Tissue Culture Studies Of *Solanum nigrum* L and Comparison of the Medicinal Activities of the Callus Extract and Plant

By

SIDRA AYYAZ

Department of Bioinformatics & Biotechnology

Faculty of Basic and Applied Sciences

International Islamic University Islamabad

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Tissue culture.
Cell "
Medicinal plants.

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Researchers

Sidra Ayyaz

130-FBAS/MSBT/F14

Supervisor

Dr. Nyla Jabeen

Assistant Professor

Department of Bioinformatics & Biotechnology

Faculty of Basic and Applied Sciences

International Islamic University Islamabad

(2016)


بسم الله الرحمن الرحيم

Department of Bioinformatics and Biotechnology

International Islamic University Islamabad

Dated: _____

FINAL APPROVAL

It is certificate that we have read the thesis submitted by Ms. Sidra Ayyaz and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the M.S Degree in Biotechnology.

COMMITTEE

External Examiner
Name: Dr. Qaiser Mahmood
Designation: Professor
Department: Environmental Biology
University: Comsats, Abbotabad

Internal Examiner
Name: Dr Jabber Khattak
Designation: Assistant Professor
Department: Bioinformatics and Biotechnology
University: International Islamic University

Supervisor
Name: Dr. Nyla Jabeen
Designation: Assistant Professor
Department: Bioinformatics and Biotechnology
University: International Islamic University

Dean, FBAS
Name: Dr. Muhammad Sher
International Islamic University, Islamabad

A thesis submitted to Department of Bioinformatics and Biotechnology,
International Islamic University, Islamabad as a partial fulfilment of requirement
for the award of the degree of MS Biotechnology

DEDICATION

I would like to dedicate my thesis to my beloved parents who devoted their lives and resources for my better future.

DECLARATION

I hereby declare that the work present in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Date:

Sidra Ayyaz.

CONTENTS

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Sidra Ayyaz.

LIST OF ABBREVIATIONS

BAP	6-Benzylaminopurine
Kin/Kn/KIN	Kinetin
IBA	Indole-3-butyric Acid
IAA	Indole-3-acetic Acid
2, 4-D	2, 4 Dichlorophenoxyacetic Acid
NAA	Naphthalene Acetic Acid
MS	Murashage and Skoog
$HgCl_2$	Mercuric Chloride
$FeCl_3$	Ferric Chloride
HCl	Hydrochloric Acid
NaOH	Sodium Hydroxide
CI	Callus Induction
SI	Shoot Induction
ddH_2O	Double Distilled Water
dH_2O	Distilled Water
UV	Ultra Violet
µM	Micro Molar
cm	Centimetre

ml	Millilitre
%	Percentage
°	Degree
C	Centigrade
i.e	That is
g	Gram
pH	Potentia Hydrogenii
AOV	Analysis Of Variance

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ABSTRACT

A current study was aimed for the *in vitro* multiplication of *Solanum nigrum* L and comparison of medicinal activities of callus extract and plant. *Solanum nigrum* L. is fairly common herb, which is the member of genus *Solanum* of family *Solanaceae* and generally considered to be native to Europe and Asia. In Pakistan it is extensively distributed in Punjab, NWFP, Sindh and Azad Kashmir in the areas of Rawalpindi, Attock, Mianwali, Gujranwala, Mansehra, Hazara, Peshawar, Chitral, Gilgit, Bannu, Quetta, Dera Nawab Khan, Sibbi, Mirpur and Muzaffarabad. Its habitat is from tropical to temperate regions and from sea-level to altitude exceeding 3500m. Several chemical compounds have been isolated from different parts of plant to cure malaria, black fever, dysentery and urinary infection. The plant is also known to have anti-inflammatory, antioxidant, antidiabetic, anti-diarrhoeal and antimicrobial activity.

For callus induction leaf and hypocotyl explants of *Solanum nigrum* L were used on different concentrations and combinations of BAP and IAA. In all media combinations different concentrations of BAP and IAA i.e 2, 3 and 4 mg/L of BAP and 0.5 mg/L of IAA were used. Best callus results were obtained on leaf explant at concentration of 4 mg/L of BAP and 0.5 mg/L of IAA followed by 3 mg/L of BAP and 0.5 mg/L of IAA and 2 mg/L of BAP and 0.5 mg/L of IAA. Calli were then transferred to MS medium containing cytokinins for regeneration which is supplemented with different concentrations and combinations of plant growth regulators. The plant showed best shooting in a media having the combination of BAP and IAA at the concentration of 6 mg/L and 0.5 mg/L respectively followed by 5 mg/L of BAP and 0.5 mg/L IAA. High frequency direct shoot proliferation was also induced in the hypocotyl explants cultured on MS medium supplemented with different concentrations of Kinetin i.e 2, 3 and 4 mg/L. Amongst the various concentrations tested, 3 mg/L kinetin proved to be the most effective.

Phytochemical analysis of callus and plant extracts of *Solanum nigrum* L was also performed. Alkaloids, flavonoids, tannins and saponins were found to be present in methanolic extract of plant. Whereas only saponins is present in aqueous extract of plant. While in methanolic extract of callus, alkaloids, flavonoids, tannins and saponins were present.

Antibacterial activity on *Solanum nigrum* L was performed with methanolic extract of both callus and leaf of the plant against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Clear zone of inhibition were observed and determined by measuring the diameter of zone of inhibition expressed in millimetre.

A standardized protocol for *in vitro* tissue culturing of *Solanum nigrum* L is successfully established which can further be used for their large scale production, irrespective of season and weather. The plants can be supplied to pharmacological industries and can be used for gene transformation as well. Also antibacterial evaluation of *Solanum nigrum* forms a primary platform for further pharmacological investigation for the development of new potential antimicrobial compounds.

Chapter 1
Introduction

1. Introduction

1.1 Medicinal Plants

For thousands of years nature has provided a source of herbal medicine and man has used its different parts for the treatment and prevention of various diseases. Nowadays based upon this; modern drugs have been extracted from the natural sources (Chah *et al.*, 2006). In developing countries 80% of world population depends on plants derived medicine (Peter *et* al., 2005).

Medicinal plants constitute the active compounds that provide plentiful resources for the pest control in the field of agriculture and food industry as well as in pharmaceutical. Due to the better compatibility with human body it is believed that the chemical compounds present in herbal product are part of physiological function of living organisms (Reddy *et al.*, 2012).

The high cost of allopathic medicine and high degree of side effect, encourage the people to use the homeopathic medicine due to the vast pharmacological properties, including anti-oxidant and anti-tumour activities (Gogoi and Islam, 2012).

Growing interests of public and extensive use of herbal medicine, open up the new area of medicinal research which is effective and safe product for human consumption (Reddy *et al.*, 2012).

1.2 Medicinal Flora of Pakistan

Pakistan has diverse range of climate and phytogeography conditions which results in wide flora having several medicinal plant species (Syed *et al.*, 2010). It is estimated that Pakistan has a diverse flora containing about 6000 plant species among them 12% are medicinally important (Shinwari and Qaiser, 2011).

In remote areas about 60% of local population depends upon medicinal herb (Qamar *et al.*, 2010). For long time the local communities used herbal products for curing various diseases (Syed *et al.*, 2010). Such flora frequently shows a broad spectrum of biological

activities, for instance they are intended to reduce inflammation, show bacterial and fungicidal properties (Cowan, 1999).

The root bark, seed and fruits extracted of their plants are exploit in the development of syrup and infusions in traditional medicine (Imtiaz and Manzoor, 2003).

The herbal market system known as "Pansara" is totally reliant on the natural flora of Pakistan. Treatment of diseases in persons and animal both are achieved by utilization of therapeutic plants (Shinwari and Qaiser, 2011).

1.3 Scientific Classification

Solanum nigrum L. is the medicinal plant. The scientific classification of this medicinal plant is (Potawale *et al.*, 2008).

Kingdom	:	Plantae
Subkingdom	:	Tracheobionta
Super division	:	Spermatophyta
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Subclass	:	Asteridae
Order	:	Solanales
Family	:	Solanaceae
Genus	:	*Solanum*
Specie	:	*nigrum*

1.4 Family Solanaceae

Solanaceae is cosmopolitan family. Though the species are spread throughout the world, they are more prevalent in tropical and warm temperate regions with centres of variety in the southern hemisphere, partially in South America. Other centre of speciation occur in Australia and Africa, with comparatively few and less dissimilar species being found in Europe and Asia (Zubaida *et al.,* 2010). It is one of the medium sized family, composed of approximately 96 genera and 3000 species occurring all over the world (Ishrat *et al.,* 2014).

From literally point of view *Solanum* means "nightshade" or "solamen" which means to comfort or soothe, referred to calming and sedative effects present in many species. Genus *Solanum* consist of 1,500 to 1,700 species including *Solanum melongena* (eggplant), *Solanum tuberosum* (potatoes), *Solanum carolinense* (horse nettle) and many more. From economic point of view large number of important vegetables, fruits and weeds are included in this family, such as *S.tuberosum* (potatoes), *Lycopersicon esculentum* (tomatoes), *Nicotiana tabacum* (tobacco) (Michael, 2003).

In Pakistan the family has 14 genera and 52 species (Ishrat *et al.,* 2014). In herbal medicines about 21 species and 01 variety of this genus were used (Zubaida *et al.,* 2013). Member of Solanaceae family are extremely diverse and contain large number of hybrid, identification based on morphological characters is quite difficult. The three medicinally important species of Solanum genus are *S. nigrum*, *S. americanum* and *S. villosum* (Zubaida *et al.,* 2010).

1.5 *Solanum nigrum* L

S.nigrum L. is fairly common herb, which is the member of genus *Solanum* of family *Solanaceae* (Jabar *et al.,* 2012). It is commonly called as Black nightshade in English, Kakamachi in Hindi and Mako in Urdu (Potawale *et al.,* 2008).

1.5.1 Distribution

It is common plant found in almost all parts of Europe and African continent but it is generally considered to be native to Europe and Asia (F.O. *et al.,* 2011). In Pakistan it is extensively distributed in Punjab, NWFP, Sindh and Azad Kashmir in the areas of Rawalpindi, Attock, Mianwali, Gujranwala, Mansehra, Hazara, Peshawar, Chitral, Gilgit, Bannu, Quetta, Dera Nawab Khan, Sibbi, Mirpur and Muzaffarabad (Zubaida *et al.,* 2010).

1.5.2 Habitat

Solanum nigrum L. grows in various types of habitats, as weeds in crops and in ruderal habitats, along the roads, fences and neglected places such as around the building and houses, under trees, on forests and grassland (Jabar *et al.,* 2012). It mainly settles at moist environment, only growing in area of low rainfall (Karschon and Horowitz, 1985). Their wide tolerance of habitat type commencing from tropical to temperate regions and from sea-level to altitude more than 3500m contributed to success as wide spread weed (Henderson, 1974).

1.5.3 Morphology

Solanum nigrum L. is a sub-glabrous to villous annual plant up to 70-75 cm in height, shielded with simple multicellular hairs having glandular or e-glandular heads. It contains tap roots with few branches and numerous small lateral roots. The stem is erected glabrous and slightly un-branched (Potawale *et al.,* 2008). The foliage is alternate and somewhat ovate with unevenly toothed wavy margin. Flowers are usually white with five regular parts. The fruit is round, fleshy berry and black when ripe. Numerous seeds are present having brown in colour (Padmapriya *et al.,* 2011) (Figure 1.1)

(a)

(b)

(c)

(d)

(e)

(f)

Figure 1.1: Morphology of *Solanum nigrum* L.

(a) Root (b) Stem (c) Leaves (d) Flower (e) Fruit (f) Seeds

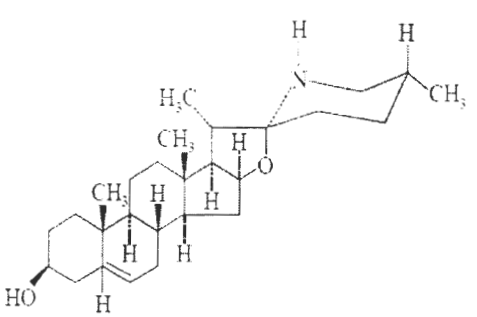
1.5.4 Chemical Constituents

Several chemical compounds have been isolated from different parts of plant that are responsible for pharmacological activities. Acetic acid, tartaric acid, malic acid and citric acid were found as major organic acids. However in adaptive responses to environmental stress; tartaric acid and citric acid were the most important (F.O. *et al.,* 2011).

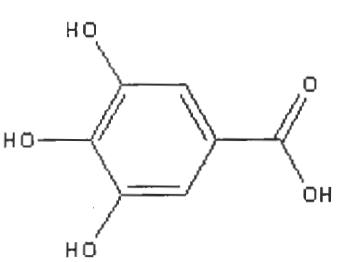
Green unripe berries contain glycoalkaloids that include solamargine, solasonine, solanine, solasodine, solanidine (Figure 1.2). Solanine is present in whole plant and its level increases as plant matures. Solasonine and solamargine are considered as two most important glycoalkaloids, responsible for anticancer activity and its concentration are highest during fruiting and flowering respectively (Potawale *et al.,* 2008).

It contains alkaloids found in all parts of plant and it is one of the major plant natural defences which are toxic even in small amount. It includes aglycone, solanidine (alkaloidial portion) and three sugar moieties (glucose, galactose and rhamnose collectively called solatriose) are present (Ramya *et al.,* 2011).

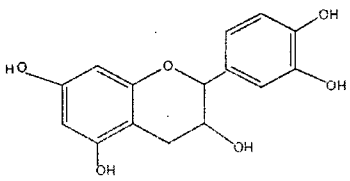
S.nigrum L. contain many other active components like glycoprotein, polysaccharides, polyphenolic compounds such as gallic acid, catechin, protocatechuic acid (PCA), caffeic acid, epicatechin acid, rutin and naringenin (Figure 1.2) (Rajani *et al.,* 2012).



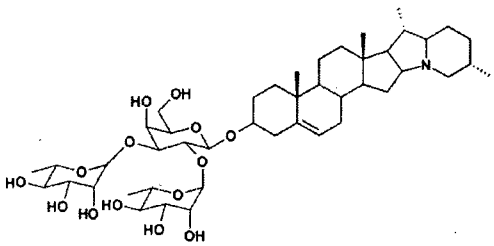
Solasodine



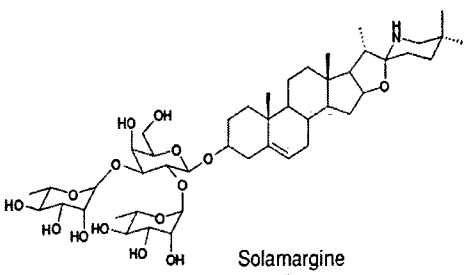
Gallic Acid



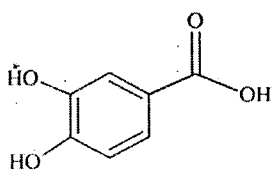
(+)-catechin



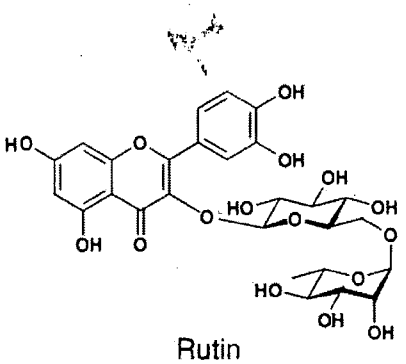
Solanine



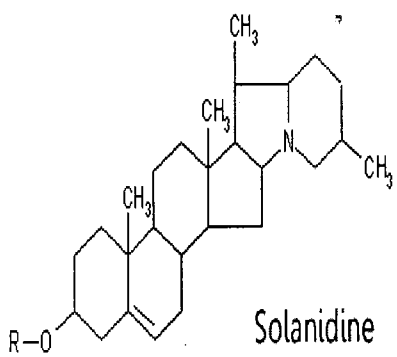
Solamargine



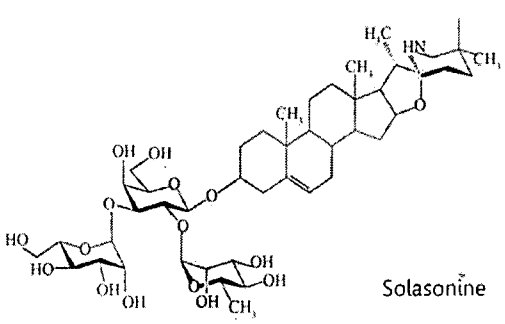
Protocatechuic acid



Rutin



Solanidine



Solasonine

Figure 1.2: Chemical Constituents of *Solanum nigrum* L.

1.6 *Solanum nigrum* L. As Traditional Folklore Medicine

Solanum nigrum L. is widely used medicinally throughout the world. It's used is recorded from the earliest times. Among great British herbals, Gerard's herbal of 1636 stated that nightshade is used for those weaknesses that need cooling and binding and it was worthy against headache, heart burning and heat of stomach. Later in Culpeper's herbal of 1649 described nightshade as cold saturnine plant, which were generally used to cool hot inflammation both outside and inside. The Arabs used fresh leaves to ease pain and reduce the inflammation. Leaf juice was helpful for the treatment of ringworm, gout and earache.

In India, it is used for the treatment of cardalgia and gripe due to its antiseptic and antidysectric properties. Freshly prepared extract of plant is effective in the cure of cirrhosis of liver and antidote to opium poisoning. Berries are used for different disease such as heart diseases, diarrhoea, ulcer and eye troubles. In Pakistan the power from the aerial portion of plant could be antiulcerogenic. In China foliage is used due to its febrifugal property. Dried aerial plant parts are used as diuretic, antihypertensive and anticancer agent. Fresh foliage is also used to cure wounds.

In East Africa it is believe that different portions of plant are helpful to treat malaria, black fever, dysentery and urinary infection. In Kenya, unripe fruits are spread over the aching teeth for pain relief. Roots are bubbled in milk and used as tonic (Chandrashekhar *et al.*, 2013).

1.7 Medicinal Importance of *Solanum nigrum* L.

For the novel pharmacologically active compounds, plants were the main source from which drugs were derived directly or indirectly (Ilyas *et al.*, 2002). *S.nigrum* is an important medicinal plant having diverse therapeutic properties.

1.7.1 Anti-proliferative Activity

On different cell lines with both crude extract and isolated components of *S.nigrum* hold anti-proliferating activity. Cell responds differently at high and low concentration of crude extracts when observed in-vitro. In high concentration of crude extract is applied on liver cancer cell, it activates apoptosis whereas at low concentration it activates autophagy in contrast to apoptosis (Ramya *et al.*, 2011).

1.7.2 Antiepileptic Activity

S. nigrum and many other traditional medicines have shown anticonvulsant and antiepileptic activity in animal model and may be a home of drugs that are antiepileptic (Ramya *et al.*, 2011).

1.7.3 Antidiabetic Activity

The blood sugar of abino rat after regular oral dose of crude ethanolic extract of S.nigrum shows major decrease in blood sugar. Thus it can be determined that S.nigrum possesses antidiabetic activity (Rajani et al., 2012).

1.7.4 Antimicrobial Activity

Methanolic and aqueous extract of leaves were treated against different bacteria. On the basis of outcomes it is determined that methanol extract produce high level of growth inhibition as compared to aqueous extract. Methanolic extract of leaves were also tested against different fungi and result shows that moderate and significant activity were observed against different fungal strains (Rajani *et al.*, 2012).

1.7.5 Antioxidant Activity

Tissue biochemical antioxidant profiling of methanolic extract of *S.nigrum* fruits exhibited significant antioxidant potential. This antioxidant activity happened in dose-independent fashion (Rajani *et al.*, 2012).

1.7.6 Anti-inflammatory Activity

The methanolic and ethanolic extracts of berries of *S.nigrum* were considered for anti-inflammatory action on animal model that has shown significant anti-inflammatory activity (Rajani *et al.*, 2012).

1.7.7 Anti-diarrhoeal Activity

The ethanolic extract of dehydrated berries of *S.nigrum* considered anti-diarrhoeal activity. The extract exhibited a prominent anti-diarrhoeal activity compared to castor oil that induces diarrhoea in mice (Rajani *et al.*, 2012).

1.8 Propagation Through Tissue Culture Technique

Medicinal plants are essential source of medicinal and a lot of other pharmaceutical products. Conventionally used breeding methods are the major source of multiplication which requires a lot of time period for development (Nalawade and Tsay, 2004).

In recent time because of increase interest in natural medicine obtained from different extracts of plant or plant parts pharmaceutical companies depends largely on medicinal plant; results in the threat of extinction of valuable medicinal plant. Modern plant tissue culture is a substitute way used for commercial propagation of many species, including a lot of medicinal plants. Biotechnology relating modern tissue culture, cell biology and molecular biology that gives the chance to become well adapted to changing demands (G.R *et al.*, 2000).

The method of cell and tissue culture *in vitro* is a substitute method of vegetative propagation commonly known as micro propagation. In the culture, the micro propagation of medicinal plants has been attained by a rapid proliferation of shoot tips and axillary buds. Due to micro propagation a large number of progeny plants are obtained that have significant impact on plant breeding, horticulture and medicine. It is a reliable method from which huge amount of genetically homogenous and healthy plant material can be obtained that can be

used further for planting. As this technique is an alternative method of propagation, so there is an enlarged propagation rate of plants, accessibility of plants all over the year and plant protection against pests and pathogen under control condition will be obtained (Padmapriya *et al.*, 2011).

1.9 Factors Affecting *in vitro* Regeneration

A number of factors have an effect on plant genetic engineering involving successful tissue culture system for plant regeneration. These factors encompass the type and structure of explants, the donor plant's genotype, the combination and concentration of all media and growth hormones. First and the most important factor among these factors is genotype (Raghavendra *et al.*, 2010).

As most of the plants are naturally enriched with polyphenolic compounds that are identified as inhibitory agents. When explants were cultured, the media turned into brown due to the oxidation of polyphenolic exuded from explants. Various therapies are present to overcome this problem concerning to polyphenols in the *in vitro* culture. One of the remedy is retaining the explants in fresh medium after every two weeks (G.R *et al.*, 2000).

1.10 Objective of Study

The present study is focused on a vital medicinal plant *Solanum nigrum* L. and is aimed on the *in vitro* propagation of curative plant by employing tissue culture technique for comparison of the medicinal activities of the callus and plant extract. In this regard the concentration and combination of different media and plant growth hormones which have been thought to play an significant role in callus induction and regeneration frequency of shooting was investigated, callus and plant extract was made to check medicinal activities and also phytochemical analysis of plant was performed.

Chapter 2

Review of Literature

2. Review of Literature

For thousands of years medicinal agents have been provided by the nature in the plants from which many of the modern drugs has been isolated now-a-days (K.F. Chah *et al.*, 2006). As the consumption of medicine from natural sources has been increasing due to which loss of genetic diversity and destruction of habitat has been occurred. The biotechnological techniques such as tissue culture play a vital role in this prospect to conserve the medicinal plants (Peter *et* al., 2005). *In vitro* multiplication brings the production of excellent quality of plant based medicine as compared to conventional breeding methods which take a long period for propagation of medicinal plants (Nalawade and Tsay, 2004).

In vitro multiplication involves cell and tissue culturing which gives the rapid mass of propagation of medicinal herbs to meet up commercial need and also for protecting of genetic erosion (Zaiats and Mitrofanova, 2013).

Multiplication of plant through *in vitro* culturing has been attained through a quick proliferation of shoot tips and axillary buds. Due to micro propagation a large number of progeny plants are obtained that have significant impact on plant breeding, horticulture and medicine. It is a reliable method from which huge amount of genetically homogenous and healthy plant material can be obtained that can be used further for planting. As this technique is an alternative method of propagation, so there is an increased propagation rate of plants, obtainability of plants during the year and plant protection against pests and pathogen under control condition will be obtained (Padmapriya *et al.*, 2011).

2.1 *In vitro* Multiplication of *Solanum nigrum* L.

Solanum nigrum L. is a vital therapeutic plant and is used due to its anti-inflammatory, antioxidant and immuno-modulating activity. In the study of *in vitro* clonal propagation of *Solanum nigrum* the fresh shoot segments were used as explant. After the surface sterilization of explants with 0.1% $HgCl_2$ for 3-4 minutes, these were then washed with autoclaved distilled water for 10-15 minutes followed to antioxidant solution (ascorbic

acid 0.1% + citric acid 0.05%) to remove all the contamination. These explants were poured in the MS media which contained 1-9 µM BAP with the combination of 0.5 µM and 1.5 µM Kin. The best shooting were observed in the media with 2 µM BAP, however the combination of 2 µM BAP and 1.5 µM Kin proved to be supportive in shooting. The formation of roots was observed on different concentration of 3-4 µM IBA. However the best rooting were observed when micropropagated shoots were rooted on the 1/2 power of MS media with 10 µM concentration of IBA (Rathore and Gupta, 2013).

Solanum nigrum is important medicinal plant and possess great medicinal importance and well known for its therapeutical properties. The commercial production of *Solanum nigrum* was achieved by growing it in *in vitro*. For *in vitro* culturing the leaves and shoots were taken as explant. The explant were surface sterilized with tap water for 25 minutes and then treated with 1% bavistin and 0.1 % mercuric chloride for 20 minutes each tracked by washing thoroughly with ddH_2O in every step to remove contamination. The explant were placed on the MS media which contain changed concentration of 2,4-D and kinef cytokinin but the best concentration on which callus formation occur at day 8-10 is 1 mg/L 2,4-D and 0.2 mg/L kinef cytokinin. The maximum shoots were witnessed in the media with 1 mg/L Kn and 0.4 mg/L 2, 4-D. The environmental conditions were optimized that is temperature were 25±2°C, humidity 60-70% and 16 hours of light and 8 hours of dark photoperiod, having light intensity 2500-3000 lux (Ashish *et al.*, 2014).

Solanum nigrum is a medicinal plant and use as a source of life saving drug by most of the world's population. In the study of *in vitro* multiplication of important pharmaceutical plant *Solanum nigrum* L. was reported to describe the standardized protocol for *in vitro* regeneration of plant. For this study the leaf as explant were used and after surface sterilization by 0.1% Tween 20 followed by ddH_2O, 70% alcohol for 2 minutes which is again followed by ddH_2O for thoroughly washing. The explant were disinfected with 0.1% mercuric chloride for 1 minute tracked by 3 time washing with ddH_2O were poured in MS media having 3-7 mg/L BAP and 0.5 mg/L IAA. The cultures were given optimized condition for 16 hours photoperiod of having 30 $\mu mol.m^{-2}s^{-1}$ of cool, white luminous light and temperature is 25±2°C with relative humidity of 5-10%. After 15 days well developed callus was obtained in the media with 4 mg/L BAP and 0.5 mg/L IAA. The well-developed shoots were observed at 6 mg/L BAP and 0.5 mg/L IAA. The root development was

witnessed in the media having 0.5 mg/L of IBA. Roots formation was inhibited with increasing concentration of IBA (Narayan *et al.*, 2010).

For direct shoot regeneration shoot tips and nodal parts were used that are surface sterilized with the 0.1% bavistin for 1 minute and then washed with tap water followed by 0.1% $HgCl_2$ for 3-8 minutes and finally rinsed with ddH_2O. The explants were poured on MS media having changed concentration of BAP such as 1-5 mg/L and KIN 1-5 mg/L individually. Under control environment condition that is 16 hours of photoperiod of 45-50 $\mu mol.m^{-2}s^{-1}$ irradiance from the cool white luminous tube light at 25±2°C temperature with 55-60% humidity. After 4 weeks of interval 2-3 leaves were emerged without multiple shooting. The best shooting results were observed in the media with 3 mg/L of both KIN and BAP individually. The rooting was observed at concentration of 0.3-1.0 mg/L NAA. However the best results were achieved at the media with 1.0 mg/L NAA. The plants grown *in vitro* were transferred into the plastic bags that are filled with equal ratio of sand and soil which developed healthy leaves after 2-3 weeks of transplantation. The plantlets were transferred to the field were they grew normally (M.S *et al.*, 2012).

2.2 *In vitro* Micropropagation of Different Species of Family Solanaceae

Several studies have been conducted on different species of family Solanaceae. *Solanum villosum* Miller is an annual and biennial herb. Due to its vital importance as ethnomedical, vegetable and fruit plant, it becomes endangered in Pakistan. For the large production of plant to use it in medicine; *in vitro* propagation has been established. The stem node, leaf and shoot tip were used as explant. After sterilization the explants were moved on MS media. For direct shoot regeneration, the MS media contain different concentration of three different hormonal combination i.e MS+BAP, MS+BAP+NAA and MS+BAP+Kin. At the concentration of 1.9 mg/L BAP and 0.1 mg/L NAA shows best response in direct shoot regeneration. For rooting the 1/2 strength MS media is supplemented with changed concentration i.e 0.05, 1 and 1.5 mg/L IBA and NAA. Successful grown plantlets were shifted to pots and hardened (Anum *et al.*, 2015).

Solanum tuberosum L. is an economic crop and most of its production is in Asia and then in Europe, South America, North America and Central America. To produce thee virus-free plants, *in vitro* procedures were developed. Tubers from two potato cultivars of potato i-e Agrija and Andrea were treated *in vivo* with GA_3 shows 100% sprout formation. These sprouts were taken as explant and after surface sterilization cultured in MS media supplemented with 4 mg/L KIN and 2 mg/L BAP separately. In one month, bud grown into plantlets having 4-5 nodal parts. For shoot and root formation, these nodal segments were taken and cultured in MS media that contain 4mg/L KIN + 1mg/L IAA and 2 mg/L BAP + 1 mg/L NAA. The environmental conditions were maintained at 25±1°C temperature, humidity 50%, 16 hours of light and 8 hours of dark photoperiod having illumination of 50 $\mu mol.m^{-2}s^{-1}$. Between the nodal part and sprout, the nodal cutting showed better microtuber formation. The cultivar Agrija showed highest potential for *in vitro* propagation with 2.14 tubers per shoot and 13.33% microtubes formation. (Koleva *et al.,* 2012).

Lycopersicon esculentum (tomato) is highly valuable and nutritious fruit food belongs to the family Solanaceae. It is grown as vegetable crop almost all over the world there are many diseases of tomato due to which productivity is affected. For *in vitro* growth of callus induction and regeneration of tomato the protocol was developed. The hypocotyl and leaf disc were used as explant, obtained from the surface sanitised germinated seeds. The explant were poured on the MS media supplemented with 2 mg/L IAA, 2 mg/L NAA, 5 mg/L BAP and 4 mg/L Kin for hypocotyl and for leaf disc 2 mg/L IAA, 2mg/L NAA, 2mg/L BAP and 4 mg/L Kin. Maximum callogenesis was obtained from the leaf which is 81.3% however hypocotyl shows 65.2% callogenesis. For the highest value of shooting 69.2% is obtained on MS media that contain 1 mg/L Zeatin and 1 mg/L IAA while rooting was done on 1/2 power MS media having 0.1 mg/L IBA and 0.0025 mg/L BAP (Zubeda *et al.,* 2010).

Solanum melongena L. (eggplant) is popular and nutritional crop. It is grown throughout the world and the major loss of this crop occurs due to biotic and abiotic stress due to which maximum loss of crop occurs. Tissue culture approach was established to make the pest's resistance, better size, shape and weight of crop. For this, seed were surface purified and poured on the MS media were seed get germinated and seedling developed that were further used for shoot regeneration. The stem and leaves were used for shoot regeneration on the MS media contained different concentration of different hormonal

combination of 2, 4-D, IAA, BAP, NAA and Kinetin in altered ratio. The direct shoot regeneration was observed was observed at day 26 on MS media having 1.5 mg/L Kinetin and 0.5 mg/L IAA and 25% of coconut milk. After 40 days of inoculation on MS media having 0.5 mg/L IAA, roots were developed. However after 10-13 days roots were developed on basic MS media which shows that it was more favourable for rooting (Khizar *et al.*, 2014).

Physalis minima are an annual medicinal herb. Nodal segment from the developed plant were used as an explant source after sterilization steps. These explants were positioned on the MS media supplemented with changed concentration of BAP (1-5 mg/L) in combination with 0.25 mg/L IAA. Maximum numbers of shoots were observed at 2 mg/L BAP and 0.25 mg/L IAA. For blossoming, the well-developed shoots were transferred on the MS media supplemented with different concentration of BAP and KIN (1-5 mg/L) in combination of 0.25 mg/L 2, 4-D and NAA. The best results were on 4 mg/L BAP and 0.25 mg/L 2, 4-D; 1mg/L KIN and 0.25 mg/L 2, 4-D. For rooting different concentration of IBA and NAA (1-5 mg/L) were used and it is observed that IBA gives better results than NAA. The best results were on 2 mg/L BAP and 5 mg/L NAA (Sheeba *et al.*, 2015).

2.3 Medicinal Activity of Family Solanaceae

To cure the incurable diseases having no toxic effect, nature provide us drugs in the form of plants, herbs and algae. It is believed that medicinal plants are important source of therapeutical effects with new source of chemical substances. The research into medicinal plants therefore is viewed as successful and reasonable approach in exploration of new anti-inflammatory and analgesic drugs (Ravi *et al.*, 2009).

2.3.1 Phytochemical Analysis

Medicinal plants contain different phytochemicals. Various studies on the *Solanum melongena* is been done. The methanol and aqueous extract from the crown and fruit revelled that *S.melongena* contains alkaloids, saponins, steroids, tannins, flavonoids, proteins and carbohydrates. Researchers revelled that crown contain number of chemical constituents which is important for pharmacological activities (Anushree *et al.*, 2009).

Solanum lycopersicum L. is the most important vegetable. It contain various phytochemicals such as lycopene, -carotene, vitamin C, quercetine glyycoside, naringenin chalcone and chlorogenic acid. The methanol, ethanol ,acetone, chloroform and ether extracts of *S.lycopersicum* were used for phytochemical analysis which indicates the presence of reducing sugar, pentoses, starch, glycogen, aromatic amino acid, tyrosine, typtophan, arginine, sterol, carotenoids, poly phenol, disaccharides, monosaccharide, ketohexose, dipeptide and flavonoids. The researchers observed that different extracts contain both antibacterial and antifungal activity because of the presence of major phytoconstituents which is phenolic and sterol compounds (Murali Krishna *et al.*, 2013).

Solanum nigrum L. is a medicinal plant and is well known for its therapeutic properties. The methanol, aqueous and hexane extracts were used and it is observed that methanol extract contain flavonoids, triterpenoids, proteins and alkaloids. However hexane extract contain tannins, triterpenoids and protein while aqueous extract contains tannins, saponins, triterpenoids and proteins. It is concluded that the best solvent is methanol which can be employed for phytochemical extraction (Rajrana, 2015).

2.3.2 Antibacterial Activity

Plant extract have numerous health related effects like antibacterial, anti-mutagenic, anti-carcenogenic and anti-thrombotic. The extracts of plant have anti-microbial compound which is effective for the bacterial control. In current study methanol, chloroform, acetone and petroleum ether of seeds were verified against four human pathogenic bacteria such as Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus and Proteus vulgaris using ager well diffusion method. Due to the presence of reducing sugar, steroid, terpenoid and flavonoid in kernel extract; the concentrations of 75, 150 and 250 µg/ml the methanolic and acetone extract of seed exhibited maximum inhibition against S.aureus, significant inhibition was observed at higher concentration of petroleum ether extract and not any inhibition was shown in chloroform extract of *S.melongena* seed extract. However, in the case of E.coli methanol extract showed greatest inhibitory effect at all concentrations while at higher concentrations of acetone and petroleum ether extract only significant activity was observed (Amutha, 2014).

Solanum lycopersicum is an important plant due to the presence of large amount of health related components. The carotene lycopene is one of its powerful components which are antioxidant. In this we investigated the antibacterial activity of aqueous extract of *S.lycopersicum* on gram negative pathogenic bacteria by well diffusion method. It was observed that aqueous extract showed maximum zone of inhibition when mixed with honey than distal water due to the presence of "inhibin" in honey which acts as antibacterial factor (Rasha *et al.*, 2014).

Medicinal plants were used to cure various diseases caused by microbial pathogen. *Solanum nigrum* is one of the important plants and has potent antibacterial activity of leaf, root and seed extracts. In this study antibacterial activity was aimed of other plant parts like stem, berries and whole plant extract against bacterial pathogen. For this methanolic and ethanolic extracts were prepared. Varying degree of inhibitory effects was observed which is directly proportional to concentration of extract. It is also observed that entire plant extract showed maximum antibacterial action as compared with stem and berries in each of the methanolic and ethanolic extracts (Parameswari *et al.*, 2012)

Chapter 3

Materials and Methods

3. Material and Methods

The research work was conducted in Applied Biotechnology and Genetic Engineering Laboratory (ABGE), International Islamic University, Islamabad.

3.1 Plant Material

The *Solanum nigrum* used in research study were obtained from the local area of Taxila, Punjab. (Figure 3.1)

Figure 3.1: (a) A mature plant of *Solanum nigrum* L as a source material from local area, Taxila, Punjab

(b)Close up view of flower of *Solanum nigrum* L

(c) Close up view of leaf of *Solanum nigrum* L

(d) Close up view of fruit of *Solanum nigrum* L

3.2 Apparatus

3.2.1 Machinery

Laminar air flow, Vertical Autoclave Fully Automatic (Classic 1050), pH meter (Martini Instruments), Electronic balance (Shimadzu), Microwave oven (Orient), Drying oven (DHG-9053A).

3.2.2 Glassware

Petri plates and beakers (Pyrex, Germany), Culture tubes (Borosil 18x150 27ml) and conical flasks (100ml) were used for callus initiation and shoot redevelopment. Culture tubes and conical flasks were plugged using non-absorbent cotton and covered it in aluminium foil. (Figure 3.2)

3.2.3 Tools

Falcon tubes, Micropipettes (Sartorius, Germany), Surgical blades, Parafilm, Filter papers, Forceps, Mortar and pestle. (Figure 3.2)

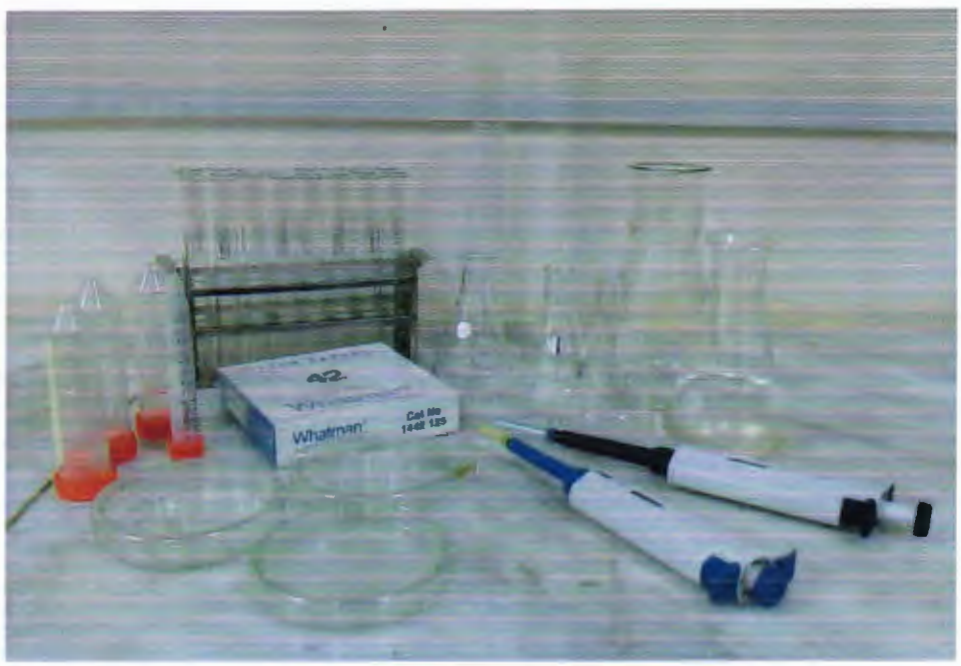


Figure 3.2: Glassware and Tools used in the experiment

3.3 Methodology

3.3.1 Stock Solution And Media Preparation

3.3.1.1 Preparation of Plant Growth Regulators Stock

The auxin used in this study was IAA and 2, 4-D while the cytokinin used was BAP and Kn. Stock solutions of all the growth regulators were prepared at the concentration of 1 mg/ml using 1N concentration of NaOH, followed up by making up the volume with distilled water, appropriately as required. All plant growth regulators were stored at 4°C and used employing sterile micropipette tips as per requirement prior to autoclaving.

Table 3.1: Preparation and Storage of Stock Solutions of Plant Growth Hormones

Growth Regulators	Auxins		Cytokinins	
	IAA (1mg/ml)	2,4-D (1mg/ml)	BAP (1mg/ml)	Kn (1mg/ml)
Mol. Weight	175.20	221.0	225.3	215.2
Solvent	1NaOH	1NaOH	1NaOH	1NaOH
Diluvent	dH_2O	dH_2O	dH_2O	dH_2O
Storage Temperature	4°C	4°C	4°C	4°C
Storage Time	1 week	2 months	2 months	2 months

3.3.1.2 Media Preparation

Synthetic MS (Murashige and Skoog) medium was employed in the study as a solid at the concentration of 0.443% (w/v). Sucrose was used as a only carbon source and was added as a solid at the concentration of 3% (w/v). Required concentrations of growth hormones were added in the medium. After that Gelrite was added as solidifying agent at the concentration of 0.25% (w/v). The pH of the medium was maintained by adding either NaOH or HCl, as required to get final value in the range of 5.7-5.8 before gelling with Gelrite. The medium was decontaminated by autoclaving at 121°C for 15 minutes at 15 psi.

For antibacterial activity nutrient agar media was used. For preparing media weighed nutrient agar up to 7 grams and add to 250 ml flask, then add distilled water up to the mark of 250 ml and covered with the lid and placed it in autoclave at 121°C for 15 minutes. Then media was taken out of the autoclave and allowed to cool at room temperature so that it could be handled easily.

3.3.1.3 Pouring and Inoculation of Plates

For pouring the media in petri plates, first clean the laminar air flow with 70% ethanol and treated with UV light for 10-15 minutes to remove any contamination. After that, poured the nutrient agar in the petri plates and allowed to cool. When the media was solidified inoculation was performed. The plates were streaked with different strains of bacteria i.e *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aerginosa* from different angles by using loop. After that the disc made up of autoclave filter paper were placed on media and extract was poured on these discs. Plates were left in incubator for overnight or two and after that checked for the activity.

3.3.2 Preparation of Explant and Surface Sterilization

Solanum nigrum, explant were collected from newly grown shoots. The collected shoots were cut into apical shoot segment and leaf segments, depending on the requirement of the experiments.

Leaf and apical shoot segments were washed thoroughly with tap water tracked by liquid detergent and shake continuously for 2, 3 minutes to remove contamination from the

explant surface and washed in dH_2O for 4, 5 times to remove the traces of the detergents to avoid the additional side effects and allowed the explant to dry.

After this step explant were surface sterilized under septic conditions in Laminar Air Flow that was carried out by using 70% ethanol (v/v) for 1-2 minutes, followed by 50% Clorox (v/v) for 2 minutes. This was followed by thorough washing with ddH_2O. The explants were then dried out on autoclaved filter paper. (Figure 3.3)

Figure 3.3: Incised apical shoot and leaf segment kept on autoclave filter paper to dry up

3.3.3 Callus Induction

In callus initiation media for initiation of callus different concentrations of 2, 4-D that are 0.5, 1.0 and 1.5 mg/L and BAP that are 2.0, 3.0, and 4.0 mg/L were used whereas 4.0 mg/L BAP in combination with 0.5 mg/L IAA was also tested. The surface dried explants were then placed in callus induction media. All cultures were maintained under 16 hours light and 8 hours dark photoperiod at 25±2°C temperature. The calli formed after a specific time of incubation from explants and then transferred to MS medium containing cytokinins for regeneration. The callus induction frequency was analysed using formula:

$$\text{Callus induction frequency (\%)} = \frac{\text{No. of calli obtained}}{\text{Total no. of explant cultured}} \times 100$$

3.3.4 Shoot Regeneration

For regeneration of calluses, different concentrations of BAP that are 5.0 and 6.0 mg/L in combination with 0.5 mg/L IAA were used whereas different concentrations of kinetin that are 0.6, 0.8, 1.0 mg/L in combination with 0.4 mg/L 2,4-D were also tested. For the direct regeneration procedure from apical stem segment different concentrations of kinetin that are 2.0, 3.0, and 4.0 mg/L were used. The culture tubes were maintained under 16 hours of light and 8 hours of dark photoperiod at 25±2°C temperature for specific period of time and then the data was recorded. The regeneration frequency was calculated by using formula:

$$\text{Regeneration frequency (\%)} = \frac{\text{No. of calli regenerated}}{\text{Total no. of calli cultured}} \times 100$$

3.3.5 Experimental Design

Completely randomized AOV was employed for each and every one of the experiment. All of the experiments were made up of 3 replications. Each replication is comprised of 08 explant per treatment for both callus induction and regeneration.

3.3.5.1 Statistical Analysis

The data recorded were analysed statistically by completely randomized AOV using statistix software version 10.0. Significance level was considered as P value <0.05.

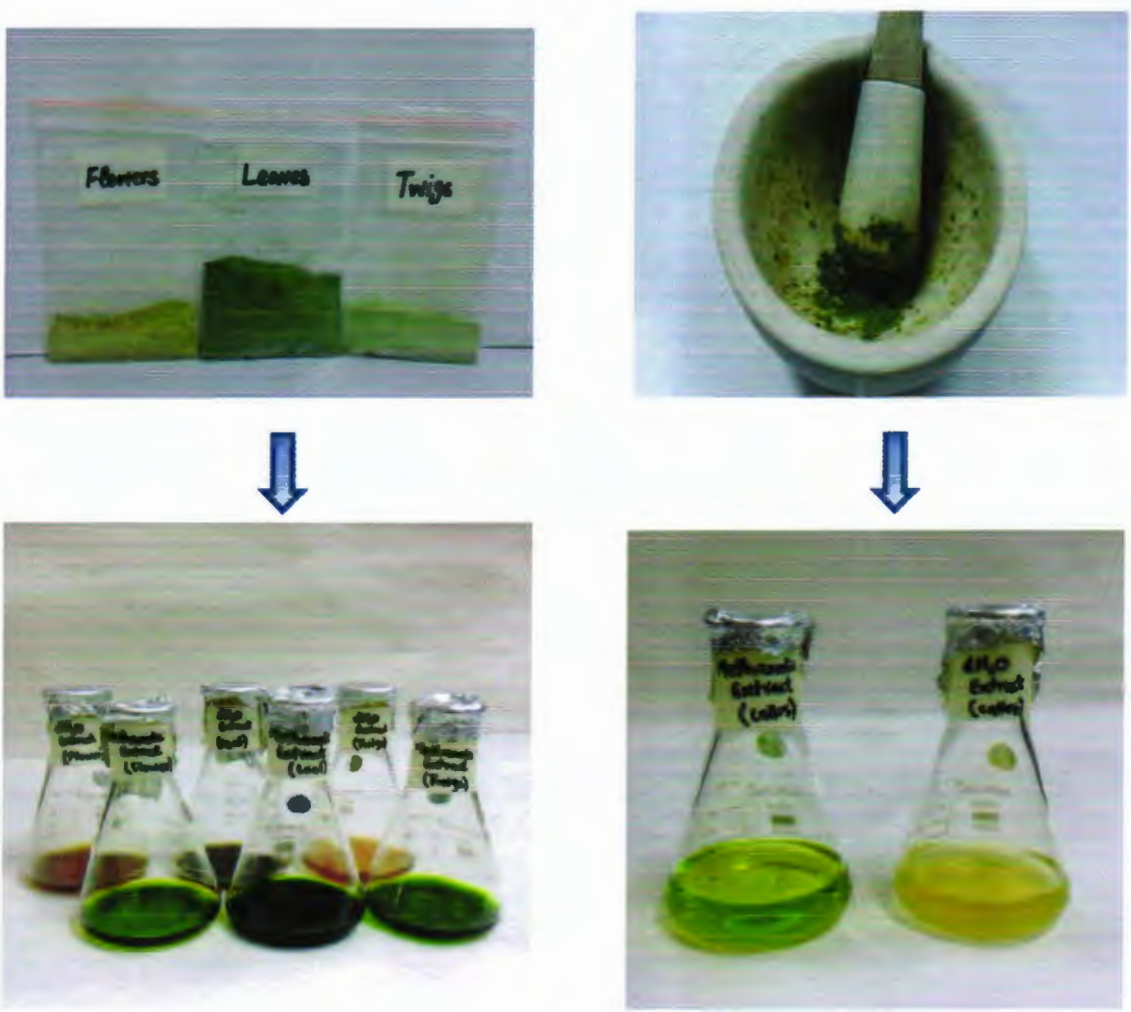
3.3.6 Phytochemical Analysis

3.3.6.1 Extract Preparation

The crude methanolic and aqueous extracts of leaf, twigs, flower and callus of *Solanum nigrum* were prepared by using method described by (Rajrana, 2015) with some modification. For the preparation of extract; fresh leaves, twigs and flower of the plant were collected from the mature *Solanum nigrum* L plant. Leaves, twigs and flowers were first washed with the tap water to remove any dust particles followed by liquid detergent to wipe out any contamination left and washed in dH_2O for 4,5 times to remove the traces of

detergents to avoid the additional side effects and allowed them for few days to dry up in open air. The dried leaves, twigs and flowers were then powdered using mortar and pestle. A total 05g of powder was obtained which was then dissolved in 3 conical flasks separately containing 50ml each of methanol and dH_2O and covered with aluminium foil for 48 hours. The extract were then filtered with the help of Whatman filter paper No.42 and used the filtrate for further phytochemical analysis (Figure 3.4)

For the preparation of callus extract, fresh calli were obtained from the preliminary experiments were grinded by using mortar and pestle. Total 01g of grinded calli was obtained which were dissolved in 50ml of methanol and dH_2O in conical flask and covered with aluminium foil for 48 hours. The callus extract were then filtered with the help of Whatman filter paper No.42 and used the filtrate for further phytochemical analysis (Figure 3.4)



(a) **(b)**

Figure 3.4: Crude Methanolic and Aqueous Extracts Prepared from:

(a) Mature Flowers, Leaves and Twigs of *Solanum nigrum* L.

(b) *In Vitro* mediated Callus of *Solanum nigrum* L.

3.3.6.2 Tests for Alkaloids

For the detection of alkaloid in crude methanolic and aqueous extract of flowers, leaves, twigs and callus of *S.nigrum*, 1 ml of each extract was added in a separate test tube and then adds few drops of 37% HCl. After that, add saturated picric acid. Formation of creamy white colour precipitate indicates the presence of alkaloids.

3.3.6.3 Test for Flavonoids

For the detection of flavonoid in crude methanolic and aqueous extract of flowers, leaves, twigs and callus of *S.nigrum*, 1 ml of each extract was added in a separate test tube and treated with few drops of NaOH, then with few drops of dilute HCl. Appearance of yellow colour which become colour less by the addition of acid indicates the presence of flavonoids.

3.3.6.4 Test for Tannins

For the detection of tannins in crude methanolic and aqueous extract of flowers, leaves, twigs and callus of *S.nigrum*, 1 ml of each extract was added in a separate test tube and treated with few drops of $FeCl_3$ solution. Appearance of blue/green colour indicates the presence of tannins.

3.3.6.5 Test for Saponins

For the detection of saponins in crude methanolic and aqueous extract of flowers, leaves, twigs and callus of *S.nigrum*, 1 ml of each extract was added in a separate test tube. After shaking, foam will appear that remains for 3-4 minutes. Appearance of stable form indicates the presence of saponins.

Chapter 4

Results

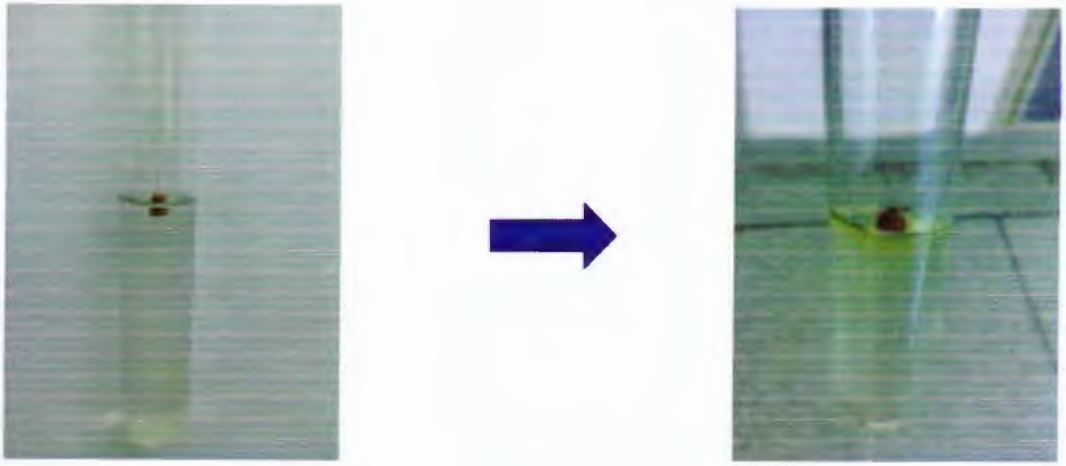
4. RESULTS

4.1 Callus Induction

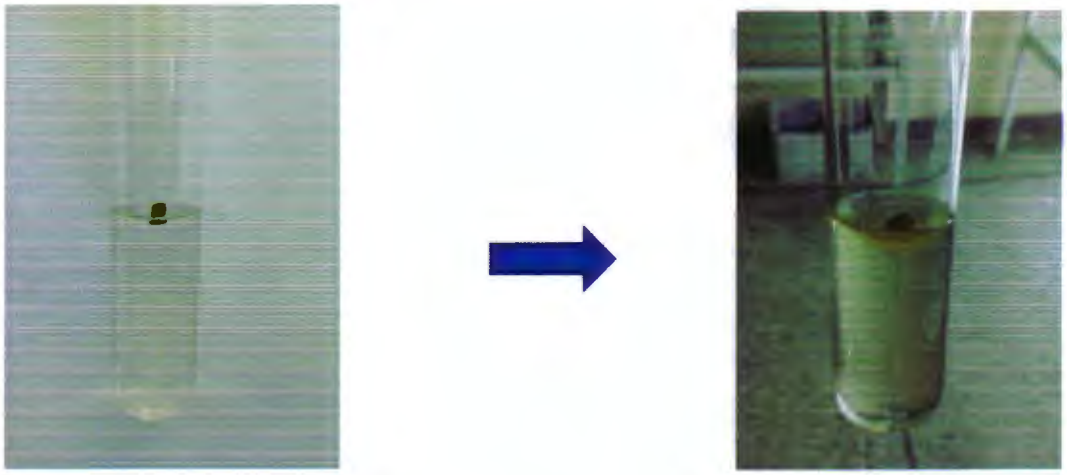
4.1.1 Effect of Different Concentrations of 2, 4-D

A total of 144 explants were cultured, 72 of each explant type, 24 on all three media combination (CI-I, CI-II, CI-III) were tested for callus induction potential. In all media combinations different concentration of 2, 4-D (0.5, 1.0 and 1.5 mg/L respectively) were tested.

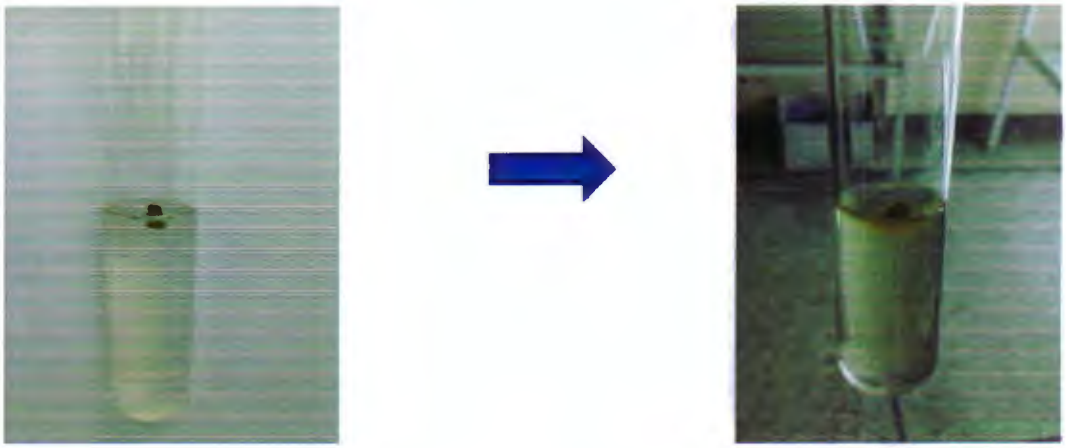
After inoculating each explant to callus induction media, explant showed constant browning from day 4th to 7th in nearly all of the callus induction media combination indicating that this media combination does not favour the callus induction (Figure 4.1)



(a)



(b)



(c)

Figure 4.1: Callus initiation of *Solanum nigrum* L. on:

(a) Callus induction media I (CI-I) supplemented with 0.5 mg/L 2, 4-D, and result in browning at day 7th in nearly all of the cultures.

(b) Callus induction media II (CI-II) supplemented with 1.0 mg/L 2, 4-D, and result in browning at day 5th in nearly all of the cultures.

(c) Callus induction media III (CI-III) supplemented with 1.5 mg/L 2, 4-D, and result in browning at day 4th in nearly all of the cultures.

4.1.2 Effect of Different Concentrations of BAP

A total of 144 explants were cultured, 72 of each explant type, 24 on all three media combination (CI-I, CI-II, CI-III) were tested for callus induction potential. In all media combinations different concentration of BAP (2.0, 3.0 and 4.0 mg/L respectively) were tested. Callus initiation was witnessed at day 19th after the cultures were inoculated on callus induction media. The plant show best results of callus induction on leaf explants at the concentration of 2.0 mg/L of BAP followed by 3.0 and 4.0 mg/L of BAP (Table 4.1) (Figure 4.2).

The different sizes of callus were produced. Higher callus induction of 33.333% was obtained for CI-I followed by 25% for CI-II and 16.667% for CI-III. Leaf explants showed greater capability to produce callus whereas not enough amount of callus was produced by hypocotyl explants. Both the explants were found to be significantly different ($P < 0.05$) from one another for callus induction frequency.

Table 4.1: Callus induction frequency at various concentrations of BAP

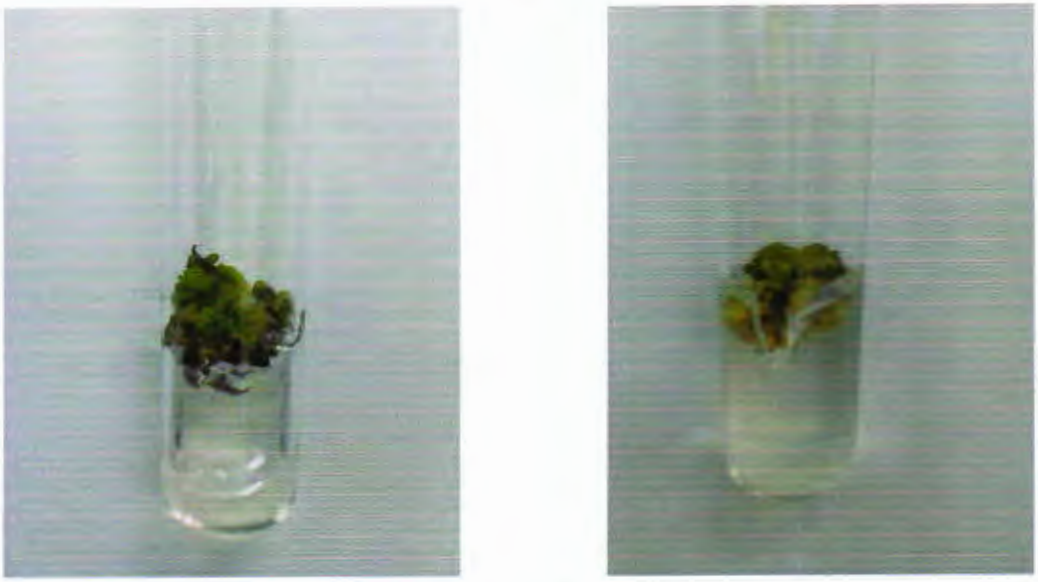
Explant	MS+BAP (mg/L)			Explant Mean
	CI-I (2.0 mg/L)	CI-II (3.0 mg/L)	CI-III (4.0 mg/L)	
Hypocotyls	4.167	4.167	8.333	5.56
Leaves	33.333	25	16.667	25
Media Mean	18.75	14.58	12.50	

Grand Mean = 1.2222

CV = 58.65

Standard Error of a Mean = 0.2390

The results were statistically significant at 5%



(a) (b)

(c)

Figure 4.2: Callus initiation of *Solanum nigrum* L. on:

(a) Callus induction media I (CI-I) supplemented with 2.0 mg/L BAP.

(b) Callus induction media II (CI-II) supplemented with 3.0 mg/L BAP.

(c) Callus induction media III (CI-III) supplemented with 4.0 mg/L BAP.

4.1.3 Effect of Media Combination on Callus Induction Frequency

Optimal callus initiation was found on MS medium supplemented with the combination of BAP and IAA, although explant types differ extensively in their abilities to start callus induction. Two types of media CI-I (MS media + BAP + IAA) and CI-II (MS media + BAP + 2, 4-D) were used for callus initiation frequency. In all media combination different concentration of BAP (2.0, 3.0 and 4.0 mg/L), IAA (0.5 mg/L) and 2, 4-D (0.5 mg/L) were tested.

Callus initiation was observed at day 13th after the cultures were inoculated on callus initiation media. The plant show best results of callus initiation on leaf explants. On CI-I type media the best callus results at the concentration of 4.0 mg/L + 0.5 mg/L of BAP and IAA respectively and on CI-II type media only the enlargement of the explant occurs, which indicates that this media combination is only aiding in increasing the size of an explant and do not favour the callus induction (Table 4.2) (Figure 4.3).

Table 4.2 illustrates that different sizes of callus were produced. Higher callus induction of 83.333% was obtained for CI-III followed by 50% for CI-II and 16.667% for CI-I. Leaf explants showed greater capability to produce callus whereas not enough amount of callus was produced by hypocotyl explants. Both the explants were found to be significantly different ($P < 0.05$) from one another for callus induction frequency.

Table 4.2: Callus induction frequency on different media combinations

Explant	MS+BAP+IAA (mg/L) CI-I			Explant Mean
	(2.0 + 0.5mg/L)	(3.0 + 0.5mg/L)	(4.0 + 0.5mg/L)	
Hypocotyls	4.167	4.167	4.167	4.17
Leaves	16.667	50	83.333	50
Media Mean	10.42	27.08	43.75	

Grand Mean = 2.1667

CV = 79.94

Standard Error of a Mean = 0.5774

The results were statistically significant at 5%

(a)

(b)

Figure 4.3: Effect of Media Combination on Callus Induction Frequency:

(a) Callus induction media supplemented with different concentration of BAP and IAA.

(b) Callus induction media supplemented with different concentration of BAP and 2, 4 –D.

4.2 Shoot Regeneration

4.2.1 Effect of 2, 4-D and Kn on Shoot Regeneration

Three different combinations of media (SI-I, SI-II, SI-III) were tested for regeneration potential. After shifting of calli to regeneration media, calli showed a constant increased in size and exhibited growth on all the regeneration media combination indicating that this media combination is aiding in shooting.

(a)

(b)

(c)

Figure 4.4: Shoot regeneration of *Solanum nigrum* L. on:

(a) Shoot regeneration media I (SI-I) supplemented with 0.6 mg/L Kn and 0.4 mg/L 2, 4-D.

(b) Shoot regeneration media II (SI-II) supplemented with 0.8 mg/L Kn and 0.4 mg/L 2, 4-D.

(c) Shoot regeneration III (SI-III) supplemented with 1.0 mg/L Kn and 0.4 mg/L 2, 4-D.

4.2.2 Effect of BAP and IAA on Shoot Regeneration

Two different combinations of media (SI-I, SI-II) were tested for regeneration potential. After shifting of calli to regeneration media, calli showed a constant increased in size and exhibited growth on all the regeneration media combination indicating that this media combination is aiding in shooting.

For shoot regeneration, SI-II (shoot induction media I) supplemented with 6.0 mg/L BAP + 0.5 mg/L IAA was found to be best showing the highest shoot induction frequency 79.167% which was followed by SI-I having 5.0 mg/L BAP + 0.5 mg/L 1AA with shoot induction frequency of 33.333% (Table 4.4) (Figure 4.5). Statistical analysis shown that a significant difference ($P < 0.05$) existed among all the media combinations for regeneration efficiency.

Table 4.4: Shoot regeneration frequency at different concentrations of BAP and IAA

MS+BAP+IAA (mg/L)	
SI-I	**SI-II**
(5.0 + 0.5mg/L)	**(6.0 + 0.5mg/L)**
33.333	79.167

Grand Mean = 4.5000

CV = 12.83

Standard Error of a Mean = 0.3333

The results were statistically significant at 5%

(a)

(b)

Figure 4.5: Shoot regeneration of *Solanum nigrum* L. on:

(a) Shoot regeneration media I (SI-I) supplemented with 5.0 mg/L BAP and 0.5 mg/L IAA.

(b) Shoot regeneration media II (SI-II) supplemented with 6.0 mg/L BAP and 0.5 mg/L IAA.

4.3 Direct Shoot Regeneration Form Shoot Tips

4.3.1 Effect of Kn on Direct Shoot Regeneration

For direct shoot regeneration, SI-II (shoot induction media II) added with 3 mg/L kinetin was found to be the best showing the highest shoot initiation frequency of 33.33% which was followed by SI-III having 4 mg/L kinetin with shoot induction frequency of 20.83% and SI-I having 2 mg/L of kinetin with shoot induction frequency of 12.50% (Table 4.5) (Figure 4.6). Statistical analysis shown that a significant difference ($P < 0.05$) existed among all the three media combinations for regeneration efficiency.

Table 4.5: Regeneration frequency of shoot tips at different concentrations of Kn

MS+Kn (mg/L)		
SI-I	**SI-II**	**SI-III**
(2.0 mg/L)	**(3.0 mg/L)**	**(4.0 mg/L)**
12.50	33.33	20.83

Grand Mean = 1.7778

CV = 26.52

Standard Error of a Mean = 0.2722

The results were statistically significant at 5%

(a)

Figure 4.6: Direct shoot regeneration of *Solanum nigrum* L. on:

(a) Direct shoot regeneration media supplemented with different concentration of Kn.

4.4 Phytochemical Screening

The aqueous and methanolic extracts of leaf, twigs, flowers and callus were employed to phytochemical test to find out the nature of phytoconstituents in the extracts of *Solanum nigrum*. Our results suggested the presence and absence of the compounds present in leaf, twigs, flowers and callus extracts of *Solanum nigrum* (Table 4.6, 4.7) (Figure 4.7)

Table 4.6: Phytochemical composition in methanolic extract

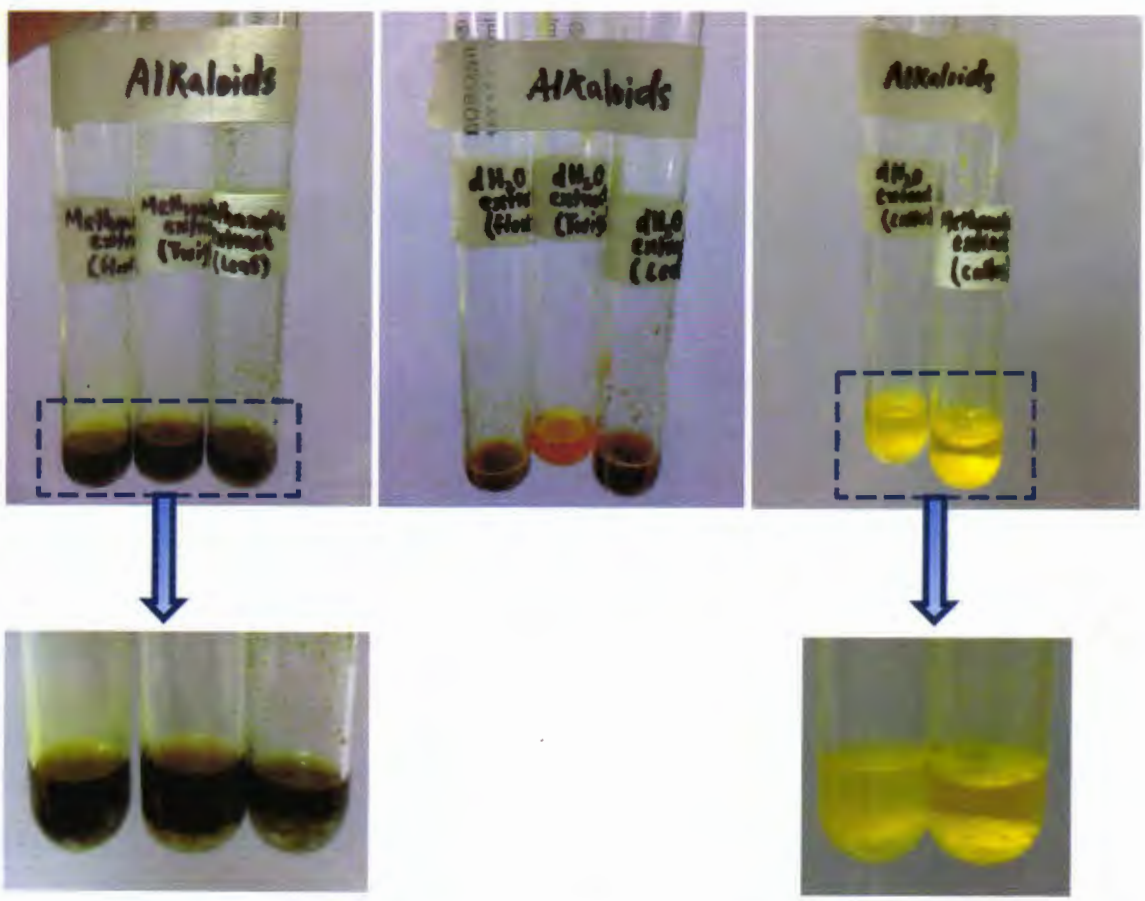
S. No	Phytochemical	Tests	Results			
			Leaf	Twig	Flower	Callus
01	Alkaloids	Picric acid	+	+	+	+
02	Flavonoids	Alkaline reagent	-	-	-	+
03	Tannins	Ferric chloride	+	+	+	-
04	Saponins	Froth formation	-	-	-	-

Key: + Present, - Absent

Table 4.7: Phytochemical composition in aqueous extract

S. No	Phytochemical	Tests	Results			
			Leaf	Twig	Flower	Callus
01	Alkaloids	Picric acid	-	-	-	+
02	Flavonoids	Alkaline reagent	-	-	-	-
03	Tannins	Ferric chloride	-	-	-	-
04	Saponins	Froth formation	+	+	+	+

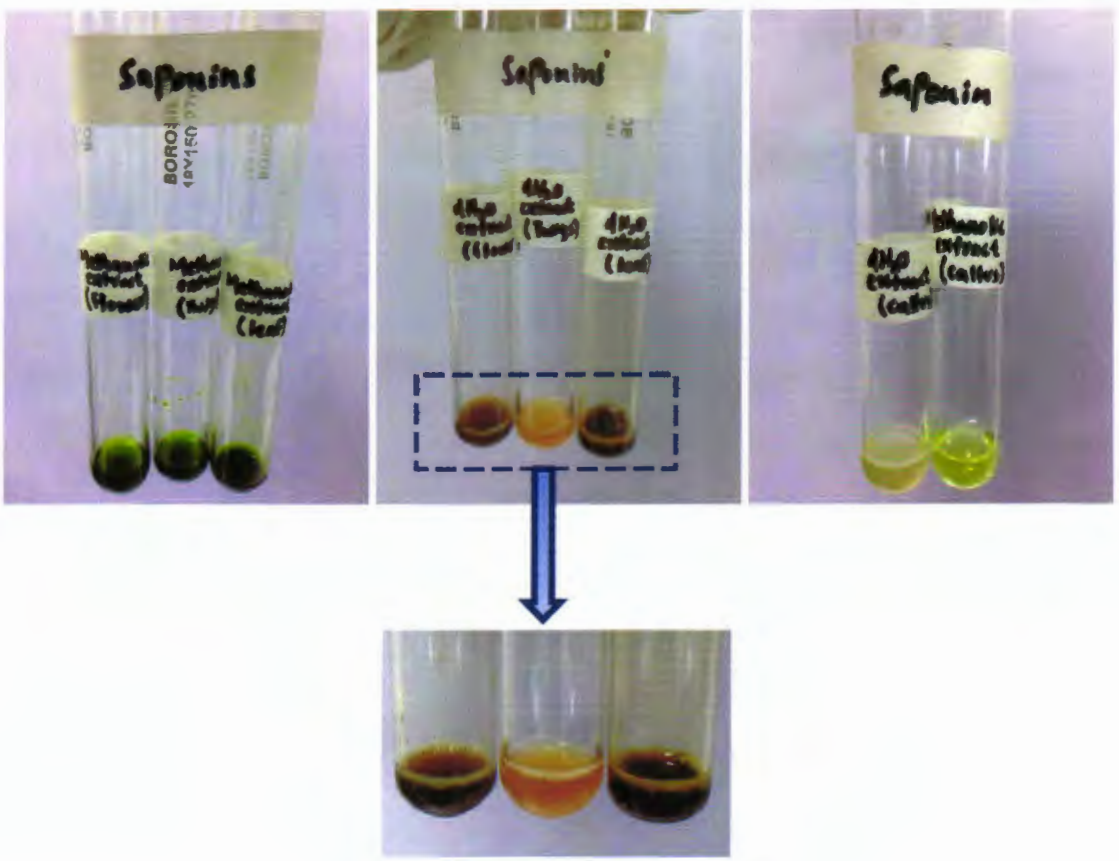
Key: + Present, - Absent



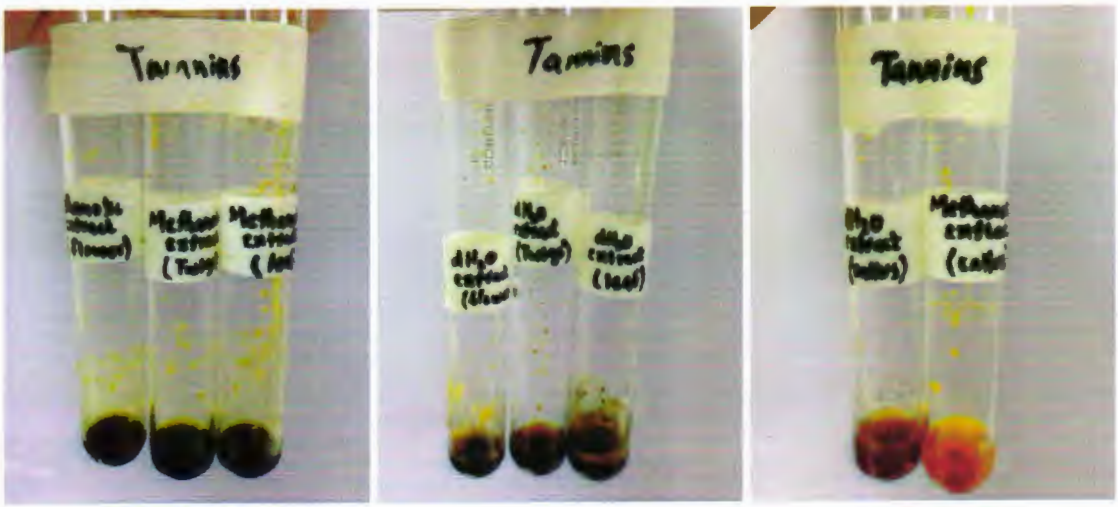
(a)


Flavonoids
Flavonoid
Flavonoid

(b)


Saponins
Saponins
Saponin

(c)



(d)

Figure 4.7: Phytochemical Screening of *Solanum nigrum* L. having methanolic and aqueous extracts of leaf, twigs, flowers and callus:

(a) Alkaloids test for methanolic and aqueous extracts of leaf, twigs, flowers and callus.

(b) Flavonoids test for methanolic and aqueous extracts of leaf, twigs, flowers and callus.

(c) Tannins test for methanolic and aqueous extracts of leaf, twigs, flowers and callus.

(d) Saponins test for methanolic and aqueous extracts of leaf, twigs, flowers and callus.

4.5 Antibacterial Activity

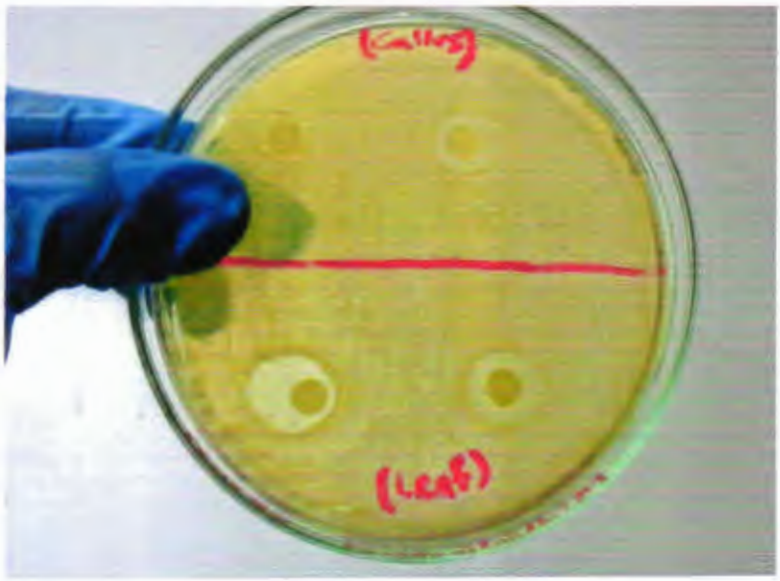
The methanolic extract of leaf and callus were employed to the test bacterial strains by disc diffusion method to find out the antibacterial activity in the extracts of *Solanum nigrum*. Our results suggested the zone of inhibition created due to the presence of compounds that is responsible for antibacterial activity of *Solanum nigrum*.

The antibacterial activity of the trial samples were determined by evaluating the diameter of zone of inhibition expressed in millimetre. The results of antibacterial activities of *Solanum nigrum* were shown (Table 4.8) (Figure 4.8)

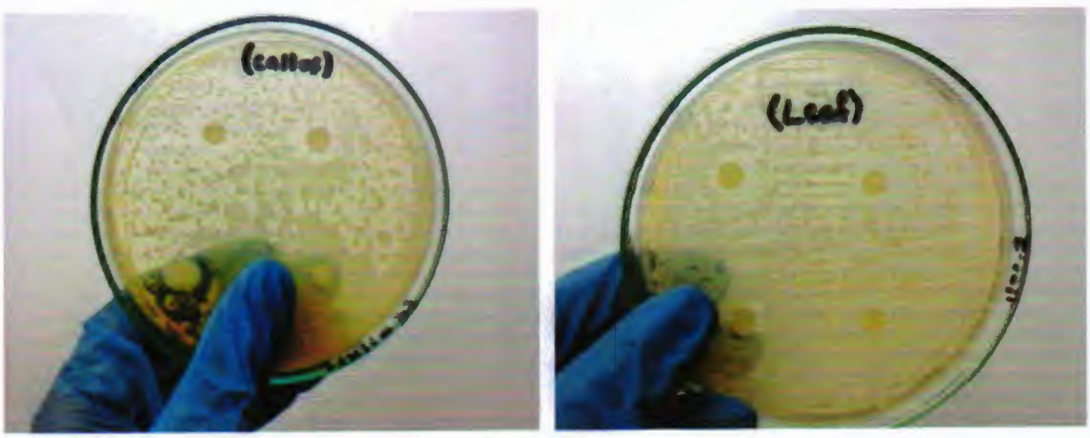
Table 4.8 illustrates that both methanolic extracts of callus and leaf showed fluctuating degree of inhibitory effect. The methanolic extract of callus showed 3mm of zone of inhibition against *Staphylococcus aureus*, 5mm against *Escherichia coli* and 4mm against *Pseudomonas aeruginosa*. However methanolic extract of leaf showed 5mm of zone of inhibition against *Staphylococcus aureus*, 7mm against *Escherichia coli* and 6mm against *Pseudomonas aeruginosa*.

Table 4.8: Antibacterial activity in methanolic extract of *Solanum nigrum*

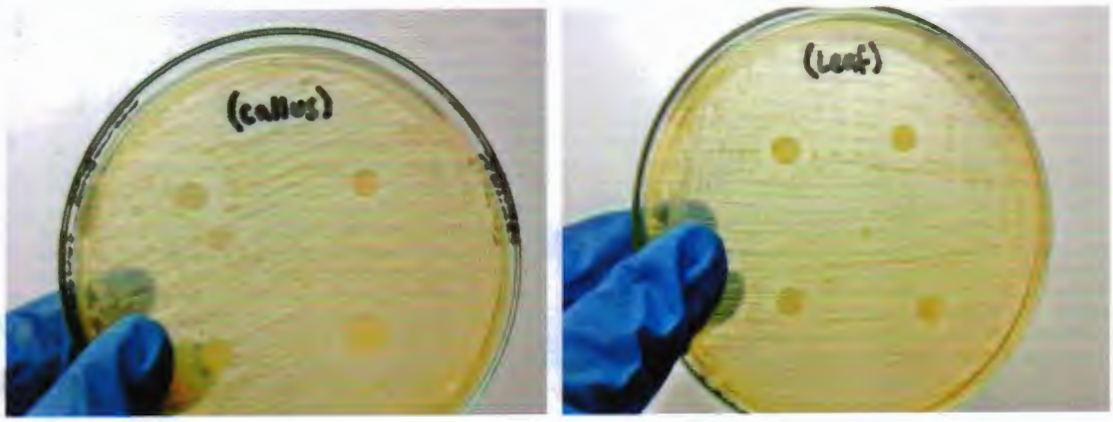
S. No	Bacterial Strains	Zone of Inhibition	
		Callus Extract	Leaf Extract
01	*Staphylococcus aureus*	3 mm	5 mm
02	*Escherichia coli*	5 mm	7 mm
03	*Pseudomonas aeruginosa*	4 mm	6 mm



(a)



(b)



(c)

Figure 4.8: Antibacterial activity of *Solanum nigrum* L. in methanolic extracts of leaf and callus:

(a) Zone of inhibition against *Staphylococcus aureus*.

(b) Zone of inhibition against *Escherichia coli*.

(c) Zone of inhibition against *Pseudomonas aeruginosa*.

Chapter 5

Discussion

5. Discussion

For thousands of years medicinal plants provide medicinal agents from which modern drugs has been isolated (Chah *et al.*, 2006). Medicinal plant comprise of constituents having curative effects, therefore have been used over a long time to treat different diseases (Arias *et al.*, 2004). In developing country 80% of the world inhabitants depend on plant derived drugs (Peter *et al.*, 2005).

Pakistan has diverse range of climate due to which several medicinal species were present (Syed *et al.*, 2010). *Solanum nigrum* is important medicinal plant having diverse therapeutic properties (Ramya *et al.*, 2011). *S.nigrum* has potent antibacterial activity of leaf, root and seed extracts (Parameswari *et al.*, 2012). It was observed that *S.nigrum* is important medicinal plant and is considered to be antibacterial, antioxidant, anti-proliferative, antiepileptic and antidiabetic activities (Rajani et al., 2012). Chemotherapeutic, antibacterial and antifungal agents are the rich source of medicinal plant (Iqbal *el al.*, 1998).

According to the literature survey different studies on *in-vitro* regeneration of *Solanum nigrum* are done throughout the world and also little work is reported on *in-vitro* regeneration of *Solanum nigrum* in Pakistan. In the present study *S.nigrum*, an important medicinal plant was evaluated for its callus induction and shooting potential and compares its medicinal activities with *in-vivo* grown plants. Phytochemical analysis was also performed to indicate the presence of important phytochemicals.

Tissue culturing is an alternative method of propagation, so that there is an increase propagation rate of plants and also defence of plants against pests and pathogens (Padmapariya *et al.*, 2011). *In vitro* culture initiation and plant regeneration was influenced by balanced concentration of phytohormone and media composition (Wen *et al.*, 1998).

In the current study explant were used for *in vitro* growth of callus and then shoot regeneration. Callus initiation and shoot regeneration was obtained on different regeneration media, examined for callus and shoot redevelopment of *S.nigrum*. The concentration of BAP were from 2-6 mg/L and IAA was 0.5 mg/L. Our results were nearly similar to that of

(Narayan *et al.*, 2010) who reported that increase the concentration of BAP at particular point with the combination of IAA at 0.5 mg/L, a well-built callus was observed and a well-developed shoot was also observed at 6 mg/L of BAP and 0.5 mg/L of IAA.

Direct shoot regeneration was observed with the addition of kinetin to the media. Three different media combinations i.e 2, 3 and 4 mg/L were tested for regeneration potential with kinetin. The best results were observed in the media with 3 mg/L of kinetin. Our results were similar to that of M.S *et al.*, 2012.

In the present study, leaf and hypocotyl explants of *S.nigrum* L were used for callus induction on different concentrations and combinations of BAP and IAA. In all media combinations altered concentrations of BAP and IAA i.e 2, 3 and 4 mg/L of BAP and 0.5 mg/L of IAA were used. Best callus results were obtained on leaf explant at concentration of 4 mg/L of BAP and 0.5 mg/L of IAA tracked by 3 mg/L of BAP and 0.5 mg/L of IAA and 2 mg/L of BAP and 0.5 mg/L of IAA. This callus was used for callus extract for phytochemical analysis. On the other hand the hypocotyl, leaves and flowers from the mature plant was used for plant extract for phytochemical analysis.

In the present study, phytochemical analysis of *S.nigrum* L showed the existence of alkaloids, flavonoids, tannins and saponins in methanolic extract of plant. Whereas only saponins is present in aqueous extract of plant which is similar to Rajrana, 2015. While in methanolic extract of callus, alkaloids, flavonoids, tannins and saponins were present. Whereas alkaloids and saponins was present in aqueous extract of callus.

In the present study, antibacterial activity on *S.nigrum* L was performed with methanolic extract of both callus and leaf of the plant against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Against *S.aureus* zone of inhibition in callus extract was 3mm and 5mm in leaf extract. Against *E.coli* zone of inhibition in callus extract was 5mm and 7mm in leaf extract. Against *P.aeruginosa* zone of inhibition in callus extract was 4mm and 6mm in leaf extract.

Conclusion

In conclusion, a quick and efficient method for *in vitro* culturing of endangered medicinal plant, *Solanum nigrum* L using leaf and hypocotyl as explants has been established. The explants were cultured on MS medium supplemented with various concentrations and combinations of plant growth regulators. The plant showed best callus induction in media having the combination of BAP and IAA at the concentration of 4 mg/L and 0.5 mg/L respectively.

After callus initiation, shoot regeneration is the next step. The callus was transferred on the MS medium added with various concentrations and combinations of plant growth regulators. The plant showed best shooting in a media having the combination of BAP and IAA at the concentration of 6 mg/L and 0.5 mg/L respectively.

High frequency direct shoot proliferation was also induced in the hypocotyl explants cultured on MS medium added with different concentrations of Kinetin. Amongst the various concentrations tested, 3 mg/L kinetin showed to be the most effective.

Phytochemical analysis revealed the existence of alkaloids, flavonoids tannins and saponins. However methanolic extracts of *S.nigrum* L possess a large spectrum against bacterial strains responsible for bacterial infection. Our research can successfully be utilized for other important species of family Solanaceae for further studies.

Future Recommendations

A standardized protocol for *in vitro* tissue culturing of *S.nigrum* L is successfully established which can further be used for their enormous production, irrespective of season and weather. The plants can be supplied to pharmacological industries and can be used for gene transformation as well. Biological and medicinal activities of plant and callus extract of *S.nigrum* can also be studied. However the present study of *in vitro* antibacterial evaluation of *S.nigrum* forms a primary stage for further pharmacological analysis for the improvement of new potential antimicrobial compounds.

Chapter 6

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6. References

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Appendix

Statistix 10.0 (30-day Trial) 03-Jun-16, 2:18:41
PM

Completely Randomized AOV for Callus

Source	DF	SS	MS	F	P
Plant	1	10.8889	10.8889	21.19	0.0003
Error	16	8.2222	0.5139		
Total	17	19.1111			

Grand Mean 1.2222 CV 58.65

Plant	Mean
1	0.4444
2	2.0000

Observations per Mean 9
Standard Error of a Mean 0.2390
Std Error (Diff of 2 Means) 0.3379

Statistix 10.0 (30-day Trial) 03-Jun-16, 2:20:04
PM

Completely Randomized AOV for Callus

Source	DF	SS	MS	F	P
Plant	1	60.500	60.5000	20.17	0.0004
Error	16	48.000	3.0000		
Total	17	108.500			

Grand Mean 2.1667 CV 79.94

Plant	Mean
1	0.3333
2	4.0000

Observations per Mean 9
Standard Error of a Mean 0.5774
Std Error (Diff of 2 Means) 0.8165

Statistix 10.0 (30-day Trial) 03-Jun-16, 2:21:25
PM

Completely Randomized AOV for Shoot

Source	DF	SS	MS	F	P
Concentra	2	14.0000	7.00000	21.00	0.0020
Error	6	2.0000	0.33333		
Total	8	16.0000			

Grand Mean 4.0000 CV 14.43

Concentra	Mean
0.6+0.4	5.6667
0.8+0.4	3.6667
1+0.4	2.6667

Observations per Mean 3
Standard Error of a Mean 0.3333
Std Error (Diff of 2 Means) 0.4714

Statistix 10.0 (30-day Trial) 03-Jun-16, 2:22:43
PM

Completely Randomized AOV for Shoot

Source	DF	SS	MS	F	P
Concentra	1	20.1667	20.1667	60.50	0.0015
Error	4	1.3333	0.3333		
Total	5	21.5000			

Grand Mean 4.5000 CV 12.83

Concentra	Mean
5+0.5	2.6667
6+0.5	6.3333

Observations per Mean 3
Standard Error of a Mean 0.3333
Std Error (Diff of 2 Means) 0.4714

Statistix 10.0 (30-day Trial) 03-Jun-16, 2:24:28
PM

Completely Randomized AOV for Shoot

Source	DF	SS	MS	F	P
Concentra	2	4.22222	2.11111	9.50	0.0138
Error	6	1.33333	0.22222		
Total	8	5.55556			

Grand Mean 1.7778 CV 26.52

Concentra	Mean
2	1.0000
3	2.6667
4	1.6667

Observations per Mean 3
Standard Error of a Mean 0.2722
Std Error (Diff of 2 Means) 0.3849