Evaluation of Antimicrobial and Antioxidant potential of native and micropropagated plants of *Tylophora indica*

A

Dissertation Report

submitted in partial fulfillment for award of the degree of

Master of Science in Biotechnology

Submitted by

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DECLARATION

I hereby declare that the work presented in dissertation entitled, "**Evaluation of Antimicrobial and Antioxidant potential of native and micropropagated plants of *Tylophora indica***" in partial fulfilment of degree of Master of Science in Biotechnology, is an authentication record of my own work done during the period of six months from January 2016 to July 2016, under the guidance of **Dr. Manju Anand**, Assosciate Professor and **Dr. Siddharth Sharma**, Assistant Professor, Department of Biotechnology, Thapar University, Patiala. I have not submitted the matter embedded in this dissertation for the award of my any other degree or diploma.

Date: 15/07/16

Place: Patiala

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CERTIFICATE

This is to certify that the dissertation entitled "**Evaluation of Antimicrobial and Antioxidant potential of native and micropropagated plants of *Tylophora indica***" submitted by **Preetika** in partial fulfilment of the requirements for the award of degree of Master of Science in Biotechnology to Thapar University, Patiala, is an authentication record of student's own carried out by her during the period of six months from January 2016 to July 2016, under my supervision and guidance. The report has not been submitted for the award of any degree or certificate in any other University or Institute.

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To God be the glory. Great things He has done!

(PREETIKA)


Dedicated to
my beloved
Parents

ABBREVIATIONS

Aº	Angstrom
BAP	Benzylaminopurine
BMS	Basal Murashige and Skoog's medium
ºC	Degree Celsius
MS	Murashige and Skoo's medium
IAA	Indole 3- Acetic acid
IBA	Indole 3-bnutyric acid
Kn	Kinetin
NAA	Naphthalene Acetic acid
Mm	Micromolar
mM	Millimolar
Mg	Milligram
Mm	Millimeter
gm/l	Gramperliter
PGR	Plant growth regulator
Zn	Zeatin
2,4 –D	2, 4-dichloro phenoxy acetic acid
2-ip	2 -isopentyl adenine
TDZ	Thidiazuron
W	Watt
WHO	World Health Organization
DPPH	2,2-diphenyl-1- picrylhydrazyl
pH	Hydrogen ion concentration
UV	Ultra-violet
MHA	Muller Hinton Agar
SDA	Sabouraud Dextrose Agar
BOD	Biological Oxygen Demand

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

The present study was conducted on an important medicinal plant namely *Tylophora indica* (Burm. f) Merrill belonging to family Asclepiadaceae with an aim to establish an efficient and reproducible *in vitro* protocol for its mass cloning and evaluation of antimicrobial and antioxidant potential of in vivo and in vitro plants.

Tylophora indica is commonly known as 'Antmool' or 'Dama Bel' and it is known to have several pharmacological effects including anti-asthmatic, anti-inflammatory, antioxidant, diuretic, antimicrobial, anti-leukemia, immunosuppressive, anti-tumor and anti cancerous. Due to the lack of adequate propagation efforts and overexploitation of natural wild populations, *T. indica* is rapidly disappearing and is now listed as one of the plant species in India vulnerable to extinction. Therefore, we present an efficient and reproducible protocol for the mass propagation of this plant under *in vitro* conditions.

Leaf explants were excised from 5 year old field grown healthy plant and thereafter planted on variously supplemented Murashige and Skoog's (MS) medium for *De novo* adventitious shoot formation directly from the leaf explants. MS medium augmented with BAP either alone or in combination with adenine sulphate was most effective in inducing *de novo* adventitious shoots through the formation of meristemoids from leaf explants, where uncountable numbers of green healthy shoots developed on subsequent subculturing.

The regenerated shoots were carefully rescued from the culture vessels and subjected to rooting on half strength MS medium or MS medium supplemented with either IBA or IAA. The best rooting response (95%) was observed on MS medium supplemented with IBA where cluster of long healthy roots was formed. Half strength MS medium also showed equally good response inducing rooting in 88% of cultures but the number of roots formed were less. Rooted plantlets were successfully acclimatized through various hardening stages and were successfully transferred to the field conditions depicting 90% survival rate with no phenotypic variations observed.

The other objective of the present study was to evaluate the antimicrobial potential of native and micropropagated plants of *Tylophora indica* against bacterial (*Staphylococcus aureus* and

Escherichia coli) and fungal strains (*Aspergillius niger* and *Penicillium* species). Antimicrobial activity of crude leaf extract was investigated using agar well diffusion method.

All the extracts showed inhibitory activity for both bacterial and fungal strains but very less inhibitory activity was observed for *Escherichia coli*. The extract prepared by method Rao and Brook, 1970 exhibited a higher inhibition against both bacterial and fungal strains at all concentrations (25 μg/ml, 50 μg/ml and 100 μg/ml) and the methanol extract exhibited a significant inhibition activity against *S. aureus*, *Aspergillius niger* and *Penicillium* species. Least activity was observed by acetone extract of both *in vitro* and *in vivo* raised plant of *Tylophora indica* at all concentrations against bacterial as well as fungal strains.

In current study, antioxidant activity (DPPH assay) has been carried out for the crude extract of roots of *in vitro* raised plant of Tylophora indica. The methanol extract of roots have exhibited significant antioxidant activity in DPPH method. The results indicates that the extracts firmly posses strong antioxidant effects. The higher free radical scavenging activity *i.e.* 75% was seen at 1000 μg/ml concentration. The results obtained from the present study indicates that the *Tylophora indica*, the plant root extract can be potential source of natural antioxidant activity. For appropriate determination of antioxidant capacity, the extraction technique, its conditions, solvent used, and particular assay methodology are important.

Introduction

Plants, since classical times have been used globally as a safe natural source of medicine for maintaining human health and improving the quality of human life. Herbal medicine is the antiquated form of health care known to man and there are about 9000 plants that the man kind is aware of which show numerous medicinal applications in various cultures and countries (Farnsworth and Soejarto., 1985). Plants synthesize and cumulate a variety of bioactive compounds which help to provide protection to the plant and are valuable to humans as a primary line of defense to combat disease. Herbal medicines have been used for the basis of treatment and aid for various diseases and other physiological conditions in traditional methods exercised such as Unani, Siddha and Ayurveda (Alam *et al*., 2011).

The age old traditional value is attached with the herbal products and has gained tremendous importance in the present century. The two largest countries of Asia i.e. India and China have the richest arrays of relatively well-known medicinal plants (Kala *et al.,* 2006). The on growing demand of medicinal plant from herbal products is due to various reasons including their effectiveness, easy availability, low cost and escalating faith in herbal medicine. Natural products derived from nature have provided the complete store house of remedies from herbal medicines to cure all elements of mankind (Kumari *et al.,* 2011). Allopathic medicines may cure a vast range of diseases but the side-effects and its high prices are causing many people to return to herbal medicines (Kala *et al.,* 2006).

According to the World Health Organisation (W.H.O), currently 4.3 billion people or 80% of world's population primarily depends on plant-derived medicines or use herbal medicines for their primary health care needs because it has no side effects. Presently about 25% of pharmaceutical recommendation in the United states contain at least one plant-derived important ingredient which shows that plants are useful source of medicines. Medicinal plants play an important role to develop various types of drugs. During 1950-1970 in USA drug market, approximately 100 drugs were introduced from the plants including deserpidine and reserpine. In modern therapeutics, plants based drugs provide outstanding contribution; for example: serpentine was isolated from *Rauwolfia serpentine* in 1953 from the root of Indian plant for the treatment of hypertension and lowering blood pressure. The numbers of plants have been found to possess significant antimicrobial, antidiabetic and antioxidant properties (Hasan *et al.*, 2009). The isolation and extraction of biological active compounds from plants are responsible for use them directly or indirectly as new drugs. As a result, there is a global triumph in the trade of medicinal plants.

Global view of Medicinal plants

Global market for medicinal plant is approximately US $14 billion per year and it is expected that the sale of medicinal plants will cross US $15 trillion in 2050. The global market for herbal medicinal plant is very large. In India, the medicinal plant related business is estimated to be around US $ 1 billion per year. The real significance of herbal medicinal plant sector has begun to be realized only during the last few decades (Srivastava *et al*., 1995). It is approximated that about 25-30% of modern medicines are derived directly or indirectly from medicinal plants. The industries of herbal products comprises a number of inter related sub sectors including Functional foods; Nutraceuticals; Herbal tears; Ethical OTC medicines; Phytochemicals; Aroma therapy; Flavours and Fragrances; Spices and Culinary herbs (Kumari *et al.*, 2011).

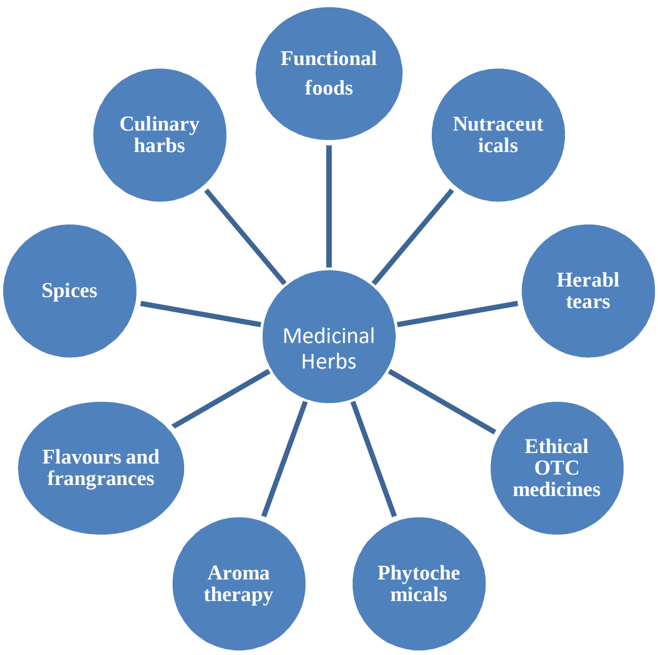


Fig.1: Overview of the Different Sub-Sectors of the Herbal industry. (Adapted from Denzil Phillips International Ltd. UK.)

According to World Bank reports, business in medicinal plants, raw materials and botanical drug products are growing at annual growth rate of 5 to 15%. The herbal products industries share approximately US $62 billion with good growth potential. Currently, India exports botanical products largely to United States accounting for about 50% of total exports. The major importer of herbal drugs is Japan, Hong Kong, Korea and Singapore making 66% share of china herbal drug export (Kumari *et al.,* 2011). Drugs derived from plants are also very popular in Germany and Russia. The herbal drugs derived in India are also exported to various countries and imported from other countries which are given in Table 1 (Hasan *et al., 2009*). The highly detailed report of world herbal products present in the market is primarily available in North America and Europe.

Table.1 Medicinal plants parts exported and imported from and in India

Exporting of Herbals		**Importing of Herbals**	
Botanical names	**Parts used**	**Botanical names**	**Parts used**
Acorus calamus	Rhizome	Aloe vera	Dried leaf
Afgemone Mexicana	Fruit	Adhatoda roots	Whole plant
Curcuma amada	Rhizome	*Cinnamomum iners*	Bark, leaf
Curcuma longa	Rhizome	Curcuma aromatic	Rhizome
Curcuma aromatic	Wild turmeric	*Garcinia indica*	Fruit
Cassia lanceolata	Leaves	*Gloriosa superb*	Tuber, seed
Glycyrrhiza glabra	Root	*Juniperus communis*	Fruit
Withania somnifera	Vegetable rennet	*Myrica nagi*	Bark
Myrica nagi	Leaf	*Strycnos nux-vomica*	Bark, seed
Piper longum	Fruit	*Phyllanthus amarus*	Fruit
Rubia cordifolia	Madder root	*Ricinus communis*	Seed
Symplocos racemosa	Bark	*Rauvolfia serpentine*	Root
Terminalia chebula	Bark, seed	*Tylophora purpuria*	Root
Swerita chirata	Whole plant	*Ocimum sanactum*	Leaf
Zinger officinale	Rhizome	*Vinca rosea*	Leaf, seed, stem
Wedelia calendula	Leaf, root	*Withania somnifera*	Tuber

The demand of medicinal plants has escalated to about $3 billion (Glaser., 1999) where as herbal remedies in European market stands at US $ 7.5 billion as 1of 1997. The import of herbal drug preparation stands at first with 46% which is escorted by 16 % for USA, 10.6% for Australia, further having Indonesia with 8.1% and at last 3.7% for India (Samy and Gopalakrishnakone., 2007).It was found that by 1990; around 2000 companies were marketing herbal medicines alone in the Europe and approximately 2223 major companies screening plants for new leads were reported worldwide (Tewari., 1996).

Status of medicinal plants in India

India records for about 8% of the total global divergence. India is the largest producer of medicinal herbs and therefore known as the 'botanical garden' of the world. (Samy and Gopalakrishnakone., 2007). Approximately 45,000 species of plants are found along the intercontinental hotspots in the province of Eastern Himalayas and Andaman and Nicobar Islands and Western Ghats. India has far reaching, safe and historic practice record of medicinal plants perceived through Siddha, Homeopathy, Ayurvedic and Unani systems of health care (Vaidya and Devasagayam., 2007 and Chaturvedi *et al*., 2007).

In India, out of 17,000 species of higher plants, nearly 7500 species find their use as medicinal plants. It is the highest proportion of medicinal plants known for medical purposes in any country of the world (Table 2). In Indian sub-continent, the oldest form of medical system Ayurveda, has alone reported around 2000 medicinal plants followed by Unani and Sidha. In Indian Himalayas, about 8000 species of angiosperms, 600 species of Pteridophytes, 44 species of gymnosperms have been reported out of which 1748 species are utilized as medicinal plants. It has been reported that the maximum species of medicinal plants found in Uttaranchal Pradesh, followed by Sikkim and North Bengal.

Table.2 Distribution of medicinal plants

Country	**Total number of native species in flora**	**% of medicinal plants**	**Number of medicinal plants species reported**	**Source**
World	297000	10	52885	Schippmann *et al*,. 2002
India	17000	14	7500	Shiva 1996
Indian Himalayas	8000	22	1748	Samant *et al*,. 1998

Source: Fictitious data, for illustration purposes only

The Himalayan region is also rich in diversity of medicinal plants. 62 species of medicinal plants are endemic to the Himalaya and over 200 species of medicinal plants of Himalaya are consumed raw, boiled, roasted, fried, cooked and also utilized in the form of spices, oils, jams or pickles (Kala *et al.*, 2006). A large number of medicinal plants which are extensively used by the pharmaceutical companies are supported by Uttaranchal for the preparation of drugs in Indian system of medicine (Dhar *et al.*, 2002).

India has rich biodiversity approximately 95% of medicinal plant species are collected from the wild or forest. The several medicinal plants are continuously exploited from the wild which has led to the substantial loss of their habitats during the past 15 years. The growing demand of medicinal plants is putting a heavy strain on the use of existing natural resources and causing a number of plant species to be either menaced or categorized as endangered species (Kala *et al.*, 2006).

Medicinal plants and Plant Tissue Culture

In growing world population, there is an uncontrolled exploitation of natural resources. As a result wild stock of medicinal plant species has been markedly dwindling. In order to meet the rising demand in pharmaceutical companies for plant-based drugs is creating heavy strain on high valued medicinal plants. To prevent this alarming situation, the unconventional biotechnological approaches have been used. Plant tissue culture an advanced biotechnology approach has been used for crop improvement and use in reforming the traditional plant breeding methods (Rani and Rana., 2010).

Most of the medicinal plants do not produce seeds or their seeds are too small which don’t germinate or have low seed viability. Moreover the seed progenies are highly heterozygous and are not true-to-type, hence mass propagation of disease free planting material is the general problem. Likewise, most of the medicinal plants are not enable to vegetative propagation by grafting or cutting. Due to lack of proper cultivation practices, destruction of plant habitats, and the illegal and indiscriminate collection of plants from natural habitats, many medicinal plants are severely threatened. Advanced biotechnological methods of culturing plant cells and tissues provide new means of conserving and rapidly propagating valuable, rare, and endangered plant species. (Faisal and Anis., 2005, Nayeem *et al.,* 2014).

Micropropagation

Micropropagation is the process of vegetative growth and multiplication from plants tissues. It is carried out in aseptic and favorable conditions on growth media, using conventional plant tissue culture techniques. Plant tissue culture technique is based on the concept of totipotency; the ability of plant cells and tissues to develop into whole new plant. In conventional cultivation many plants do not germinate, flower and produce seed under certain climatic conditions or have long periods of growth and multiplication. Micropropagation ensures a good regular supply of medicinal plants, using minimum space and time.

The advantages of *in vitro* propagation or micro propagation of medicinal plant are listed below:

1. Enhances the rate of multiplication.
2. Environment can be controlled or altered to meet specific needs of the plant.
3. Plant available all year round for nursery production.
4. Identification and production of clones with desired characteristics.
5. Production of secondary metabolites.
6. New and improved genetically engineered plant can be produced.
7. Stocks of germplasm can be maintained for many years.
8. Preservation of genetic material by cryopreservation. (Sidhu., 2010)

Techniques of Micropropagation:

Three main techniques used for plant propagation under *in vitro* conditions are:

1) ***Enhanced axillary shoot proliferation:*** Micropropagation through apical and axillary shoot proliferation which is the most popular method for commercial production of plants. It is the most efficient and reliable method for clonal propagation because the cells of meristems are diploid and are very least susceptibility to genetic changes. Hence, ensures the genetic stability of clones.

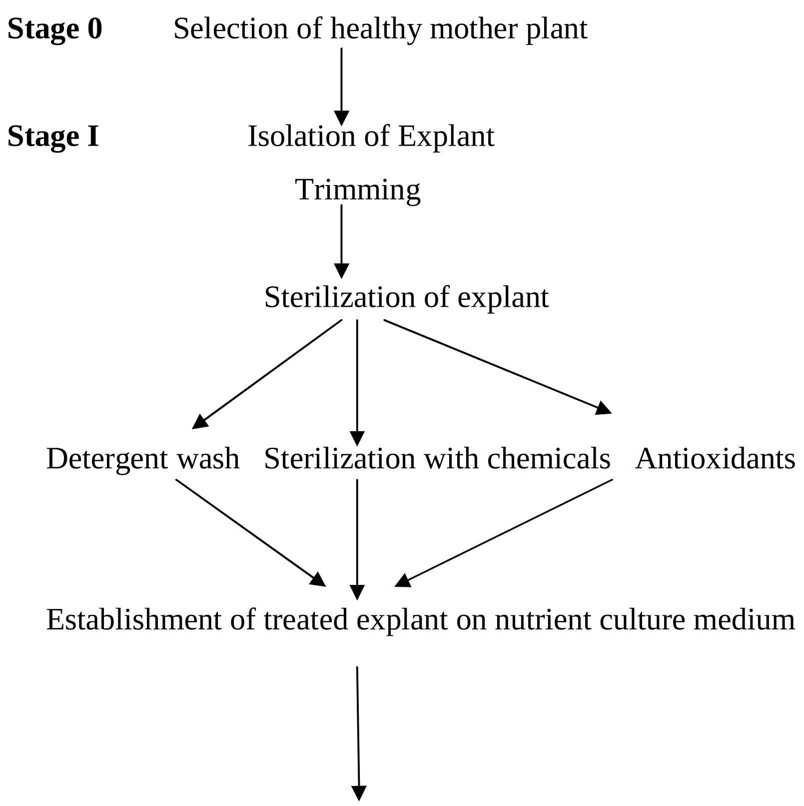
2) ***De novo formation of adventitious shoots***

- Directly from the explants like root, stem, petiole, leaf lamina, flower parts **or**
- Indirectly from callus cultures obtained from these explants. Plants obtained through calli may not be true elites because of high incidence of polyploidy and aneuploidy associated with callus cells and plants obtained from it.

3) ***Somatic or non-zygotic embryogenesis***

Somatic embryogenesis is the process of a single cell or a group of cells initiating the developmental pathway that leads to the reproducible regeneration of non- zygotic embryos, capable of germination to form complete plantlets. These embryos like structures are bipolar units containing root and shoot axis and can develop into fully functional plants under appropriate conditions.

Major steps in Micropropagation:



Stage II Transfer to multiplication medium

Multiplication of Shoots →
- From nodal explant and shoot tip
- Directly from explants
- Indirectly from callus
- Somatic embryos formation from calli or explant

Stage III Developed shoots were transferred to rooting medium

↓

Plantlets

↓

Stage IV Acclimatization by various hardening process

↓

Transferred to natural field conditions

Fig. 2 Schematic representation of various stages of Micropropagation

Antimicrobial activity of medicinal plants

Medicinal plants have been used for treatment of several human diseases because they contain components which are having therapeutic potential. Currently, traditional medicines are accepted as an alternative form of promoting health care (Nostro *et al.*, 2000). The clinical potency of the already present antibiotics is being jeopardized by the upcoming multi drug resistance pathogens. The bacterial and the fungal pathogens have emerged a number of new defense mechanisms against antimicrobial agents as well as resistance to the old and the newly produced drugs are also accelerating day by day. The increasing breakdown of chemotherapeutics and antibiotic resistance which is displayed by the pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity. There are several reports in the literature with regard to antimicrobial activity of crude extracts prepared from plants. There are various reports in the literature about the characterization of medicinal plants that may inhibit the bacteria, for example: *Escherichia coli, Baccillus species, and Staphylococcus aureus.* Natural products provide unlimited opportunities for new drug leads, either as pure compounds or as standardized plant extracts, because of the unmatched availability of chemical diversity (Parekh and Chanda., 2007).

Antioxidant activity of medicinal plants

Free radicals causing a number of disorders in humans like arthritis, atherosclerosis, central nervous system injury, cancer, gastritis and AIDS. Free radicals cause depletion of immune system antioxidants and induce abnormal proteins. They are generating due to polluted environment, chemical toxins, radiations, spicy and deep fried foods. The enzymes catalase and hyperoxides present in human body function as natural antioxidant because they convert into non radical forms. Butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), and gallic acide esters are synthetic antioxidants and have been suspected to cause negative health effects to humans. Synthetic antioxidants also have low solubility and moderate antioxidant activity. Therefore, strong restrictions have been placed on their usage and people are shifting towards natural antioxidants produced by medicinal plants. Medicinal plants have therapeutic potential of antioxidant activity in reducing free radicals. Tea, wine, fruits, vegetables and spices are well known traditionally used antioxidants are already exploited commercially (Pourmorad *et al*., 2006).

Antioxidant compounds present in various medicinal plants and used as natural antioxidants. The compounds which can inhibit the oxidation of other molecules and play vital role as health promoting factors are known as antioxidant compounds. The demand of antioxidants has been increasing due to its high capacity of scavenging free radicals related to several diseases. The working of antioxidants is that the electrons are donating to free radicals and are convert them to harmless molecules. This harmless molecule protects cells from oxidative damage which further leads to aging and various diseases. Phenolic acids, polyphenols and flavonoids present in plants scavenge free radicals (Bhatia *et al*., 2013). A rapid, easy and sensitive method for antioxidant activity of plant extracts is free radical scavenging assay by using 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical spectrophotometrically. DPPH radicals obtained one more electron and the absorbance of sample decreases in the presence of an antioxidant (Pourmorad *et al*., 2006).

OBJECTIVES

The present investigation was carried out on an important medicinal plant *Tylophora indica*.
The main objectives of the present investigation are:

- To do mass cloning of *Tylophora indica* by *De novo* adventitious shoot induction from leaves
- To compare the antimicrobial potential of native and micropropagated plants of *T. indica*
- To evaluate the antioxidant potential of the root extract of micropropagated plants of *T. indica*

Review of Literature

The clonal propagation of selected phenotypes is an essential step in most of the plant breeding programs to produce plants with desirable characteristics. The plants which are sexually propagated produce a high degree of heterogeneity showing lot of variations in habit, growth and yield. The plants produced through asexual reproduction are genetically identical to the parent plants and therefore maintain the special characters of the cultivar. The *in vitro* tissue culture technique has an innovative access to morphogenetic investigations and permits a living system to be studied under controlled environmental conditions. The advanced Plant Tissue Culture technique has come out as a promising technique to obtain genetically true elites rather than having indifferent populations.

Micropropagation or *in vitro* propagation is the miniature adaptation of conventional propagation technique which is conducted under aseptic conditions. The technique of micropropagation is based on the concept of totipotency that every cell of the body is capable of giving rise to new plant under appropriate nurture conditions as envisaged by Gottlieb Haberlandt. The clonal propagation technique is the backbone of plant tissue culture in today's scenario and emerged as a powerful technique for the improvement of forest, horticulture, field and plantation crops producing high quality planting material. The accelerated and mass production of number of important plants such as *Populus, Fressia, Eucalyptus, Albizzia* and various medicinal plants and fruit tress has been done by well established technique of micropropagation. In this technique a small piece of tissue can be used to produce thousands of plants.

Three main techniques of Micropropagation are:

1. Enhanced axillary shoot proliferation from apical buds and axillary buds
2. *De novo* formation of adventitious shoots either directly from explant or indirectly through callus
3. Somatic or non-zygotic embryogenesis

***De novo* adventitious shoot formation**

Directly from explants

De novo adventitious shoot formation directly from root, petiole, stems, floral parts and leaf lamina is considered as the most reliable and valuable technique for clonal propagation. This technique has been successfully used for *in vitro* propagation of many horticulture, ornamental and medicinal plant species. Choice of explants and hormone regime to which the explants are subjected to, are two important factors in the initiation of adventitious shoot formation.

De novo adventitious shoot formation through direct organogenesis is regarded as the most reliable method for mass propagation because it upholds genetic uniformity among the progenies. Many medicinal plants such as *Withania somnifera* (Kulkarni *et al.,* 2000), *Ophirrhiza (*Beguma *et al.,* 2006*)*, *Embelia ribes* (Annapurna and Rathore., 2010), *Psorela corylifolea* (Baskaran and Jayabalan., 2010) and *Cassia angustifolia* (Siddique *et al*., 2010) have been prosperously propagated *in vitro* by *de novo* direct adventitious shoot formation.

Bera and Roy 1993 standardized the protocol for *in vitro* propagation of *Tylophora indica* by *de novo* adventitious shoot formation from mature leaf explants subjected on MS medium supplemented with 22 µM in combination with 0.65 µM Adenine sulphate. Chaudhari *et al.,* 2004 standardized the protocol for the formation of organogenic nodular meristemoids from root explants of *T. indica* when subjected to MS medium in combination with 2-ip or 6-Benzylaminopurine. The meristemoids showed two type of responses- direct formation of somatic embryogenesis in 39 % of root explants and shoot bud formation in 42% of explants. Waseem *et al.,* 2009 described the regeneration capability of *Chrysanthemum* from leaf discs by using different types of auxins.

Kaur *et al.,* 2011 b & c established a protocol for *de novo* adventitious shoot formation from the root and stem explants of *T. indica* on 8.8 µM BAP, whereas leaf explants gave the best results on 9.8 µM BAP in combination with 1.35 µM adenine sulphate (Kaur *et al*., 2011 a).The use of cytokinins showed promotory effect on a number of medicinal plants species for the formation of *de novo* adventitious shoot formation employing different kind of explants (Hiregoudar *et al*., 2003; 2006 and Loc *et al.,* 2005).

Pretto and Santarem., 2000 described BAP to be most effective in promoting shoot regeneration from leaf segement of *Hypericum perforatum.* Thangavel *et al.,* 2011 reported that in *Plectranthus barbatus,* 6.9 µM of Kinetin was most effective in inducing an average number of 19.7 ± 2.08 shoots per explants whereas MS medium + 8.8 µM BAP in conjunction with 7.35 µM NAA produced 15.0 ± 2.20 shoots per explant.

According to Yildrum and Turker., 2009 extensive shoot regeneration was observed in *Filipendula ulmaria* from root, leaf and petiole explants when TDZ was used in combination with IAA and GA3. In *Gaultheria fragrantissima,* thidiazuron (TDZ) alone was most effective in inducing direct shoot regeneration from leaf and internode explants (Ranyaphia *et al*., 2011).

Antimicrobial activity of medicinal plants

It is admitted that the study on medicinal plants as antimicrobial agents is important to know the real value of medicinal flora but the use of inexpensive and standard efficient methods are essential for investigation. As we all know that, the use of medicinal plants for treating various types of diseases is as old as human species. The major morbidity and mortality cause among the population is due to infectious diseases in developing countries. This is the reason that pharmaceutical companies have been inspired to produce new antimicrobial drugs, especially due to constant development of microbes resistance to traditional antimicrobials.

Medicinal plants (Eight) which are traditionally used in South Africa reported by More *et al*,. 2008 and reported that these eight medicinal plants have antimicrobial activity against oral infections. It is found that for plant extracts, gram negative bacteria was more challenging as compare to gram positive bacteria. *Baccharis trimera* (Carqueja), a medicinal plant also shows antimicrobial actions against both gram positive such as *Streptococcus uberis* and *Staphylococcus aureus* and gram negative bacteria includes *Escherichia coli* and *Salmonella gallinarium* and are sensitive to this herb (Avancini *et al*., 2000 and Silva and Junior., 2010).

Furthermore, study carried out an essential oil of *Pelargonium graveolens* (geranium) which shows low values of MIC against various bacteria such as Staphyloccous aureus (0.72 mg/ml), bacillus subtilis (0.72 mg/ml) and Bacillus cereus (0.36 mg/ml). However, oregano oil also effective against the same bacteria, but also show antimicrobial activity against Escherichia coli. (Rosato *et al.,* 2000).

Hasan *et al.*, 2008 reported the antimicrobial activity of ethanolic extracts and hot water of six medicinal plants, which were used to prevent liver damage. The extracts of these medicinal plants (*Acacia Arabica, Emblica offficinlis, Cardus marianum, Sphareranthus hirtus, Nymphaea lotus* and *Cinchorium intybus*) were tested against two fungal strains and seven bacterial strains by agar well diffusion and microdilution method. Most inhibited microbes are found to be *S. typhi, E.coli and P. aeroginosa*.

Reddy *et al*., 2009 investigated the antimicrobial, antifeedant and antifungal activity of pure and crude extracts of *Tylophora indica*. The highest antifeedant potential was observed by crude extracts of leaves against *Spodoptera litura* rather than root and stem extract. The highest antibacterial activity against *S. aureus, B. subtilis, P. aergenosa* and *M. luteus,* was exhibited by crude extract of leaves. The crude extract or pure compound of *T.indica* was found to be not effective against *E.coli* even at higher concentrations. On the other hand, antifungal activity was showed by both pure compounds and crude extract against *Aspergillus fumigates, Aspergillus niger* and *Trichoderma viridae.* The strong antifungal activity was shown by pure compound as compared to crude extract.

Ponnanikajamideen *et al.,* 2013 reported that crude extracts were prepared by suing different solvents such as Ethyl acetate, Isopropyl alcohol and Benzene from *Tylophora asthmatica* leaves which showed the antibacterial activity against infectious bacteria included *E.coli, S. typhi* and *S. aureus* at different concentrations. *S. typhi* was found to be more inhibited in all extracts as compared to others.

Deshwal *et al.,* 2013 selected alcoholic and aqueous extracts obtained from both micropropagated and parental *Tylpohora indica* plant for evaluating the potential activity by agar well diffusion method against various fungal strains included *Candida parapsillosis, Candida krusei, Aspergillus flavus, Aspergillus niger, Candida albicans, Penicillium* species, *Fusarium* species, *Alternaria* species and *Rhizopus* species. The significant activity of leaf extract of micropropagated raised plants was seen against *Candida parapsilosis.* The alcoholic stem extract and aqueous leaf extract do not show significant activity against fungal strains.

Antioxidant Activity

Since the classical times, various types of herbal plants have been used to maintain health and for prevention from primary diseases. Oxidative stress has developed as a major player in the pathogenesis of various diseases in model organisms as well as humans. As we all know that, number of medicinal plants produce antioxidant activity and represent as a source of new compounds with favorable antioxidant property. These days there has been an escalating interest in worldwide to found medicinal plants with antioxidant activity that are having lesser side effects.

Singh *et al.*, 2013 evaluated the strong antioxidant activity of six medicinal plants (*Lantana camara, Acacia Arabica, Cinnamomum zeylanicum, Saraca indica, Vaccinium oxycoccos* and *Glycyrrhiza glabra*) with their pharmacological and phytochemical aspects.

Ju *et al.,* 2014 reported that different extracts (water, methanol, acetone and ethanol) of *L. aromatica* showed antioxidant activity at various concentrations i.e. 50%, 75% and 100%. The ethyanol extract at 100% concentration showed maximum antioxidant activity, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity and reducing power which suggests that *Limnophila aromatic* can be used to reduce oxidative stress.

Pourmard *et al*., 2006 reported relative antioxidant activity in some Iranian medicinal plants. Extract of *M. officinalis* showed four times maximum antioxidant activity (IC50= 0.018 mg/ml) than antioxidant BHT (butylated hydroxy toluene) by DPPH assay. The presence of higher amount of phenolic compounds in *M. officinalis* leads to potent free radical scavenging activity.

Bhatia *et al*., 2013 standardized the protocol for investigation of antioxidant activity of alcoholic and aqueous leaf extract of medicinal plant *Tylophora indica* by different type of assays (LPO, SOD and CAT). The aqueous extract showed better activity than alcoholic extract. *Tylophora indica* represents higher amount of superoxide dismutase and catalase activity with lesser LPO level concluded the strong antioxidant activity.

Mini *et al.*, 2012 determined the potential antioxidant activity of *Tylophora indica* by *in vitro* radical scavenging DPPH assay. The crude extract prepared in different solvents included petroleum ether, Dichloromethane, ethyl acetate, methanol and aqueous extract. Out of all extracts, ethyl acetate and methanol extract showed highest activity i.e. 81µg/ml and 87µg/ml IC50 values respectively. The presence of tylophorinidine hydrochloride in methanol extract showed favorable antioxidant activity with 25 µg/ml IC50 value.

According to Gunasekaran *et al.*,, 2015 Extract of *Tylophora indica* was considered for its antioxidant potential using Nitric Oxide (NO) and DPPH assay. Methanol extract at all concentrations (50 µg/ml, 100 µg/ml and 150 µg/ml)showed strongest activity and least activity was seen by hexane extract. Moderate antioxidant activity was observed by both Chloroform and ethyl acetate. By this study, it was suggested that leaf extract of *Tylophora indica* has potent free radical scavenging activity and has suitable for future research.

Materials and Methodologies

Choice of material

Tylophora indica (Burm.f.) commonly known as 'Antmool' or 'Dama Bel' a medicinally important plant belonging to family Asclepiadaceae was selected as an experimental material. It is a threatened medicinal climber shrub found in the plain and hill forests of Southern and Eastern India overspread up to an altitude of 900m (Gupta., 2003). This indigenous medicinal plant has multifarious uses and has been used traditionally as a folk remedy for the treatment of various ailments like bronchitis, bronchial asthma, dysentery, inflammation, dermatitis and allergies (Singh *et al*., 2009, Rani and Rana., 2010, Kaur *et al*., 2012 and Anjum *et al*., 2014).

Morphology

The plant is a perennial branched climber having slender, twining stem with long fleshy roots. The stems are glabrous and elongate in nature. The leaves are ovate oblong to elliptic oblong and acute at the tip. The size of leaves is 5-10 cm by 2.5-5.7 cm. Flowers are bisexual, minute, greenish yellow from outside and purplish from within and grow in umbellate cyme (Figs. 3 & 4). The roots are stout and cord like. (Kirtikar and Basu., 1991 and Nema *et al*., 2004).

Fig.3 *Tylophora* plant in vegetative stage

Fig.4 *Tylophora* plant in blooming stage

Habitat

Tylophora indica, an indigenous medicinal plant is found in the forests, plains, hilly slopes and outskirts of the forests with optimum temperature ranging from 5- 35°C and annual rainfall of 500-2500 mm (Faisal and Anis., 2007). In open hills and narrow valleys this plant forms dense patches in the forest with moist and humid conditions. The plant shows undersized growth in the areas with lesser rainfall (Nadkarni., 1976).

Distribution

It is indigenous to India, native to the plains and hill forests and habitats up to an elevation of 1260m in the sub Himalayan tract from Uttar Pradesh to Meghalaya and in Central and Peninsular India. It also harbors in North-East and Central India, Eastern, Bengal and parts of South India. *Tylophora* comprises about 50 species which are distributed in tropical and sub-tropical, Africa and Australia and approximately 35 species are found in China (Nema *et al*., 2007). Some of the species found in India are *Tylophora rotundifolia*, *Tylophora indica/asthmatica*, *Tylophora apiculata*, *Tylophora hetero-phyla*, *Tylophora anomala, etc.*

Propagation of *Tylophora*

The conventional propagation of *T. indica* occurs through seeds but seeds are too small and have low seed viability and germination. The plant is not amenable to vegetative propagation through grafting or stems cuttings as they fail to produce proper roots (Thomas and Philip., 2005). Due to the shortage of high quality planting material, commercial cultivation of this valuable plant *T. indica* is uncommon and its overexploitation and indiscriminate use have rendered the species highly vulnerable to extinction (Faisal and Anis., 2007).

Medicinal Importance

Tylophora indica seems to be good remedy in traditional medicines for the treatment of bronchial asthma, inflammation, bronchitis, and allergy. It is used for different types of treatments such as whooping cough, cold, dysentery, hay fever and arthritis. It is also used as anti-psoriasis. The roots and leaves are useful as a source of bioactive compounds and are known to have expectorant, laxative, purgative diaphoretic, stimulant and cathartic properties. (Verma *et* *al.*, 2010). This plant is also prominent as a blood purifier and observed as one of the best indigenous substitute for ipecauahna (Rani *et al.*, 2015).

Active Ingredients

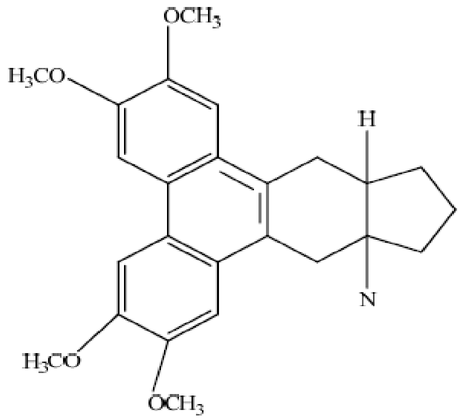
The leaves and roots of this medicinally important plant contain various active alkaloids like 34 epatoprote ($C_{24}H_{27}O_4N$), tylophorinine ($C_{23}H_{25}O_4N$) and anticancerous tylophorinidine ($C_{22}H_{22}O_4N$). These potent alkaloids exhibit various biological and pharmacological activities and endowed this plant with a variety of medicinal value to cure various ailments (Verma *et al.*, 2010).The alkaloid tylophorine which is the major constituent is responsible for strong anti-inflammatory and immunosuppressive action (Goplakrishnan *et al.,* 1979). The other chemical constituents found in this plant are septicine, isotylocrebrine, sterols, wax, resins, 34epatoprot and tannins. The non alkaloidal compounds are also isolated from *Tylophora indica* which include quercetin, kaempferol, sigmasterol, tetratriacontanol, octaosanyloctacosanoate tyloindane, coutchone, pigments, glucose, calcium salts, potas)O 9—msium and chloride. The alcoholic extract of dried root powder by process of steam distillation gave p- methoxy salicyaldehyde and a little amount of oily matter (Vivean *et al.*, 2015).

Pharmacological activities

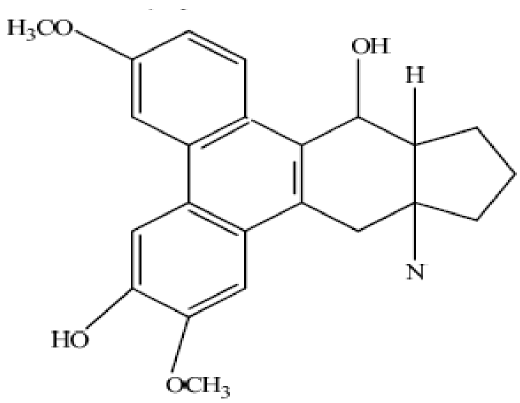
Tylophorine which is the major alkaloid of *Tylophora indica* has various pharmacological activities like anti-tumor and cancer cell inhibition both *in vivo* and *in vitro* (Donaldson *et al*. 1968 and Li *et al*., 2001), anti-leukemia properties (Gellert *et al*., 1962). It is the key component in the process of inflammation. It is traditionally use in the treatment of asthma and also in anti-allergic medication.

The various extracts of *Tylophora indica* such as aqueous and alcoholic show 34epatoprotective activity (Gujrati *et al*., 2007). Studies revealed that the alkaloids of *Tylophora* inhibit the cellular immune responses and thus show immunomodulatory activity, stimulant of adrenal cortex (Udupa *et al*., 1991), diuretic activity (Meera *et al*., 2009), anti-amoebic (Bhutani *et al*., 1985), anti-bacterial , anti-fungal properties (Reddy *et al*., 2009) and antioxidant activity (Bhatia *et al.*, 2013)

Chemical structure of alkaloids



Tylophorine



Tylophorinidine

Toxic effects

The plant may also produce some unwanted secondary effects like giddiness and drowsiness. In some cases, loss of taste for salt, tightness in throat or chest, mouth pain vomiting, upset stomach, temporary nausea, rashes or itchy or swollen skin may occur. The prior studies showed that the extracts of *Tylophora indica* are producing toxic effect only in extremely high doses. The smaller doses produce therapeutic effect and are safe for consuming. This plant or herb should not be used by the pregnant lady or nursing women, children or individuals with severe liver or kidney diseases (Gupta *et al.*, 1979).

Experimental Requirements

Glassware

All glassware used for experimental work was made up of borosilicate glass. The glassware used for work included test tubes (25 x 125; 25 x 150 mm), conical flasks (100 ml, 150 ml, 250 ml, 500 ml, 1000 ml, and 2000 ml), tissue culture bottles, beakers (100, 200, 250, 500 and 1000 ml), glass pipettes (1 ml, 2 ml and 5 ml), round bottle flask (250 ml and 500 ml), separating funnel (125 ml and 250 ml), reagent bottles (100 ml, 200 ml and 500 ml) and Round bottom flasks (125 ml and 250 ml). In addition other glassware included Petri dishes, measuring cylinders and volumetric flasks. The other material used for experimental work comprised Rotary flash evaporator, Elisa plate reader, magnetic stirrer, 96 well titer plate, cotton, tissue roll, aluminum foil, surgical blades, forceps, disinfectants, pestle & mortar and spatula.

Before use, the glassware was thoroughly washed with teepol detergent (10 %) and then washed in running tap water. These were then treated with chromic acid solution (mixture of $K_2CR_2O_7$ + H_2SO_4 1:3ratio) for 24 hours, followed by thorough washing under running tap water. Distilled water was poured in every culture vessel which was tightly plugged. Plugs were made out of absorbent surgical cotton wrapped in muslin cloth. Glassware was steam sterilized in an autoclave at a pressure of 1.1 kg/cm2 for 15 - 20 minutes and oven dried prior to use.

Culture media

For micropropagation work, Murashige and Skoog (1962) medium (MS), was used. Media stocks were prepared in double distilled water (Table 1). Generally 4 times major elements, 100 times minor elements and 10 times organic constituents of stock solutions were made. All the stock solutions were stored in refrigerator at 4°C. These stocks were mixed in desired proportions only before use. Stock solutions of auxins like naphthalene acetic acid (NAA), 2, 4-dichloro phenoxy acetic acid (2, 4- D), indole 3-acetic acid (IAA), indole 3- butyric acid (IBA) and cytokinins i.e. kinetin (K), benzyl amino purine (BAP) and adenine sulphate were prepared and stored at 4º C. The stock solutions were stored only for 15 days.

Table.3 Composition of Murashige and Skoogs medium (1962)

Ingredients	Concentration(mg/ml)
Major elements:	
$(NH_4)NO_3$	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2POÚ	170
$FeSO_4,4H_2O$*	27.8
Na_2EDTA	37.3
Minor elements:	
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	8.3
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Organic constituents:	
Myoinositol	100
Glycine	2.0
Nicotonic acid	0.5
PyridoxineHCL	0.5
ThiamineHCL	0.1
Sucrose	20,000
Agar	10,000

*Ferric Na EDTA is added freshly to the medium (i.e. 0.04 gm/l) because it act as a chelating agent and keeps iron in dissolved form so that it does not get precipitated in the medium.

All the constituents were mixed in definite amounts except agar and volume was adjusted by adding distilled water. The pH of the solution was adjusted to 5.8 by using 0.1N HCL or NAOH depending upon high or low. In each conical flask (250ml) definite aliquots of medium were distributed, generally 100ml medium was dispersed in each flask. About 20mland 40 ml medium was added in each culture tube and culture bottles respectively. Vessels were plugged with non absorbent cotton plugs and autoclaved at 15 lbs/in² (121°C) for 15-20 minutes. After autoclaving test tubes were placed over racks in a slanting position to give slanted surface to the agar media. MS medium enriched with different concentrations and combinations of auxins and cytokinins was used for adventitious shoot formation and rooting of microshoots. The details of plant growth regulators (PGRs) combinations used are given below:

1. MS medium
2. MS medium + NAA (5.37µM- 21.48µM)
3. MS medium + NAA (5.37µM- 21.48µM) + K (4.65µM- 18.6µM)
4. MS medium + NAA (5.37µM- 21.48µM) + BAP (4.44µM- 17.76µM)
5. MS medium + 2, 4-D (4.55µM- 18.2µM)
6. MS medium + 2, 4-D (4.55µM- 18.2µM) + K (4.65µM- 18.6µM)
7. MS medium + 2, 4-D (4.55µM- 18.2µM) + BAP (4.44µM-17.76µM)
8. MS medium + BAP (4.44µM- 22.2µM)
9. MS medium + BAP (4.44µM- 22.2µM) + Adenine sulphate (1.35µM- 5.4µM)
10. MS medium + Kinetin (4.65µM-18.6µM)
11. MS medium + Kinetin (4.65µM-18.6µM) + Adenine sulphate (1.35µM- 5.4µM)
12. Half strength basal MS medium
13. MS medium + IBA (4.92µM- 19.68µM)
14. MS medium + IAA (5.71µM- 22.84µM)

Inoculation

To maintain aseptic conditions, all the experimental works were carried out in laminar air flow fitted with a bactericidal ultraviolet tube (15W, peak emission 2537Å). The floor of the inoculation chamber was cleaned or scrubbed with tissue paper dipped in alcohol. The all other accessories used in experimental work such as vessels and instruments (spatula, blade, forceps and scalpel etc.), matchbox, tube containing 70% alcohol etc. were also cleaned with spirit to remove all contaminants. The Petridish containing fresh material to be inoculated was covered with a piece of black paper to protect it from the harmful radiations or effects of ultraviolet rays. The Ultraviolet (UV) tube was kept on for one hour.

Surface sterilization of Inoculum

Plant tissues were also sterilized before they were placed over the medium. Explants such as leaves were taken from healthy mother plant growing in the field (both native and *in vitro*) and washed thoroughly under running tap water for 30 minutes by placing them in culture bottles covered with net to remove all microbes and dust particles from the surface of explants. It was followed by washing with teepol solution 1% (v/v) for 10 minutes followed by thorough washing with tap water. Thereafter, bavistin (0.1% w/v) treatment was given for 10-12 minutes to remove fungal contamination followed by repeated washings with tap water. Final sterilization was carried out by giving treatment with 0.05-0.1% (w/v) aqueous solution of mercuric chloride for 2-5 minutes in laminar air flow followed by3-4 washings with autoclaved distilled water.

Cultural or Growth Conditions:

Inoculated cultures were maintained in plant growth room in controlled conditions of temperature at 25±2°C. The source of illumination was provided by 4 feet wide cool white fluorescent tubes of 40W with intensity of 50μmol/m^2/s^1. The intensity of illumination was 12 hours light regime followed by 12 hours of darkness.

Adventitious Shoot induction

For *De novo* adventitious shoot formation and proliferation, the leaf explants were cultured on MS medium enriched with different auxins and cytokinins. Best results occurred on 6-benzylamineopurine either alone or in conjunction with adenine sulphate. Initially the nodular meristemoids were formed from the cut ends of the leaf. After some time, the entire surface of the explant was covered. Furthermore, these meristemoids developed into green leafy shoots and proliferated. The number of shoots increased after subsequent subculturing on the same medium. The number of shoots formed per explant was recorded after 3, 5, 7 and 11weeks respectively

Rooting

Root induction was carried out on half strength basal MS medium and MS medium supplemented with IBA (19.68µM). Regenerated shoots were rescued from tissue culture bottles and placed upward in the half strength MS medium or MS medium augmented with IBA.

Acclimatization

For acclimatization, plantlets were carefully taken out from the culture medium and washed under tap water to remove agar sticking to them. The plantlets were then transferred to plastic cups containing potting mixture of soil: vermicompost (1:1) and covered with perforated plastic bags to maintain internal humidity and aeration and were kept inside the growth room for another 15 days. The plastic bags were removed from cups and kept as such in the growth room for another one week. The harden plantlets were then transferred into poly bsags containing same potting mixture and kept in the growth room for further 15 days. By this time, the plants formed new leaves and become photosynthetically active. After that, the plants were transferred into earthen pots containing mixture of soil: vermicompost (3:1) and maintained in greenhouse for another 2 weeks before their final transfer to open field conditions.

Extraction of Plant extract

Leaf extract was prepared from leaves of 5 year old healthy *in vitro* raised field established plants maintained at Thapar University Campus at TIFAC and also from *in vivo* raised plants maintained at Punjab agricultural university, Ludhiana. The roots were taken from *in vitro* raised plants of *Tylophora indica*. The collected leaves and roots were thoroughly washed under running tap water to remove dust particles, microbes and other impurities. The dried leaves were finely grounded using pestle and mortar and sieved through mesh size of 1 mm. Leaf extract from dried powder was made using different extraction protocols which are as follow

Extraction Procedure 1 (Bhatia *et al*., 2013)

The leaves of *Tylophora indica* were taken from both *in vitro* and *in vivo* plants and were washed with running tap water. The leaves were dried in hot air oven at 37°C for 3 days. The dried leaves were crushed to powdered form. 6g of dried powder was put in 80 ml of methanol and acetone each for obtaining extracts. The mixture was placed on the magnetic stirrer for 48 hours. The extract obtained was filtered using Whatmann filter paper and then sterilization was done by using Millipore filters of pore size 0.22µm. The obtained extract was evaporated by using a rotary evaporator (rota evapo) to get the crude extract. The extract was then stored at 4°C for further use. The similar procedure was repeated for extraction of roots of *in vitro* raised plant.

Extraction Procedure 2 (Rao and Brook method, 1970)

Method of Rao and Brook, 1970 was used for making leaf extract. **100**g of dried powder was washed twice with 50 ml hexane to remove the oily components. The powder was then extracted thrice in cold conditions by 100 ml of 1% acetic acid in methanol, with each extraction lasting for a day. The rotary flask evaporator was used for methanolic fraction at 50°C. The concentrated fraction was obtained. The concentrated fraction was further extracted by using equal proportion of ethyl acetate: HCL (1:1) three times. The two immiscible layers were formed after shaking in the separatory funnel. Acid layer was collected from the two layers and pH was adjusted to 8.5-9.0. The acid layer was also extracted thrice with chloroform (50ml each). Aqueous layer formed contained the yellow pigments and alkaloids passed into the chloroform layer. The chloroform extracts were concentrated using rotary flash evaporator and digested with 50 ml of hot methanol. The mixture was cooled, filtered and further analyzed.

Antimicrobial testing

Antimicrobial activity of leaf extract was tested against different types of bacterial and fungal strains by using Agar well diffusion method.

Preparation of Muller Hinton Agar (MHA) and Saboured Dextrose Agar (SDA)

Table.4 Composition of MHA and SDA

Ingredients for MHA	gm/l	Ingrdients for SDA	gm/l
Beef extract	2 gm	Dextrose	40.00 gm
Acid hydrolysate of casein	17.50 gm	Mycological peptone (enzymatic digest of casein and animal tissue)	10.00 gm
Starch	1.50 gm	Agar	15.00 gm
Agar	17.00 gm	Distilled water	1000 ml
Distilled water	1000 ml		

Procedure

1. 38g of MHA and 65g of SDA was suspended in one liter of distilled water respectively.
2. Suspended medium was heated with frequent agitation and boiled the medium completely till the clear solution was formed.
3. pH adjusted to 5.6 at 25^0 C for SDA and 7.3 ± 0.1 at 25°C for MHA medium.
4. Autoclave at 121°C for 15 minutes and then cooled to room temperature.
5. Sterilized petri plate was taken and poured MHA and SDA into in on a level, horizontal surface to give uniform depth.
6. Allowed it to cool at room temperature.
7. Stored the plates at 2-8 °C for antimicrobial testing

Bacterial strains

Antibacterial testing was done against one gram positive bacterial culture such as *Staphylococcus aureus* and one gram negative bacterial culture *Escherichia coli.*

Escherichia coli

Domain : Bacteria

Phylum : Proteobacteria

Class : Gammaproteobacteria

Order : Enterobacteriales

Family : Enterobacteriaceae

Genus : *Escherichia*

Species : *coli*

Staphylococcus aureus

Domain : Bacteria

Phylum : Firmicutes

Class : Bacilli

Order : Bacillales

Family : Staphylococcaceae

Genus : *Staphylococcus*

Species : *aureus*

Test for antibacterial susceptibility

Muller Hinton agar medium was used for the antibacterial activity. The three sets of plates were made for methanol extract, acetone extract and the extract prepared by using Rao and Brook method of both *in vitro* and *in vivo* raised plants. The suspension culture of using micro-organisms was made in normal saline and then sterilized. The prepared suspension was adjusted it to 0.5 Macfarland standard (108Cfu/ml) (NCCLS, 2000). From the stock solutions of 5mg/ml serial dilutions were made to 25µg/ml, 50µg/ml and 100µg/ml. All MHA plates were labeled properly and were uniformly inoculated with test micro-organisms by using a autoclaved cotton. The cotton was dipped into the suspension and swabbed uniformly on the surface of all MHA plates so that lawn can observed. Wells of 0.5mm diameter was made by using sterile cork borer and 0.1 ml of each concentration of different extract were dropped into each appropriate well. The solvents which were used for extraction were tested as control for each organism. The plates were kept in refrigerator for one hour so that extract can diffuse properly into the agar. After that plates were incubated at 37°C for 24 hours to observe the zone of inhibition. By measuring the diameter of zone of inhibition antimicrobial activity was determined. (Aibinu *et al*., 2007).

Fungal strains:

Antifungal testing was done against *Aspergillus niger* and *Penicillium species*.

Test for antifungal susceptibility

Sabouraud Dextrose Agar (SDA) medium was used for antifungal susceptibility testing. Similarly antibacterial testing, the three sets of plates of methanol, ethanol and acetone extract respectively and one set plate for Rao and Brook method extract were prepared and agar well diffusion method was used for testing.

The fungal strains maintained on SDA plates and stored at 4°C after growth for further use. The sets of SDA plates were inoculated with fungal suspensions by using sterile cotton swabs. The wells of 5mm diameter were made on each plate by using sterile borer. From the stock solution of 5 mg/ml serial dilutions were made to 25µg/ml, 50µg/ml and 100µg/ml. 20µl of each extract was poured into each well with the help of micropipette. Blank control was also taken depending upon the plant extract. All the plates were kept in the upright position to diffuse the solution into the medium. After 5-10 minutes, the plates were incubated aerobically at 25°C in BOD incubator for 24-72 hours. The diameter of zone of inhibition was measured against fungal strains (Deshwal *et al*., 2013).

Estimation of Antioxidant activity

***In vitro* DPPH free radical scavenging assay:**

The crude extract of roots of *in vitro* raised plant of *Tylophora indica* was prepared for antioxidant activity by DPPH assay. The ability of antioxidant activity was investigated using the 2,2-diphenyl-1- picrylhydrazyl (DPPH) assay, described by Blois (1958). The DPPH estimation was performed at different concentrations of the sample (200µg/ml, 400µg/ml, 600µg/ml, 800µg/ml and 1000µg/ml) by using methanol to make up the volume. Each concentration was added in 96 well microtiter plate and 0.1mM freshly prepared DPPH was added to each well. The blank was prepared without using sample. Plates were wrapped with the aluminum foil and incubate at 25°C for 30 minutes. Ascorbic acid was used as a positive control. The absorbance of reaction mixture was measured at 517 nm by using ELISA plate reader (Molyneux., 2004). The scavenging activity was measured in percentage of inhibition by using following formula:

$$\text{\% of Inhibition} = \frac{(\text{A of control}) - (\text{A of Test}) \times 100}{(\text{A of Control})}$$

Results and Observations

Objective 1: To do mass cloning of *Tylophora indica* by *De novo* adventitious shoot induction from leaves

Leaf explants of 3-5mm in size were excised from mature healthy field grown plants of *Tylophora indica*. The leaf explants were planted on MS medium supplemented with different growth regulators either alone or in conjunction with each other for direct *de novo* adventitious shoot induction.

De novo adventitious shoot formation

Murashige and Skoog's medium augmented with different auxins like NAA (5.37µM-21.48µM), 2, 4-D (5.37µM- 21.48µM) either alone or in conjunction with cytokinins like Kinetin (4.65µM- 18.6µM) or BAP (4.44µM- 22.2.2µM)or Adenine sulphate (1.36µM- 5.4µM) were used for *De novo* direct adventitious shoot induction. Likewise MS medium supplemented with different cytokinins like Kinetin (4.65µM- 18.6µM) or BAP (4.44µM- 22.2.2µM) either alone or in combination with Adenine sulphate (1.36µM- 5.4µM) was used for regeneration of adventitious shoots from leaf segments. Out of all these supplements, best results were observed on MS medium supplemented with BAP either alone or in conjunction with Adenine sulphate. Forty replicates were used for each treatment and each experiment was repeated thrice.

Tylophora indica exhibited high propensity of *De novo* adventitious shoot formation from the leaf explants on MS medium supplemented with 8.8 µM BAP. Nodular meristemoids developed from the cut ends as well as abaxial and adaxial surface of leaf explant after 10 days of culturing (fig.5). After 2 weeks, the entire surface of leaf explant was covered with these nodular meristemoids (fig.6). Initially, 8-9 microshoots emerged from these nodular meristemoids after 21 days (fig.7) leading to the formation of 17-20 shoots after 5 weeks of culturing (figs. 8 & 9). The shoots multiplied prolifically forming 50-55 shoots per flask after 11 weeks (fig.10). The proliferation rate of nodular meristemoids and shoots increased tremendously after first subculturing forming innumerable number of shoots (fig.11 & 12). The shoots elongated further forming numerous green leaves (fig.13). Figure 14 depicts large number of healthy green leafy shoots formed ready to be transferred to the rooting media.

Fig.5 Formation of nodular meristemoids (arrow heads) from leaf segment on MS + BAP (8.8 µM) after 10 days of culturing **Fig.6** Nodular meristemoids (arrowheads)covering the entire surface of leaf explants after 3 weeks **Fig.7** Sprouting of shoots (arrowheads) from the meristemoids **Figs.8 & 9** Further proliferation of shoots with well developed leaves after 5 weeks

Fig.10 Prolific shoots formation after 11 weeks of culturing **Figs.11 & 12** Subculturing and proliferation of numerous shoots on MS + BAP (8.88 µM) **Fig.13** Elongation of shoots developing numerous leaves **Fig.14** Large number of healthy green leafy shoots formed

Leaf explants cultured on MS medium augmented with 4.4 µM and 17.76 µM BAP also induced the formation of nodular meristemoids but the number of meristemoids formed were less and sprouting of shoots took much longer time.

The leaf explants cultured on MS medium supplemented with BAP (22.2 µM) in combination with lower concentration of Adenine sulphate (1.35 µM) produced the largest number of shoots. Nodular meristemoids differentiated from the entire cut surface of leaf explant after 8-10 days of culturing (fig.15) and whole surface of leaf lamina was covered with nodular meristemoids after 4 weeks of culturing in 100% of the cultures (fig.16). Initially, only 2-3 shoots were sprouted after three weeks of culturing because the formation of nodular meristemoids was slow initially. By 4^{th} week the entire surface of the leaf segment was covered with nodular meristemoids leading to vigorous shoot induction (figs. 17 & 18) forming 18-20 shoots per flask after 5 weeks (fig.19). Thereafter the shoots multiplied prolifically forming more than 70 shoots per flask after 11 weeks (fig.20). These shoots were proliferated further on subculturing and developed into new leaves (figs.21 & 22). The shoots elongated further forming numerous shoots in 100% of the cultures (fig 23). Repeated subculturing accelerated the formation of shoots in large number and there was no decline in shoot proliferation even after many subcultures (Fig.24)

Fig.15 Formation of nodular meristemoids (arrow heads) from leaf segment on MS + BAP (22.2µM) + Ad.sul.(1.35µM) **Fig.16** Nodular meristemoids covering the entire surface (arrow heads) of leaf explants on MS + BAP (22.2µM) + Ad.sul.(1.35µM) after 4 weeks **Figs.17 & 18** Sprouting of shoots (arrow heads) from meristemoids after 3 & 4 weeks respectively **Fig.19** Prolific shoot formation after 5 weeks.

Fig.20 Prolific shoots formation on MS medium + BAP (22.2µM) + Ad.sul.(1.35µM) **Figs.21 & 22** Subculturing and proliferation of numerous shoots on MS + BAP (22.2µM) + Ad.sul.(1.35µM **)** **Fig.23** Elongation of shoots developing numerous leaves **Fig.24** Formation of large number of healthy leafy green shoots.

Effect of different concentrations of BAP either alone or in combination with adenine sulphate on direct *de novo* adventitious shoot formation from leaf explant is depicted in **Figure 25** and **Table. 5**

Growth Regulators (µM)	**No. of explants cultured**	**% of Explant showing shoot induction**	**Average no. of shoots per explants**			
			3 weeks	5 weeks	7 weeks	11weeks
BAP (4.44)	40	80	5	15	23	32
BAP (8.88)	40	100	9	17	25	54
BAP (17.76)	40	95	7	24	39	37
BAP (22.2) + Adenine Sulphate(1.35)	40	100	2	19	50	More than 70

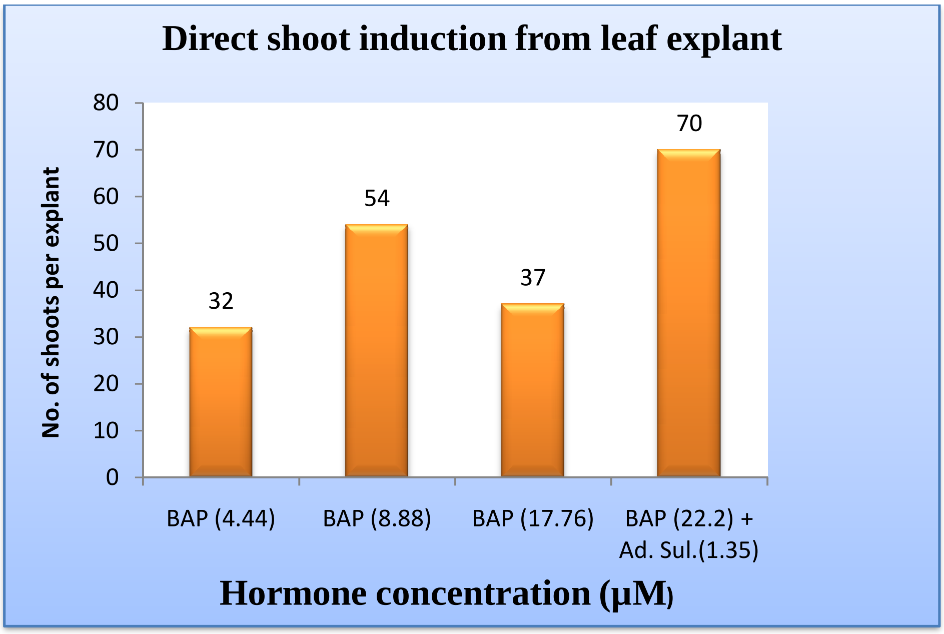


Fig. 25

Rooting of Microshoots

Elongated shoots were carefully rescued from the culture bottles and each shoot was carefully inoculated upright in the half strength MS medium or MS medium supplemented with either IBA (4.92µM- 19.68µM) or IAA (5.71µM- 22.84µM). Best rooting occurred on 19.68 µM IBA, where rooting initiated after 13 days of culture (Fig.26). The roots multiplied further forming a bunch of short, thick roots in 95% cultures (Fig.27). The roots elongated further and a cluster of 30-35 thick, stout roots was formed (Fig.28). Roots were also formed on half strength basal MS medium, where 2-3 roots emerged in 88% of the cultures (Fig.29). Further elongation of the roots occurred but the roots formed were long and thin (Figs.30 &31). Figure 32 depicts a complete plantlet with well developed shoot and root system ready to be transferred to the soil.

Effect of culture media on rooting from regenerated shoots is depicted in Table.6 and Figure 33.

Growth regulators	**% age of shoots forming roots**	**Average no. of roots/ explants**			**Average length of roots/ Explant (cm)**		
		One week	Three weeks	Five weeks	One week	Three weeks	Five weeks
Half strength MS medium	88%	1	3	5	2.7	6.5	7.7
MS medium with IBA (19.68µM)	95%	11	27	35	1.3	2.8	7.5

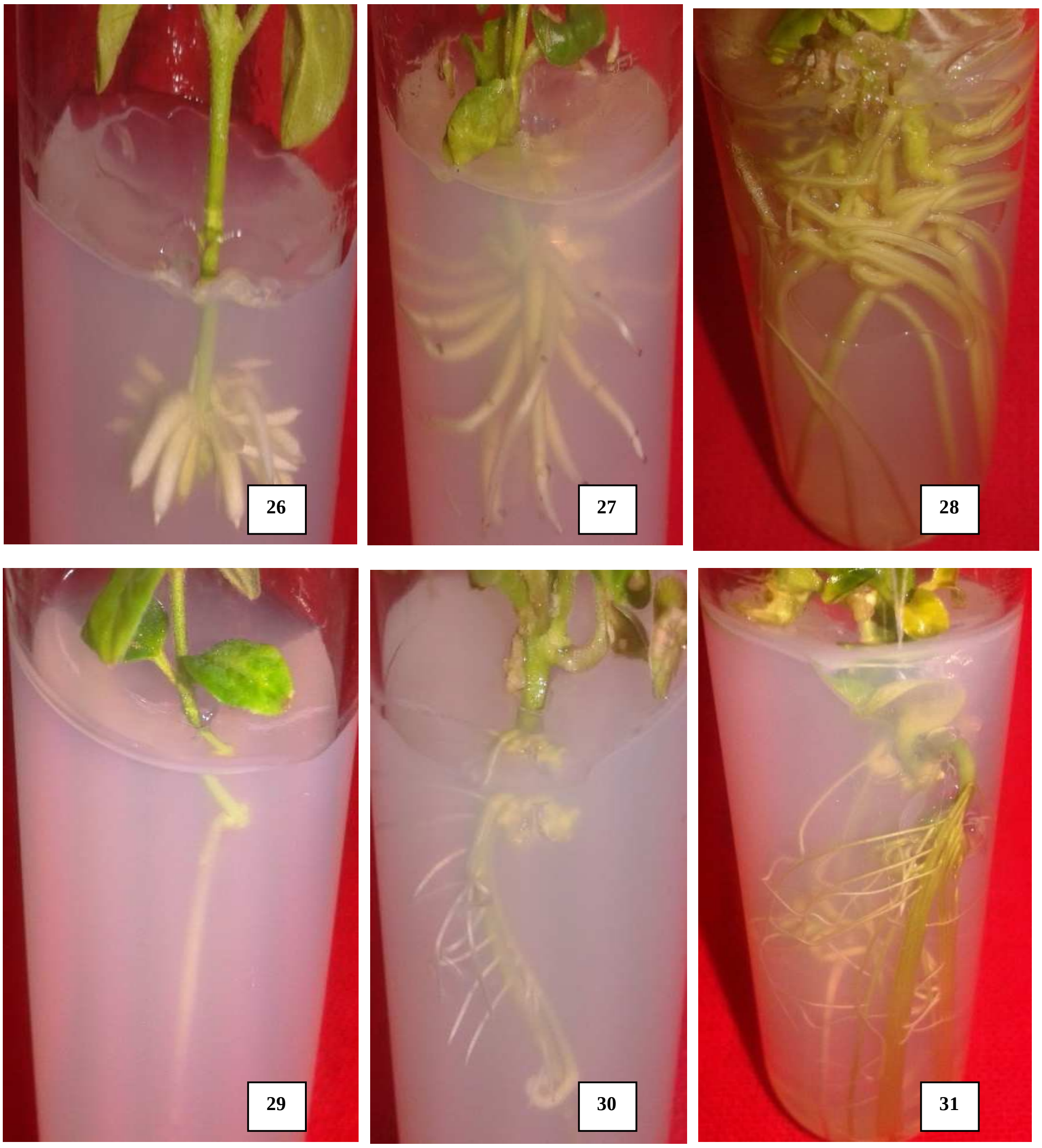


Fig.26 Formation of thick-branched roots on MS medium with IBA (17.24 µM **)** **Fig.27** Further growth and elongation of roots **Fig.28** Clusters of roots formed at the base of shoot **Fig.29** Initiation of roots from basal end of regenerated shoot on half strength MS medium **Fig.30**. Further elongation of roots after 3weeks **Fig.31** Formation of numerous roots on half strength MS medium

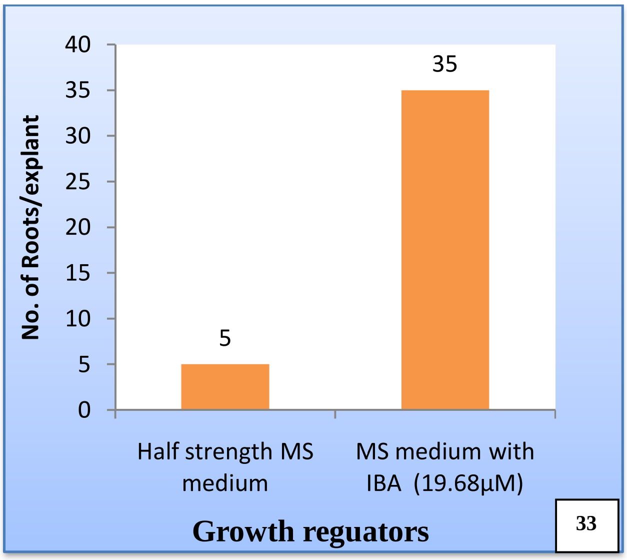


Fig.32 An isolated complete plantlet showing well developed root system **Fig.33** Effect of different growth media on rooting

Acclimatization and Hardening

After the complete plantlet formation, the regenerents were established in the soil. In order to acclimatize, the rooted plantlets were carefully removed from the culture vessels using forceps to avoid any mechanical damage to roots. The plantlets were washed with distilled water to remove any agar sticking to them. Plantlets were transferred to plastic cups covered with perforated plastic bags to maintain aeration and internal humidity and kept under the culture conditions for 15 days (Fig.34). The plastic bags were removed from the cups which were kept as such in the growth room for another one week (Fig.35). The plants were monitored and watered after every 3rd day. Thereafter, the plantlets were shifted to poly bags containing the same potting mixture and w kept in the growth room for another 2 weeks (Fig. 36). The hardened plantlets were then transferred to green house for 2 weeks before their final transfer to full sunlight outdoor (Fig.37). 90% survival rate was observed when plants were transferred to field conditions (figs.38 & 39).

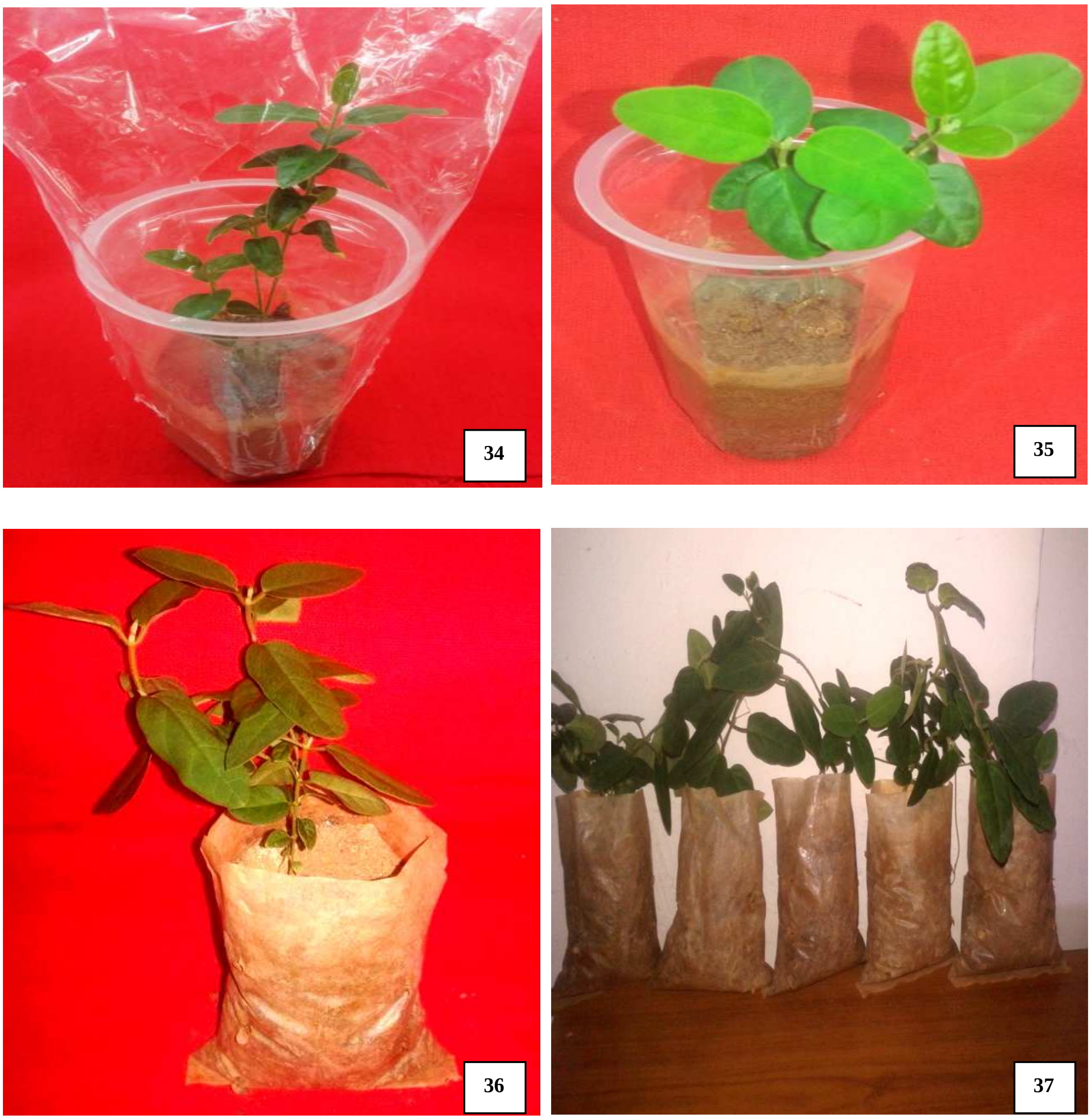
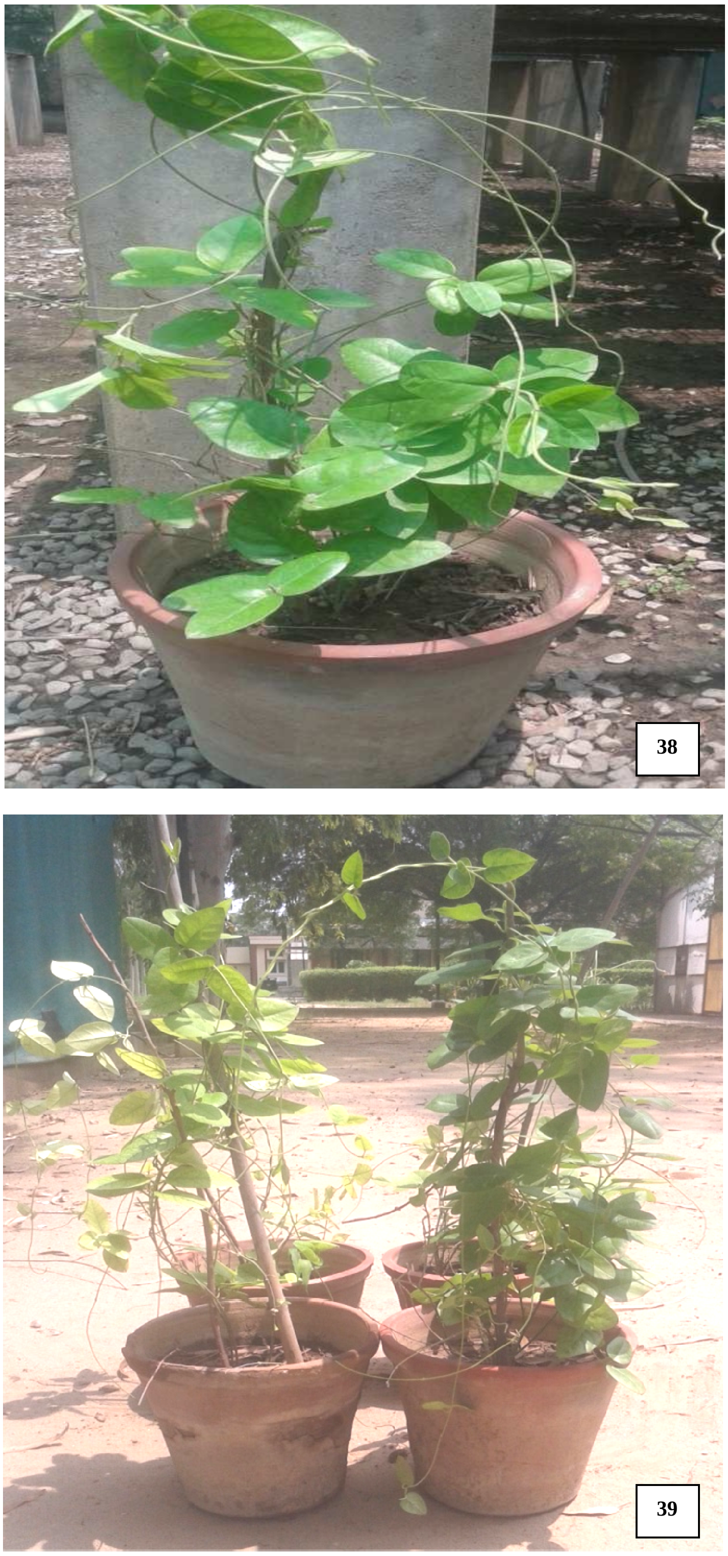


Fig.34 Plantlet transferred to plastic cups covered with perforated plastic bags for initial acclimatization **Fig.35** Plantlets in plastic cups containing potting mixture and kept at growth room **Fig.36** Plantlet in poly bags under growth room conditions **Fig.37** Hardened plantlets in green house.



Figs (38 & 39) Well acclimatized plants in open field conditions

Objective 2: To compare the antimicrobial potential of native and micropropagated plants of *T. indica*

Preparation of crude extract of *Tylophora indica*

The leaf extracts of *in vitro* and *in vivo* plant of *T. indica* were prepared by using two methods, one method was according to Bhatia *et al.*, (2013) in which methanol and acetone solvents were used for extraction of leaves. The obtained concentration of extract was largely dependent upon the solvent used. The extraction yield of leaf extract of both *in vitro* and *in vivo* by using methanol and acetone solvent was different. The concentration of yield obtained by methanol extract for *in vitro* and *in vivo* raised plant was 250 mg/ml and 120 mg/ml respectively and yield of acetone extract of both *in vitro* and *in vivo* was 130 mg/ml and 125 mg/ml respectively. The root extract was also prepared by same method and methanol solvent was used for extraction of roots. The extraction yield of root extract of *in vitro* raised plant was 170mg/ml. The other method used for extraction of leaves was Rao and Brook's method, 1970. The extraction yield by this method was very high as compared to first method. The yield obtained for *in vitro* raised plant was 532 mg/ml and for *in vivo* was 392 mg/ml.

Antimicrobial testing

Results obtained in the present study indicate that the tested medicinal plant extracts of *Tylophora indica* exhibited potential activity against tested bacteria such as *Staphylococcus aureus* and very less activity shown against *Escherichia coli.* The antifungal activity was also observed against *Aspergillus niger* and *Penicillium spp.* When tested by Agar well diffusion method, the methanol extract of *Tylophora indica* showed highest activity against bacteria and fungus.

Antibacterial susceptibility test

The crude extract was prepared by using different solvents, out of which the extract prepared by Rao and Brook method shown highest overall activity of *in vivo* raised plants against *S. aureus* *i.e*. showed 25mm diameter of Zone of inhibition(Fig.41), followed by the methanol extract and

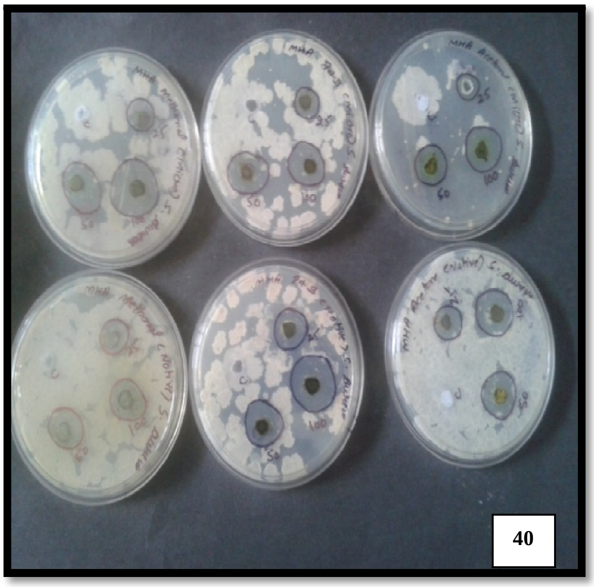
Acetone extract. The least activity of crude extract, prepared in acetone and extract prepared by Rao and Brook method of *in vitro* raised plants having concentration 25µg/ml concentration with zone of inhibition 12mm and 11 mm respectively. The leaf extract of both *in vitro* and *in vivo* prepared in acetone solvent showed similar activity at concentration 100µg/ml against *S. aureus* of 20mm zone of inhibition. The extract prepared by Rao and Brook method also showed significant activity against *S. aureus* at all concentrations *i.e.* 19mm at 25µg/ml, 20mm at 50µg/ml and 25mm at 100µg/ml (Fig.40)

The crude extract of *Tylophora indica* represented very less activity against *Escherichia coli.* The methanol extract and acetone extract of *in vivo* raised plants did not showed any zone of inhibition but the extract prepared by Rao and Brook method, 1970 of both *in vivo* raised plants showed activity against *E.coli* at concentration 50µg/ml and 100µg/ml of 12mm and 17mm respectively (Fig.42). The methanol extract of *in vitro* raised plants of *Tylophora indica* showed highest activity of 16mm against *E.coli* at concentration 100µg/ml followed by acetone and extract prepared by Rao and Brook method (Fig.43).

Table.7 Zone of inhibition (mm) of different concentrations of various extracts against *Staphylococcus aureus* and *Escherichia coli.*

Extracts	Concentrations (µg/ml)	***Staphylococcus aureus***		***Escherichia coli***	
		Zone of inhibition (mm)		Zone of inhibition (mm)	
		In vitro raised plants	*In vivo* raised plants	*In vitro* raised plants	*In vivo* raised plants
Methanol	25	18±1.5	12±2.0	0±0	0±0
	50	19±1.0	18±2.0	12±2	0±0
	100	23±2.1	19±2.0	15±1.5	0±0
Acetone	25	12±1.5	14±1.0	0±0.6	0±0
	50	16±1.0	18±1.0	0±0	0±0
	100	20±2.0	20±0.6	11±1	0±0
Rao and Brook	25	11±1.0	19±2.0	1±1.2	1±1.2
	50	21±1.5	20±2.0	9±1.5	12±1.5
	100	22±1.5	25±1.0	12±1.5	17±1.5

Values are mean of Zone of inhibition (mm) ± Standard deviation of mean values



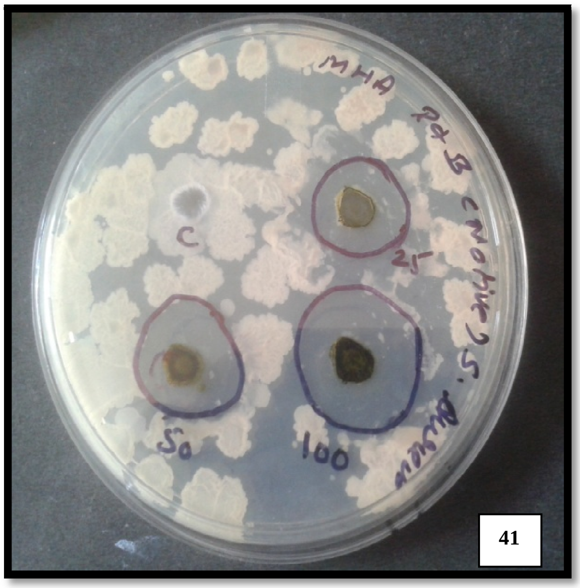
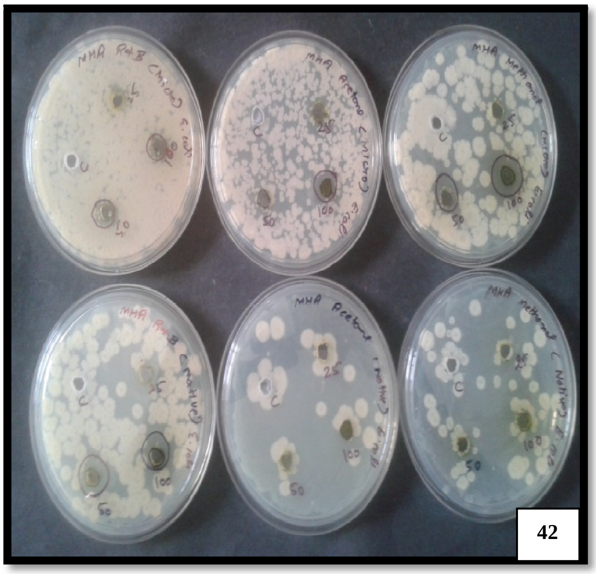


Fig.40 Zone of inhibition of different concentrations (25µg/ml, 50µg/ml and 100µg/ml) of methanol, acetone extract and the extract prepared by Rao and Brook method of both *in vitro* and *in vivo* plants of *T. indica* against *S. aureus*. Fig.41 Zone of inhibition of different concentrations (25µg/ml, 50µg/ml and 100µg/ml) of extract prepared by Rao and brook method of *in vivo* plant of *T. indica*



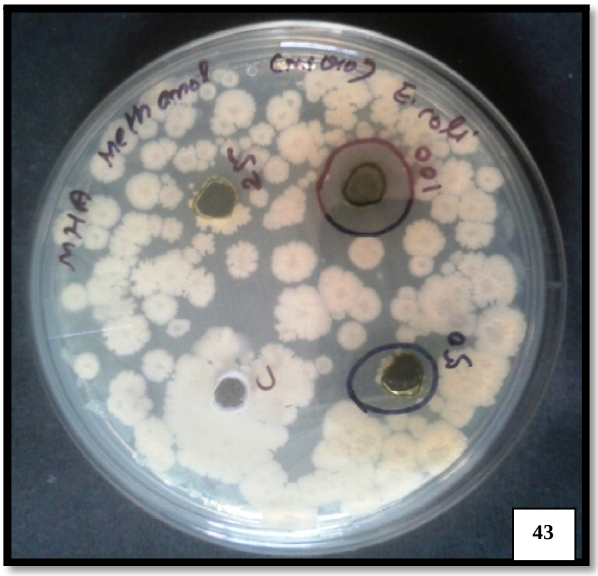


Fig.42 Zone of inhibition of different concentrations (25µg/ml, 50µg/ml and 100µg/ml) of methanol, acetone extract and the extract prepared by Rao and Brook method of both *in vitro* and *in vivo* plant of *T. indica* against *Escherichia coli.* **Fig.43** Zone of inhibition of different concentrations (25µg/ml, 50µg/ml and 100µg/ml) of methanol extract of *in vitro* plant of *T. indica*

Antifungal susceptibility test

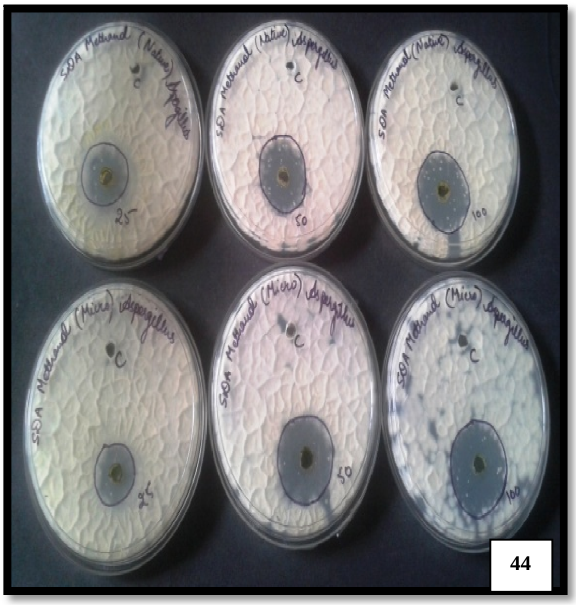
Antifungal activity of leaf extract of *Tylophora indica* showed significant activity against *Aspergillus niger* and *Penicillium* spp. The crude extract prepared in different solvents showed significant activity at different concentrations. Overall highest activity was shown by methanol extract of both *in vitro* and *in vivo* raised plants at all concentrations (25µg/ml, 50µg/ml and 100µg/ml) against *Aspergillus niger*. The maximum activity was shown by the extract prepared by Rao and Brook method of *in vitro* raised plants at concentration 100µg/ml *i.e*. the zone of inhibition made was of 38mm against *Aspergillus niger*. The second largest activity against *Aspergillus niger* was shown by methanol extract of *in vivo* raised plants at concentration 100µg/ml and measured zone of inhibition of 36mm followed by Acetone extract of *in vitro* raised plants of around 35mm. The methanol extract prepared of *in vivo* raised plants recorded higher zone of inhibition *i.e.* 30mm, 31mm and 36mm against *Aspergillus niger* as compared to *in vitro* raised plants at 25µg/ml, 50µg/ml and 100µg/ml concentration respectively. The minimum zone of inhibition measured was around 10 mm of acetone extract of *in vivo* raised plants against fungus *Aspergillus niger*.

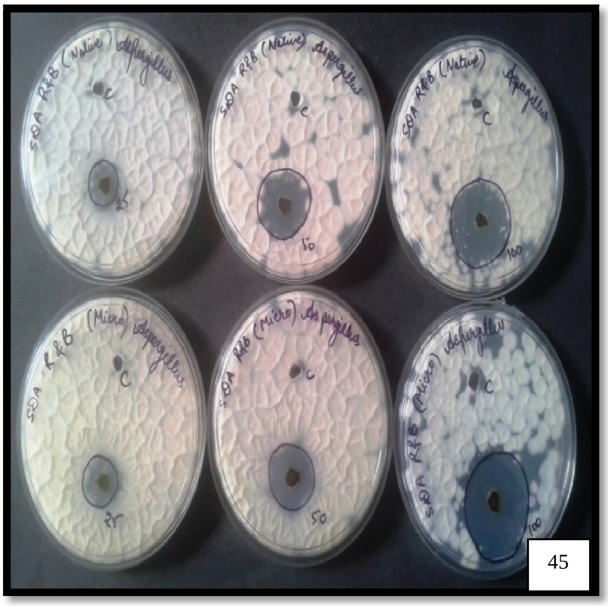
The crude extract of *Tylophora indica* against *Penicillium* spp. shows significant activity. The extract prepared by Rao and Brook method of *in vitro* raised plants shows powerful activity as compared to other extracts at all concentrations, the maximum activity of 33mm observed at concentration 50µg/ml followed by 100µg/ml and 25µg/ml. The prominenet activity of methanol extract of *in vivo* raised plants was also recorded against *Penicillum* spp. at concentration 100µg/ml of around 32mm, 27mm at 50µg/ml and 23mm at 25µg/ml. The least activity was measured 8mm at concentration 25µg/ml of acetone extract of *in vitro* raised plants against *Penicillium* spp.

Table.8 Zone of inhibition (mm) of different concentrations of various extracts against *Aspergillus niger* and *Penicillium* spp.

Extracts	**Concentrations (µg/ml)**	***Aspergillus niger***		***Penicillium spp.***	
		Zone of inhibition (mm)		Zone of inhibition (mm)	
		In vitro raised plants	*In vivo* raised plants	*In vitro* raised plants	*In vivo* raised plants
Methanol	25	19±1.2	30±1.0	13±1.0	23±1.2
	50	27±1.0	31±1.2	17±1.0	27±1.5
	100	30±1.5	36±0.6	14±0.6	32±1.5
Acetone	25	19±1.5	10±1.5	8±1.0	10±1.0
	50	27±1.0	15±1.5	13±1.5	12±0.6
	100	35±1.0	30±1.5	28±1.0	15±1.0
Rao and Brook	25	16±1.5	19±1.0	30±0.6	15±2.1
	50	22±0.6	26±0.6	33±1.2	20±1.5
	100	38±1.0	31±1.2	32±1.0	25±0.6

Values are mean of Zone of inhibition (mm) ± Standard deviation of mean values





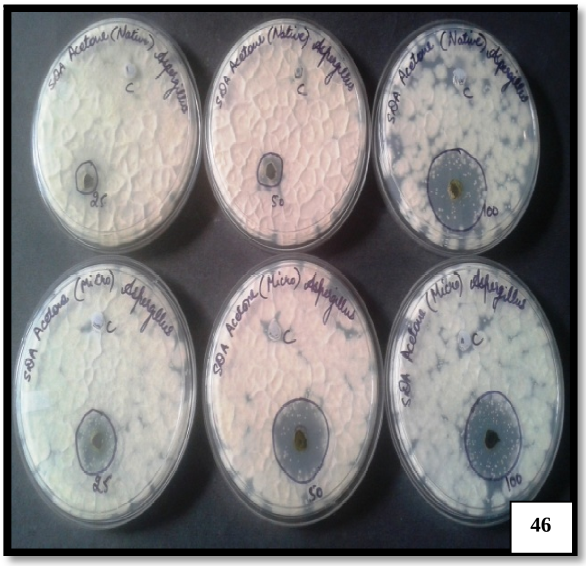
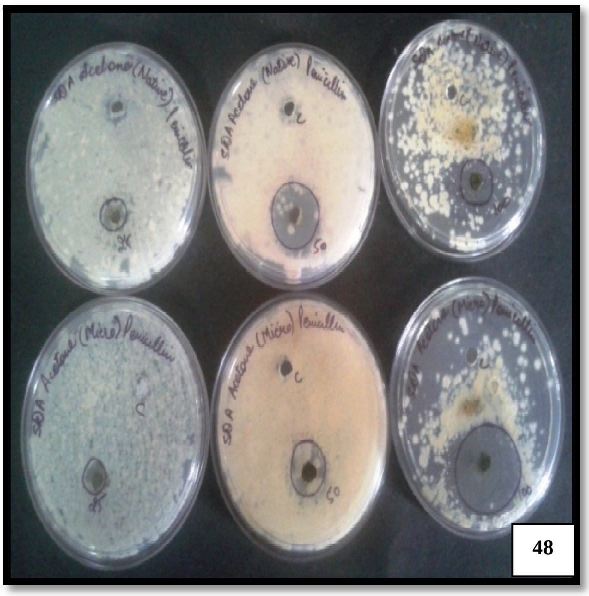


Fig.44 Zone of inhibition of different concentrations (25µg/ml, 50µg/ml and 100µg/ml) of methanol extract of both *in vitro* and *in vivo* plant of *T. indica* against *Aspergillus niger*. **Fig.45** Zone of inhibition of different concentrations (25µg/ml, 50µg/ml and 100µg/ml) of acetone extract of both *in vitro* and *in vivo* plant of *T. indica* against *Aspergillus niger* **Fig.46** Zone of inhibition of different concentrations (25µg/ml, 50µg/ml and 100µg/ml) of extract prepared by Rao and Brook method of both *in vitro* and *in vivo* plant of *T. indica* against *Aspergillus niger* **Fig.47** Zone of inhibition of extract prepared by Rao and Brook method of *in vitro* plant of *T. indica* at concentration 100µg/ml against *Aspergillus niger*.



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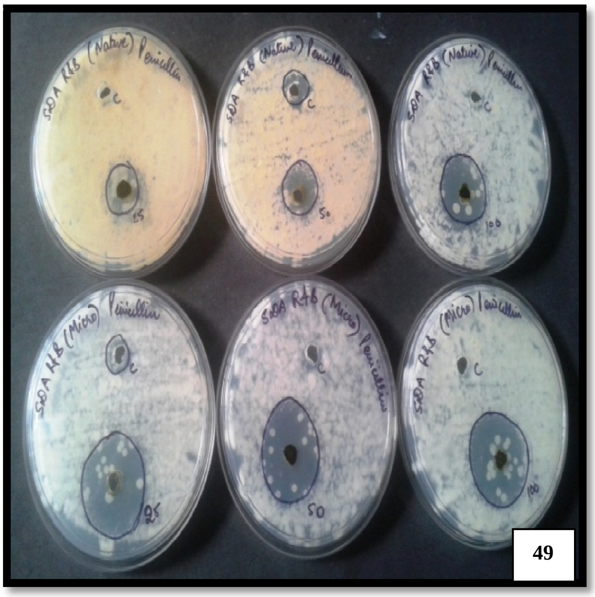

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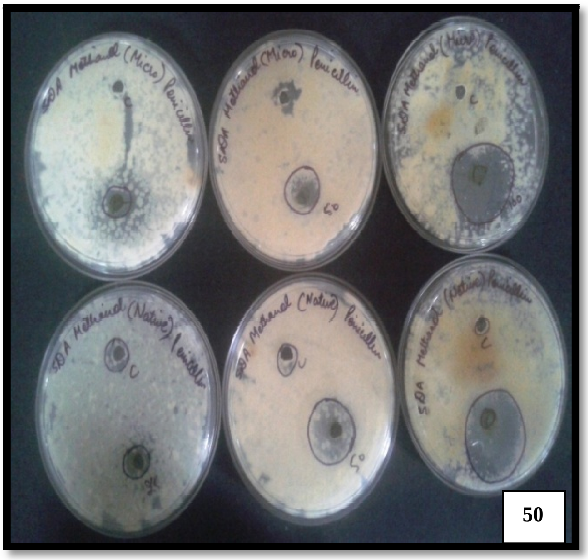

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Fig.48 Zone of inhibition of different concentrations (25µg/ml, 50µg/ml and 100µg/ml) of acetone extract of both *in vitro* and *in vivo* plant of *T. indica* against *Penicillium* spp. **Fig.49** Zone of inhibition of different concentrations (25µg/ml, 50µg/ml and 100µg/ml) of extract prepared by Rao and Brook method of both *in vitro* and *in vivo* plant of *T. indica* against *Penicillium* spp. **Fig.50** Zone of inhibition of different concentrations (25µg/ml, 50µg/ml and 100µg/ml) of methanol extract of both *in vitro* and *in vivo* plant of *T. indica* against *Penicillium* spp.**Fig.51** Zone of inhibition of extract prepared by Rao and Brook method of *in vitro* plant of *T. indica* at concentration 100µg/ml against *Penicillium* spp.

Objective 3: To evaluate the antioxidant potential of the root extract of micropropagated plants of *T. indica*

Antioxidant activity

The root extract of *in vitro* raised plant of *Tylophora indica* showed promising antioxidant activity. Ascorbic acid (Positive control) exhibited 76% scavenging activity which is the promising activity for scavenging of free radicals. The root extract was analyzed at five different concentrations *i.e*. 200 µg/ml, 400 µg/ml, 600 µg/ml, 800 µg/ml and 1000 µg/ml. the results of the DPPH assay are shown in Table.9 and depicted in figure.52. As the concentration of root extract increases the scavenging activity was also found to be increases. Highest scavenging activity was 75% at concentration 1000 µg/ml and lowest activity was observed 63% at concentration 200µg/ml. This shows that the scavenging activity of root extract of *Tylophora indica* was found to enhance e in a concentration dependent manner.

Table.9 Antioxidant activity of root extracts of *T. indica*- DPPH assay

Concentration (µg/ml)	**Scavenging activity (%)**
Ascorbic acid + DPPH	76
200 + DPPH	63
400 + DPPH	69
600 + DPPH	70
800 + DPPH	74
1000 + DPPH	75

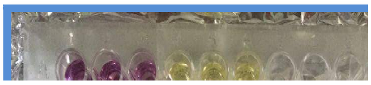

Fig.52 96 wells micro titer
antioxidant activity (% of Sca

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