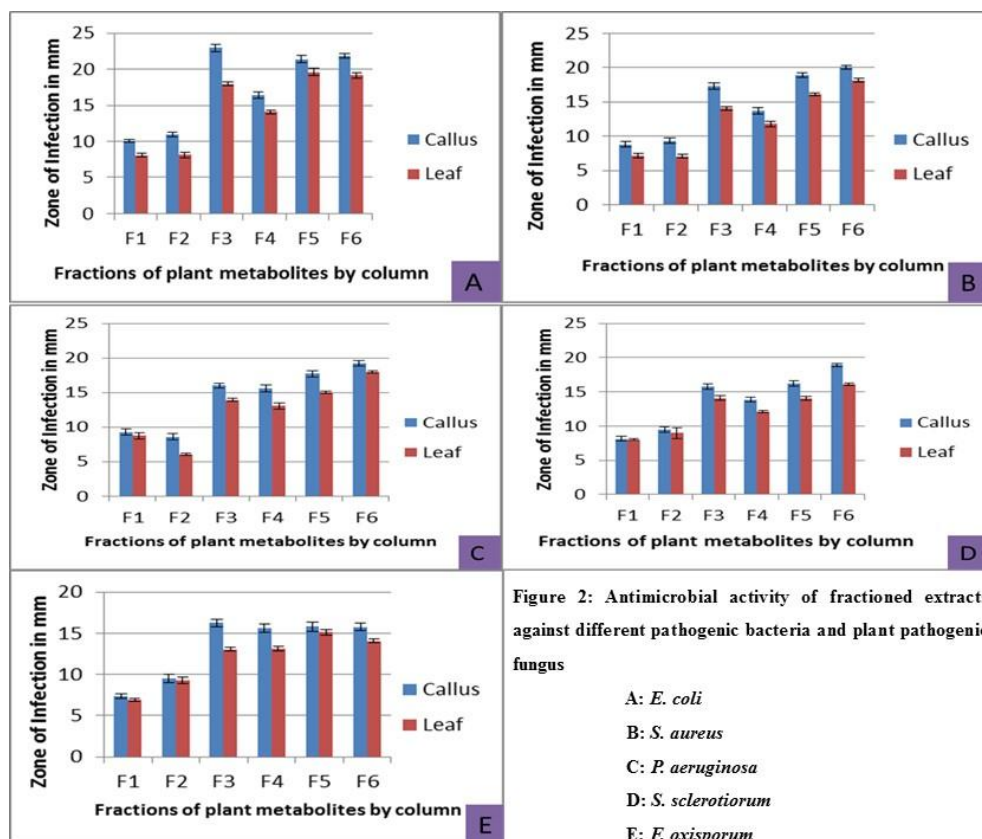


Figure 2- Antimicrobial activity of fractionated extracts against different pathogenic bacteria and plant pathogenic fungus

- A: *E. coli*
 B: *S. aureus*
 C: *P. aeruginosa*
 D: *S. sclerotiorum*
 E: *F. oxysporum*

Several reports suggest that the *in vitro* shoot culture show maximum accumulation of bioactive compounds (Sharda et al. 2007; Jain et al. 2011). In the present study the callus culture showed maximum ZOI may be due to higher concentration of secondary metabolites in the extract than that of the extract obtained from field leaves. Plant cell cultures supplemented with auxins have tendency to increase the endogenous auxin levels in the callus and hence decrease the morphogenetic potential. This results in higher accumulation of bioactive compounds in the callus (Das et al. 2013).

CONCLUSION

The callus was produced from inter nodal segments of *Tinosporacordifolia* for regular supply of bioactive compounds. Secondary metabolites were isolated and fractionated by

using column chromatography by *in vitro* produced callus as well as *in vivo* leaf samples. The antibacterial activity of these fractionated extracts from *Tinosporacordifolia* callus and leaf were seen against multi drug resistance bacteria and plant pathogenic fungus. All fractionated extracts showed antimicrobial activity but callus extracts were proved to be best in compare to leaf extracts. Further, we are trying to analyze different bio active compounds through GCMS.

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