In-vitro multiplication Antimicrobial Activity,and Phytochemical Analysis of Medicinal Plant Malva*sylvestris* L.

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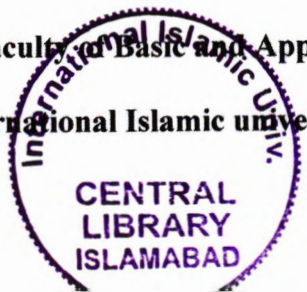

Medicinal plants

Phyto chemical analysis

Genus malvaceae.

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بِسْمِ اللهِ الرَّحْمٰنِ الرَّحِيْمِ

BISMILLĀHIR-RAḤMĀNIR-RAḤĪM

In the Name of Allah, the Most Gracious, the Most Merciful.

Department of Bioinformatics and Biotechnology

International Islamic University Islamabad

Dated: 26-10-16

FINAL APPROVAL

It is certify that we have read the thesis entitled "Basic Principle and protocol for tissue culture studies of Malva sylvestris L morphological characters, phytochemical analysis and antimicrobial activity of Malva sylvestris, submitted by Ms.AdeebaRani 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.

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A thesis submitted to department of Bioinformatics and Biotechnology ,International Islamic university, Islamabad. As a fulfillment of requirement for the award of the degree MS Bio technology.

DEDICATION

I dedicate this efforts to my beloved parents my mother Mrs.Rehana Gul and my father Muhammad Ashraf (late), who has inspired me to work hard for my better future and taught me the best kind of behavior and help to gain knowledge and after my father my mother always sustained with me through each and every moment of my life.

May Allah bless both of them always and forever. (Ameen)

DECLARATION

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

Date ----------

Adeeba Rani

Adeeeba Rani

All work should be done in Applied Biotechnology and Genetic Engineering Laboratory (ABGE).International Islamic University, Islamabad.

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First and foremost, all praises and thanks to Allah (SWT), the most merciful, the most beneficent, who gave opportunity us to study in MS biotechnology and blessed us with courage to complete this desired task in the required time frame.

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I would extend my gratitude to my brother and sisters for their encouragement and motivation .I am also really thankful to my mother to always motivated me for higher studies.

Last but not least ,I recognized that this research would not have been possible without financial academic and technical support of Applied Biotechnology and Genetic Engineering lab (ABGE) and International Islamic University Islamabad.

LIST OF ABBREVATION

2,4 D	2,4 Dichlorophenoxyacetic acid
ABGE	Applied Biotechnology and Genetic Engineering Lab
BAP	6- Benzylaminopurine
CI	Callus Induction
ddH_2O	Double distilled water
HCL	Hydrochloric acid
IAA	Indole-3-acetic acid
IBA	Indole -3-butyric acid
Kn	Kinetin
Mins	Minutes
MS	Murashige and skhoog
NAA	Naphthalene acetic acid
NaOH	Sodium hydroxide
NARC	National Agricultural Research Council

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CHAPTER NO 1

INTRODUCTION

1. INTRODUCTION

1.1 Medicinal plants

From ancient times, plants have been used for many purposes, including edible food, herbal medicine, flavoring agents, cosmetics and other uses (Husain et al., 2008). The medicinal plants include are the plants which were use in a medicinal activities. The medicinal plant to be used as high resources for various ingredients which may be use in drugs making. Although these plant play vital part in the green culture of the world. Moreover, some of these plant consider to be the important source of dietary substances so these plants recommended for their therapeutic values in the whole world.(Bassam Abdul Rasool Hassan ,2012)

Plants that have therapeutic activities and exert important pharmaceutical effects on the body of human generally considered as medicinal plants. Medicinal plants considered as our natural wealth. In the history uses of medicinal plants to treat different diseases probably goes back to the beginning of human life. (Muhammad kamal Hussain et al(IUCN) 2011).

The medicinal plants mostly use in the treatments of different diseases ,wounds cold and influenza stomach, kidney diseases and diabetes (13.4%) (Sukran kultur 2007).

In the present time plant medicines became more common in traditional culture, it focus on using plants for medicinal purpose. Even most of medicines we

are use in our daily life taken from plants. Medicinal plants are used for the extraction of many active products, which can be used in the synthetic applications of numerious drugs..(Qamar Zaman Qamar et,al 2010)

1.2. IMPORTANCE OF MEDICINAL PLANTS IN PAKISTAN:

Out of 258,650 species of higher plants from the world more than 10% are used to cure ailing communities.

Most of known drugs (e.g. tubercurarine, reserpine, aspirin and morphine etc) were discovered on traditional based knowledge. Majority of the people in Pakistan rely on medicinal plants to find treatment for their minor, even in some cases major diseases.(Zabta k.Shinwari 2010)

Most of research practices done invitro on plants research in Pakistan are under the documented level. The research is mostly conducted in the universities and that in other research center as ethno botanical level of resources. The different regions of our country have been very old knowledge for traditional uses of medicinal plants. This old knowledge of plants have been transferring from generation to generation. These plants were used to treat any kind of diseases from headache to cut and wound (Bhardwaj and Gakhar,2005).

The most important plants are harvested for the use of different types of active drugs. Different cultures of unani, Ayurvedic are mostly have the medicinal activities of plants.(Shinwari and Gilani.2003) and (Hamayun et al. 2006).

In Pakistan a huge drug market system known as "Pansara" is totally relient on the natural flora of Pakistan. Treatment of diseases in person and animals both are achieved by utilization of therapeutic plants (Shinwari and Qaiser,2011).

1.3. Future of Medicinal Plants:

Plants have important future due to the medicinal plants in the world ,and the mostly of the medical values have not yet investigated , and their medical values should be under the treatment future studies. Therefore its have most important point for the encouragement of researchers , perform new practices in vitro and field for the production of the active constituent which should be taken from plants have medicinal values. Also clear their roles in the relief of various diseases and how these could be useful to develope and synthesied effective drugs.

1.4. GENUS MALVACEAE:

Malvaceae are worldwide family of herbs, shrubs and small trees comprised 88 genera and 2,3000 species are distributing in tropical ,subtropical and temperate regions. (Willis,1973;Mabberley,1987) .There are 19 genera with 94 specific and intra specific taxa of this family in pakistan. (Abedin,1979). These are mostly found in

native Europe, North Africa and Asia. The most accounted ones are Malva ambigua Guss., Malva erecta .Fourn., Malva sylvestris, Malva neglecta Wallr. (Jaradat et al .2015).

1. 4.1.MALVA SYLVESTRIS LINN:

Malva sylvestris is the species of genus malva and belong to the family Malvaceae. Its a monocot angiosperm. In Pakistan Malva sylvestris was illustrated by (M. Rafique in 1967). It have the common name Khabazi and English name is common mallow, round leaf mallow, button weed. (Yeole et al., 2010)

Malva sylvestris is consider as a weed , The leaves of plant is consumed as food and the seeds, which contain 21% protein and 15.2% fat. (Chevallier,1996; Blumenthal, et al., 2000)

Fig : 1.1 Malva sylvestris L.

1.4.2 WORLD WIDE DISTRIBUTION:

Malva sylvestris Linn is known as common mallow mostly present in whole world native to Europe, North Africa and Asia. (Prudente et al;2013) (John Hilty; 2012)

1.4.3 DISTRIBUTION IN PAKISTAN:

Malva sylvestris L. is a herb and spread in mostly all areas as a common weed. In Pakistan its mostly occurs from plains to 1400ft (Nighat shaheen et al 2013).

In Pakistan it is frequently distributing in all provinces but mostly occurs in Punjab and KPK Tehsil katlang ,district Mardan ,The Siran Valley, District Mansehra, , a part of the internationally recognized Western Himalayan. (Habib Ahemad et al 2008)(Musharaf khan et al 2014)

1.4.4 MORPHOLOGICAL DISCRIPTION:

This species have long time history about the edible and medicinal uses in the Mediterranean region .(Gasparetto-et-al-2011).

Malva sylvestris L. (Malvaceae), is an annual to short-lived perennial plant, most abundantly distributing in temperate and tropical regions. (Podlech, 1996; Kintzios, 2001). Mostly this plant prefers clayey and clayey- loamy soils, and is the weedy member of mallow family. (Davis;1966) .

Glandular hairs are present on the stem, petiole and leaf. Leaves are dorsoventral, shallowly lobed, pilose. Stems are ascending to erect, pilose is simple setae and 10-60 cm long. Flowers are fascicles in the leaf axils. Petals colors are pink to white and 8-15 mm. Fruits can be bears from March to June.(Ozturk et al.(1997).

1.4.5 MEDICINAL IMPORTANCE OF MALVA SYLVESTRIS:

Most of the studies involved in the use of the importance of Malva sylvestris Linn as a traditional medicine in the world. Flowers extracts have traditional used to treats of various skin alliments, anti bacterial and anti-inflammatry. Extract of Malva sylvestris L flowers was also used for the wound healing activity.(Abdullah Ghasemi 2011)

Malva sylvestris L. leaves are particularly have been reported anti-inflammatory , anti cancer ,antioxidant , anti-complementary, and skin tissue integrity. An anti-ulcerogenic effects were recently proved , and also used to treat gastric ulcers.(Gasparetto- et al. 2011)

Malva sylvestris Linn are traditionally used for the treatment of liver diseases, but sufficiently based pharmacological literature is not available to its used in liver.(Choi et al .,2011; Rehman et al .,2014)

Ancient time this was used by Greek and Romans for its laxative properties and as a famous medicine.(Guarrera,2005; Schulz et al.,2003).Malva sylvestris L. has

many properties in treatment of ulcer, antioxidant ,anticancer, skin treatment ,antimicrobial and anti inflammation.(Shirin Hajyani, 2015)

1.4.6 MALVA SYLVESTRIS L. AS A FOLK MEDICINE

Malva sylvestris L. stands as folk medicine due to variety of uses, it was reported have been originate in 3000 BC in the region of Syria. Archaeological studies have shown the presence of Malva sylvestris seeds in dental calculus human fossils. Researcher concluded that the consumption of this species are very ancient ,due to its edible and medicinal properties .(Gasparetto et al 2011)

1.4.7 BIOACTIVITY OF MALVA SYLVETRIS L:

Malva sylvstris L have been considered as a chemotherapeutic and antiseptic agent. There are many research on phytochemicals activities from Malva sylvestris some of these reports indicated the presence of monoterpenes Malvone A, various other known chemicals naphthoquinone, tetrahydroxylated diterpenese and aromatic compounds, . (Seyed Mehdi Razavi ;2011)

1.4.8 PHYTOCHEMICAL ANALYSIS:

Anti oxidant activity of natural compounds in food and in dietary supplements plays important role in healthy life.(Nidal Amin jaradat ; 2015)

Phytochemical research of this medicinal plant indicate the presence of terpenoid , monoterpenes diterpenes ,polysaccharides, mucilage anthocyanins, coumarins, tannins, flavones, anthocyanidines ,leucoanthocyanidines,. (Cutillo et al., 2006.,Gasparetto et al., 2012)

1.4.9 ANTIBACTERIAL ASSAY:

Anti bacterial assay of Malva sylvestris plant extracts were used against Staphylococcus aureus, Escherichia coli, Erwinia carotovora, Entrococcus faecalis ,Streptococcus aureus ,Staphylococcus agalactiae. Antibacterial assay shows that ethanolic extracts of leaves and leaves of Malva sylvestris have good bacterial inhibition zone. (Razavi et al ,2009).

1.4.9.1 ANTIFUNGAL ASSAY:

Antifungal assay of mallow extracts were resolute beside Candida albicans, Candida ,Aspergillus niger, Pincillum SP, and Sclerotinia sclerotiorum. The result of antifungal assay showed that ethanolic extracts of the flowers have highest antifungal action (Lorain 1996).

1.4.9.2 DERMATOLOGICAL EFFECT:

Malva sylvestris L. have been studied for Hand eczema in human and as a herbal its indicates vital results. But the common therapeutic such as anti-histamines have numerous side effects.(Barikbin et al 2010)

1.4.10 PLANT TISSUE CULTURE TECHNIQUES:

Invitro multiplication techniques of plant are important to a lot of types of Lab intellectual inquiry, and to most of the functional aspects of botanical studied. From history, tissue culture techniques have been used for university investigations and the roles of hormones in organogenesis. This technique is also play vital role in inventive areas of applied plant science such as biotechnology and agriculture.(Mineo, L.1990)

This technique also involves the use of minute pieces of leaves and other part of plant (explants). which are cultivated in a nutrient medium in the given hygienic conditions. The appropriate growth conditions for each explants , new shoots can be produce from induced plants, and with supplementation of suitable hormones roots will be produced. Now these plantlets are placed in soil and grown under the normal conditions. (Garcia et al; 2009).

In vitro multiplication are involved in cell and tissue culturing techniques, which provide increased mass multiplication of medicinal herbs to fulfill commercial need and protect the genetic erosion (Zaiasts and Mitrofanova ,2013).

The plant species are multiplying by culturing in-vitro for regeneration. It is an important way to have a enormous amount of plants in a short time period. Invitro state such as media culture ,light , temperature should be optimize to the explants for variety of identical plants by using tissue culture technique(leal et al.,2011)

The in vitro multiplication of plants are done in a sterilized environment in which culture recipient have no gaseous exchange and due to this ethylene (gaseous plant hormone) are formed. This gas in the culture helps the embryo and shoot regeneration and development. In vitro Ethylene indicates negative effects on the Callus development and shoot initiation . The other gas silver nitrate is the real inhibitor of ethylene activity. In most of studies have highlighted the beneficial role of silver nitrate when added to the tissue culture medium of plants (Cristea et al,.2012)

CHAPTER = 2

REVIEW OF LITERATURE

2 REVIEW OF LITERATURE:

Since early time plants have been play very vital role in human life. Now a days, the edible use of plants and as way of plant treatments are still beneficial for human beings (Baytop ,1999).

Ayurveda is an old medicinal system also practiced in Pakistan and have been known for approximately 5000 years. Its included dietary fibers and also as herbal remedies , while emphasized on the body in the prevention of disease and treatment (Hamayun et al ;2006)

In vitro multiplication of this plant involve in tissue culturing techniques which give increase multiplication of medicinal herbs to fulfill profitable need and for defense of genetic erosion (Zaiasts and Mitrofanova ,2013).

The plant species are multiplying by culturing in-vitro for regeneration. It is an important resources to have a enormous number of plants in a short time period. Invitro provision such as media culture ,light , temperature should be optimize to the explants for variety of identical plants by using tissue culture technique(leal et al.,2011)

Plant tissue culture involves the use of small pieces of plant tissue (explants). which are cultured in a nutrient medium under the given sterile conditions. The appropriate growth conditions for each explants , new shoots can be produce from induced plants, and with supplementation of

suitable hormones roots will be produced. At the present these plants are placed in soil and full-grown under the normal position. (Garcia et al; 2009).

The in vitro multiplication of plants are through in a untainted environment in which way of life recipient have no gaseous exchange and due to this ethylene (gaseous plant hormone) are formed. This gas in the culture helps the embryo and shoot regeneration and development. In vitro Ethylene indicate harmful property on Callus growth and shoot initiation . The additional silver nitrate also is the actual blocker of ethylene activity. In the majority of studies have demonstrated the positive role of silver nitrate when supplementary to the tissue culture medium of plants (Cristea et al,.

2.1 IN VITRO MULTIPLICATION OF malva sylvestris L

Malva sylvestris L. (Malvaceae) is remedial herb , an annual to short-term persistent plant in our country. There is only a modest available information about in vitro propagation of Malva species (Kintzios,et al 2001). According to the present sequence study , only 1 study can be conduct on the callus development and in vitro propagation of Malva sylvestris .No work have been made on the in vitro proliferation on this plant inside Pakistan.

The designed of studied to prove the hormonal effectiveness of the BAP(6-Benzilaminopurineacid) ,and NAA(naphthalene acetic acid) to proliferate the species as of recognized in vitro stem segments. These stem segments were inoculate inside MS medium supplement with different conc and combinations of NAA and BAP, addicted to 8 procedure with 60 repetes each one of them, Explants of the Malva sylvestris are set aside into the growth room, and later than one week the resultant seedling were detached and regard the amount of leaves, sum of height. The seedlings were now set under the "Big bio" the seedling are transfer into the green houses. The the same plants obtain in MS medium supplement by the conc of 2.0mg/L BAP and 0.5mg/L NAA are at the maximum standard variety advocate for production of plantlet .(Bras et al, 2014).

It was investigate that the light strength and relative contact of light and dark state generate property on the term of somatic embryogenesis of the common mallow. In this study Petiole used as explants and were inoculated on Murashige and Skoog medium which were supplement with 200 mg/L casein hydrolysate. The photosynthetic proton flux concentration (PPDF) of 150 µmol m-2 sec-1 were used to incubate or helps in improved darkness of one week. The callus initiation of spherical somatic embryos, were observed every day. The effect of light capacity on the somatic embryogenesis was evaluate through incubated the medium cultures under different light quantities (50, 150 or 250 µmol) for 7 days. The evaluate consequences show that improved in light concentration have considerable effects relation with increase propagation rate of mallow embryogenesis (J. Konstas, et al. 2003)

2.2 IN VITRO MULTIPLICATION OF OTHER GENUS OF MALVACEAE:

Abutilon indicum (L.) is also the plant from the family Malvaceae. In vitro callus restoration of Abutilon indicum from leaves, intermodal and nodal explants on MS medium supplement by diverse conc and mixture of PGRs BAP and Kn were experienced and B5 vitamins also supplementary . The Callus initiation was obtain from leaves, nodal and Inter nodal explants with mixture of BAP (8.88 µM). The shoot regeneration was obtained from leaves, nodal and Internodal explants on BAP (8.88 µM), and Kn (0.578 µM). (K. Ramar and V. Ayyadurai 2014)

This revision was complete over the sterile state by using cotyledonary explants f of Althea rosea for callogenesis. unlike conc with the mixture of auxins and cytokinins i-e IAA, NAA, BAP and Kinetin were experimentation. After 10days initiation maximum Callus Index (CI= 560) was observed with 0.03mg/L 2, 4-D , 8 mg/L and 10mg/L BAP (CI= 320) and BAP+ NAA 4:3.0mg/L and BAP + 2, 4 D 4:0.03 mg/L were shows better response (CI= 480). Protocol of this study should be indicated that it fulfill the requirement of medicinal ornamental, and industrial demand of A. rosea by the advance technique of tissue culturing.(Mubashrah Munir 2012)

2.3 PHYTOCHEMICAL STUDIES:

The previous studies of chemical constituents of plants have desirable, knowledge for the new discovery of therapeutic agents ,Previous information are useful for discovering new sources of such economical important materials.(faraz mojab et al ;2003)

Phytochemical studies of the Malva sylvestris herb revealed the presence of various polysaccharides, Malvon A ,anthocyanins, tannins, flavones, anthocyanidines, leucoanthocyanidines, essential oils and also described the presence of terpenoids such as monoterpenes and,diterpenes. .(Cutillo et al., 2006.,Gasparetto et al., 2012).

Phytochemical study of Malva sylvestris L. leaves and petioles were analyze the nearby constituent of , fatty acid ,mineral content , starch ,flavnoids ,mucilage tannin and nitrate were also present. The samples were predictable for oxidant

capacities by using Folin-Ciocalteu (FC), Trolox comparable antioxidant capacity (TEAC). Result indicates that palmitic ,linolenic, linoleic, and oleic acids were quantify more than 82% .(Tabaraki et al ;2011)

The aerial parts of Malva sylvestris were use for photochemical study there result indicates the presence of phytocopounds like mucilage, carotenoids flavonoids, phenolic compounds tannin, ascorbic acid, tocophrerols, and antocyanines (Modaresi et al ;2015)

Results of ethanolic extract of fruits, flowers, leaves and roots showed the presence of anthocyanidines, Malvon A, naphthaquinones, or mucilaginous polysaccharides which are in high amounts. The M. sylvestris extract should be a good preserver for the configuration of biopesticide.(Syed Mehdi Razavi 2011)

The seed and stem were used for triphytochemical test analysis after extracting the solvents (ethanolic and aqueous).The ethanolic extract indicates the occurrence of Flavonoids , Tannins, Alkaloids sterols and steroids and aqueous extract indicates the occurrence saponins and starch.(Fatima Zohra et al ;2012)

2.4. ANTIMICROBIAL ACTIVITY OF MALVA SYLVESTRIS

Malva sylvestris dried flowers leaves, and roots were used for the training of extracts. The antibacterial activity of M. sylvestris plant extracts were used against the three bacterial strains Staphylococcus aureus (Gram-positive), Pseudomonas

aeruginosa and Escherichia coli (Gram- negative). Malva sylvestris extracts show maximum zone of inhibition against Escherichia coli (3.7mm) ,S. aureus (3.1 mm) at 15 mg/ml. The bacterial strains were collected from the Pathology Laboratory of Pakistan Institute of Medical Sciences, Islamabad. (Cynthia Walter et al., 2011)..

The methanolic extracts of aired dried , leaves and flowers were extracted through the soxhlet apparatus by n-hexane, dichloromethane and methanol. Antimicrobial, and phytochemical analysis of the plant extracts were evaluated by following disk diffusion method respectively. All the extracts of plant were used to determined the antibacterial activities against Escherichia coli (PTCC 1047), Staphylococcus aureus (PTCC 1112), Erwinia carotovora (PTCC 1190), Streptococcus agalactiae (PTCC 1321), and Staphylococcus aureus (PTCC 1138) (Persian Type Culture Collection) .The result indicates strong higest zone of inhibition against Escherichia coli and Erwinia carotovora, a common plant pathogenic bacteria, for both leaves and flowers extracts . (Razavi et al, 2011).

CHAPTER # 3

MATERIAL AND METHODS

3 MATERIALS AND METHODS

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

3.1 PLANT MATERIAL

3.1.1 COLLECTION OF SAMPLES:

Plant samples used in the research were collected from different localities of pakistan including wah cantt and District Haripur.(Fig)

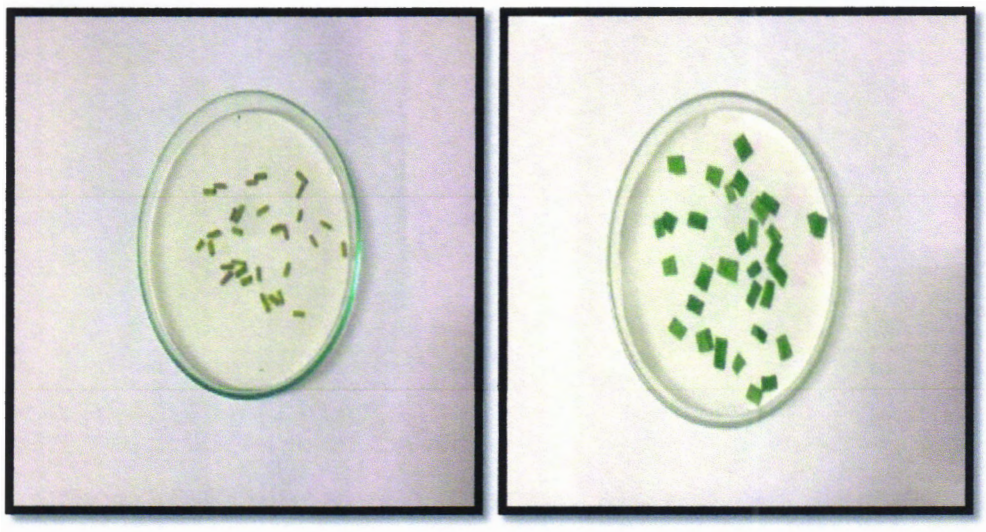


Fig :1.2 Surface sterilized Stem and Leaves explants used for experiment

3.1.2 APPARATUS:

3.1.2.1 MACHINERY

Laminar airflow cabient , fully automatic autoclave (Classic 1050), pH meter (Martini Instruments), Electronic balance (shimadzu), Microwave oven (Orient), Drying oven (DHG-9053A),Shaker (HY-4 speed adjusting multipurpose vibrator).

3.1.2.2 GLASSWARE

Petri plates and beakers (Pyrex ,Germany), Culture tubes(27ml) and conical flask (100ml) were used as glassware for the callus induction, multiplication, and regeneration Culture tubes and conical flasks were pluged using non –absorbent cotton wrapped in muslin cloth.

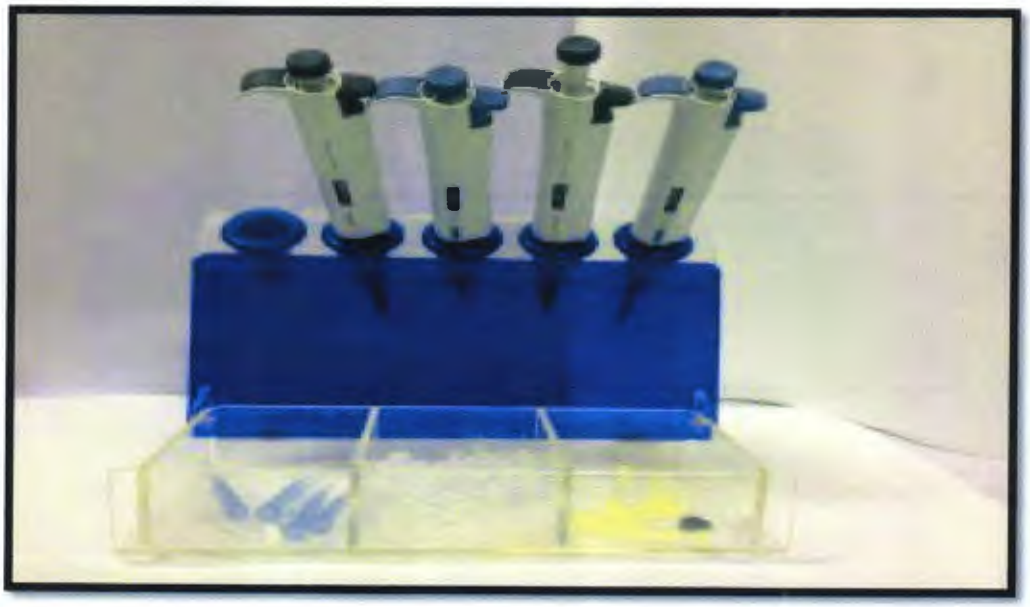


Fig 1.3 Tools and Glass ware using in Experiments

3.1.2.3 TOOLS

Falcon tubes ,Micropipettes (Biohit,Fnland) surgical blades , forceps and scalpels.

3.1.2.4 CHEMICALS

MS salts ,nutrient agar , sucrose (table sugar), NAOH, HCL, H2SO4 picric acid Chloroform Acetic acid Fecl3

3.2 METHODOLOGY:

3.2.1 PREPARATION OF STOCK SOLUTION OF PLANT GROWTH REGULATORS:

The auxin in the present study were 2,4-D and IAA while cytokinins used were BAP and kinetin. Stock solutions of all the plants growth regulators were prepared at the concentration of 0.1mg/ml using 1N concentration of NAOH ,followed by making up the volumes with double distilled water , appropriately as required. However stock solution 2,4-D prepared at the concentration of 1mg/ml using 1N concentration of NaOH, All plant growth regulators were stored at 4°c and used employing sterile micropipette tips as per requirement prior to autoclaving.

Table 1 .1 Preparation and Storage of Stocks of Plant Growth Hormones

Growth Regulators	Auxin		Cytokinins	
	2,4-D (1mg/ml)	IAA (0.1 mg/ml)	Kn (0.1mg/ml)	BAP (o.1mg/ml)
Mol. Weight	221.0	175.20	215.2	225.3
Solvent	1NaOH	1NaOH	1NaOH	1NaOH
Diluvent	ddH2O	ddH2O	ddH2O	ddH2O
Storage Tempe.	4°c	4°c	4°c	4°c
Storage time	2 month	1 week	2 months	2 months

3.2.1.2 MEDIA PREPRATION:

Synthetic MS medium with vitamins was employed in the study (Murashige and skoog,1962). Sucrose was used as a sole carbon source and was added as a soild at the concentration of 3 % (w/v).The ph of the media was adjusted by adding either 0.5M NaOH or 0.5M HCl,as required to get a final value in the range of pH 5.7-5.8. Gelrite was used as a solidifying agent at the concentration of 0.3%.The media was sterilized by autoclaving at 121°C for 15 minutes .

3.2.1.3 PREPRATION OF EXPLANT AND SURFACE STERILIZATION :

Malva sylvestris , explants (stem and leaves) were taken from collected newly grown plants shoots. The stems and leaves were cut into small segments depending on the requirement of the experiments.

Stem and Leaves were fistly washed throughly with tap water by using liquid detergent 1-2 mins, and then washed with dH2O for 4-5 times to remove the traces of detergent. After this explants were surface sterilized under aseptic conditions in Laminar Air Flow Hood. Firstly explants were washed with 70% ethanol (v/v) for 2 mins and then washed 50% Clorox (v/v) for 5-10 mins and then rinsed the ddH2O 3-4 time .The explants were than dried out on autoclaved filter place in the autoclave Petri plate in the hood.

3.2.1.3 CALLUS INDUCTION :

For Callus induction the MS media used to contain different concentrations and combinations of PGRs like IAA(Indole 3 acetic acid) and BAP(6-Benzilaminopurine acid) were tested. Different concentration of BAP i. e. 1.0, 1.5, 2.0 ,2.5 ,3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10 mg/L with the combination of 0.5mgL IAA were supplemented to MS medium. Both surface sterilized leaf and stem explants taken from mother plant 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. Out of 16 tested media for Callus induction, only 3 media (2.0mg/L, 3.0mg/L, 4.5mg/L) were showed effective Callus induction from stem explants only three media (2mg 4.0mg/L, 5.0mgL) were showed effective callus induction from leaf explants. The callus induction frequency was analyzed using the formula:

$$\text{Callus induction frequency (\%)} = \frac{\text{Total number of explants cultured}}{\text{Total number of explants cultured}} \times 100$$

3.2.1.4 REGENERATION:

For the shoot regeneration of calluses, MS media was supplemented with two different specific growth hormones BAP and kinetin which are responsible for shooting. Different concentration of BAP 2mg/L ,3mg/L ,4mg/L with the combination of kinetin 0.5mg/L, 1mg/L, 1.5mg/L were also tested.

3.2.2 EXPERIMENTAL DESIGN:

Factorial design was employed for each and every day one of the experiment. All of the experiments were made up of 3 replication. Each replication for callus induction comprised of 24 stem and leaves explants and nodal segments for shoot regeneration will also comprised 24 replication.

3.2.2.1 STATISTICAL ANALYSIS:

All the data was recorded timely and analyzed statistically by two factorial Analysis of Variance (ANOVA) using Statistix software version 8.1 significance level was considered as P value .

3.2.3 PHYTOCHEMICAL ANALYSIS

3.2.3.1 EXTRACT PREPARATION:

The crude ethanolic and aqueous extracts of leaf and callus of Malva sylvestris L were prepared by using the method described by (Wadood et al .,2013).

3.2.3.2 PREPARATION OF LEAF EXTRACT:

For the preparation of leaf extract, fresh leaves of the plant were collected from a mature Malva sylvestris plant.After that the leaves were washed with running tap water to wipe out any dust particles and then were kept in a drying oven for 3-4 days to dry up. The dried leaves were then grinding by using mortar and pestle to make the powdered. The powdered was weighted i.e 10g of the dried powered were dissolved in 100ml of ethanol ,ddH2O and put on the shaker for over night. Same procedure should be followed for Aqueous extract.

3.2.3.3 PREPARATION OF CALLUS EXTRACT:

For the preparation of callus extract, fresh calli were obtained from the preliminary experiments and calli were grinded by using mortar and pestle. The grinded accumulate calli was weighted as 0.5g and then dissolved in 50ml of methanol. The extract was covered and put on the shaker for overnight. Same procedure should be followed for Aqueous extract. Both the leaf and callus extract

solutions were then filtered with the help of Whatman paper No. 1 and used for further phytochemical analysis .

3.2.3.4 TEST FOR PHENOLIC COMPOUNDS

3.2.3.4.1 TEST FOR ALKALOIDS:

For the detection of alkaloids in the crude ethanolic extracts of leaf and callus of Malva sylvestris L 2ml of each extract was added in a separate test tube and then a small amount of picric acid was poured in both test tubes having extracts. Formation of white precipitate indicates the presence of alkaloids.

3.2.3.4.2 TEST FOR FLAVONOIDS:

For the detection of Flavonoids in the 5ml ethanol extract 1 ml of concentrated sulfuric acid and o.5g of Mg were added. Formation of red or pink coloration that disappear on the standing of 3-4 mins indicates the presence of flavonoids.

3.2.3.4.3 TEST FOR SAPONINS:

For the detection of saponins in 1ml of Aqueous extract few volume of distilled water was added . The solution was shaken vigorously and observed for a stable persistent froth for 20 mins.

3.2.3.4.4 TEST FOR TANNINS:

For the detection of tannins in 1 ml of ethanol extract 2-3 drops of diluted ferric chloride solution was added and observe blue –green blue -black color.

3.2.3.4.5 TEST FOR STEROL AND STEROID:

For the detection of sterol and steroid in 200ml of each extract was added in 2ml acetic acid. Solution was cooled was cooled well in ice and then mix with small amount of conc. H2SO4 .Ring of blue green showed presence of sterol and steroids.

3.2.3.4.6 TEST FOR STARCH:

The Aqueous extract 5ml was treated with reagent of starch (iodine).Any shift to blue violet indicates the presence of starch.

3.3 PLANT SAMPLE COLLECTION:

The ethanolic extracts of leaves and callus of Malva sylvestris L. used to check the anti bacterial effects against the different pathogenic strains.

3.3.1 BACTERIA:

Pathogenic strains of Staphylococcus aureus, Escherchia coli , klebsiella pneumonia were obtained from Microbiology Department of Al-Shifa Hospital Islamabad.

3.3.1.2 PREPRATION OF ETHANOLIC EXTRACT:

The method of jameela et al.(2011), was used to obtain both leaves and callus extracts. In which 20 gram of a leaves and callus were extracted separately and dissolved in 80% ethanol as a solvent for 24 hours .Then the extracts was filtered with what,s Man filtered paper NO. 1 and allowed to evaporate in oven at 45°c for 2-3 mins .

3.3.2 PLANT EXTRACTS ACTIVITY ASSAY:

3.3.2.1 PAPER DISK DIFFUSION ASSAY:

For preparation of media 14g of Nutrient agar should be dissolved in 1L of dH2O and allowed to boiled the medium in oven at 45 °c for 2-3 mins. After that medium were placed in autoclave for 1 hour . when autoclave circle should be completed take the medium and medium were spread on petri plates work should be done under the sterilized environment.

The use filter paper discs (5mm in diameter) was placed on the agar plates which was inoculated with the tested microorganisms and then impregnating with 20µl of plant extracts . The plates were subsequently incubated at 37°C for 24 Hrs.

After incubation the growth inhibition zone were quantified by measuring the diameter of the zone of inhibition in mm(kumar et al., 2009).

CHAPTER NO 4

RESULTS

4 RESULTS

4.1 EFFECTS OF DIFFERENT CLOROX CONCENTRATION:

For the prevention of microbial growth in the culture medium ,explants were gone through several washing steps and to boost the information of different microorganisms. These steps should be carried out very carefully. The time for every washing step was appropriately recorded. Clorox (commercial bleaching agent) is usually employed in the tissue culture labs for the sterilization of explants. Our results illustrate that 50% Clorox in combination with 70% ethanol and with 10% Clorox liquid detergent was sufficient to prevent the contamination. The rate of contamination is enhanced rapidly with 10% of Clorox concentration. . The Contamination frequency rate with 50% Clorox is 14.44% while with 10% Contamination frequency rate is high up to 75.00% . Therefore 50% Clorox was proved to sufficient for the eradication of microbial contamination.

Reason behind high contamination rate of this Plant

The *Invitro* multiplication of Malva *sylvestris L* is very difficult to maintain because it's a one of the fungus best host plant. Endophytic fungi may survive with simple surface sterilization of explants and at initially it become dormant and multiplying later in the medium. Another reason of high contamination rate is difficulty in surface sterilization of plants leaves due to their dorsoventrally structure and are very shallowlly lobed.

Table ; 1.2 Contamination frequency at different Clorox concentrations

Clorox Concentration	Contamination Frequency
10%	75%
3o%	50%
50%	25%

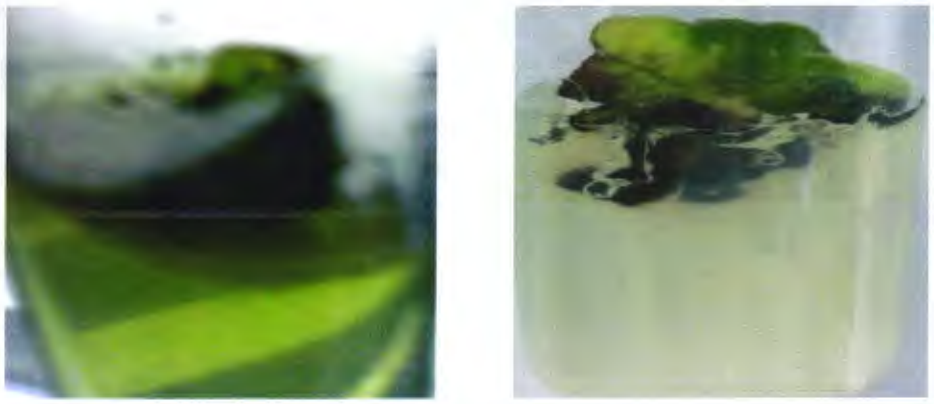


Fig: 1.4 Contaminated medium culture by fungus

4.1.1 EFFECT OF BAP AND IAA ON THE NUMBER OF DAYS TO CALLUS INDUCTION:

Total 120 samples of explants were cultured , 60 of each explants type, 20 on all 3 media combinations. Both of the explants took about 10- 15 for the induction calli. Best callus was induced on the stem explants at the concentration of 2.0mgL and 4.0mgL with the combination of 0.5mgL IAA.

A friable yellowish callus in different sizes were produced. Higher callus induction frequency was obtained CI-I and CI-II .All three callus induction media (CI-I, CI-II, CI-III) were not significantly different (P>0.01) and both of explants were found to be significantly different (P>0.01) from one another for callus induction frequency.

Table :1.3Callus induction frequency at various concentrations of BAP and IAA

Explants	MS , + BAP and IAA			Control
Hormonal Concentration	CI-I 4.5mg/L+0.5mg/L	CI-II 3.0mg/L+ 1.0mg/L	CI-III 2.0mg/L+1.5mg/ L	
Hypocotyle	1.3333a	1.1250a	1.0000a	0.2500b
Media Mean	1.0000A	1.0000A	0.7917A	0.2500B

LSD for Media= 0.4943

LSD for explants = 1.3734

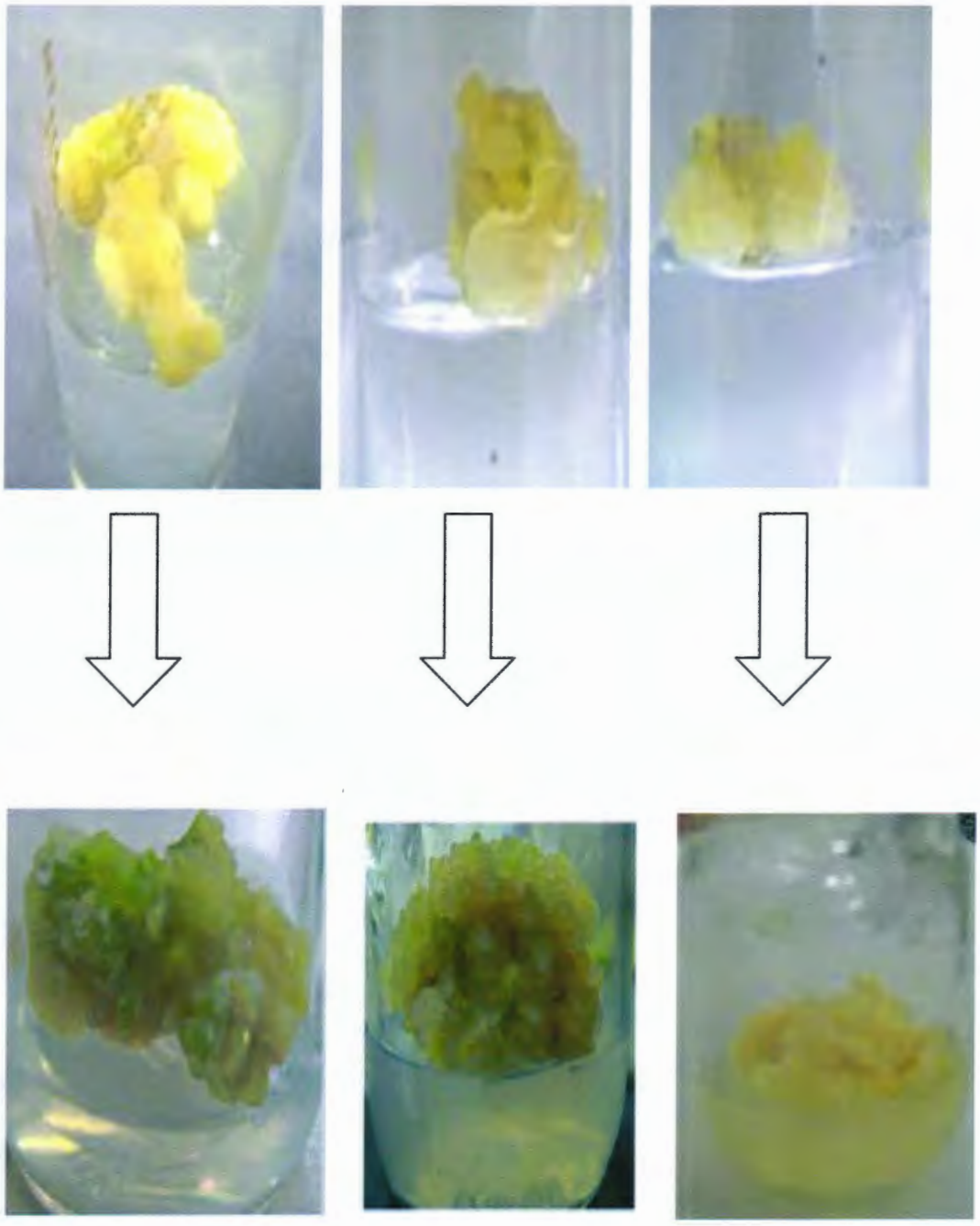
(Data followed by Capital alphabets is designated to the explants mean and small Alphabets is designated to the media mean.Each replicates consisted of 10 treatments).

Table1.4 Size of Callus induction frequency at various concentrations of BAP and IAA

Explants	MS , + BAP and IAA			Control
Hormonal Concentration	CI-I 5.0mg/L+0.5mg/L	CI-II 4.0mg/L+1.0mg/L	CI-III 2.0mg/L+1.5mg/L	
Hypocotyle	1.0333a	1.0000a	1.0000a	0.2933b
Media Mean	0.3300A	10.2700AB	0.2133B	0.0600C

LSD for media = 0.0837 LSD for explants = 0.0946

(Data followed by Capital alphabets is designated to the explants mean and small Alphabets is designated to the media mean.Each replicates consisted of treatments).



(a) (b) (c

4.5mg/L+0.5mg/L 3.0mg/L+ 1.0mg/L 2.0mg/L+1.5mg/L

Fig : 1.5 Callus Induction of Malva sylvestric L.from stem explants on MS medium supplemented with different concentration of BAP and IAA

Table:1.5 Callus induction frequency at various concentrations of BAP and IAA

Explants	MS , + BAP and IAA			Control
Hormonal concentration	CI-I 4.5mg/L+0.5mg/L	CI-II 3.0mg/L+ 1.0mg/L	CI-III 2.0mg/L+1.5mg/ L	
Leaves	1.0000a	1.0000a	0.7917a	0.1667b
Media Mean	1.1000A	1.0200A	0.7917A	0.1667B

LSD for media =0.3464

LSD for explants = 1.389

(Data followed by Capital alphabets is designated to the explants mean and small Alphabets is designated to the media mean.Each replicates consisted of 10 treatments).

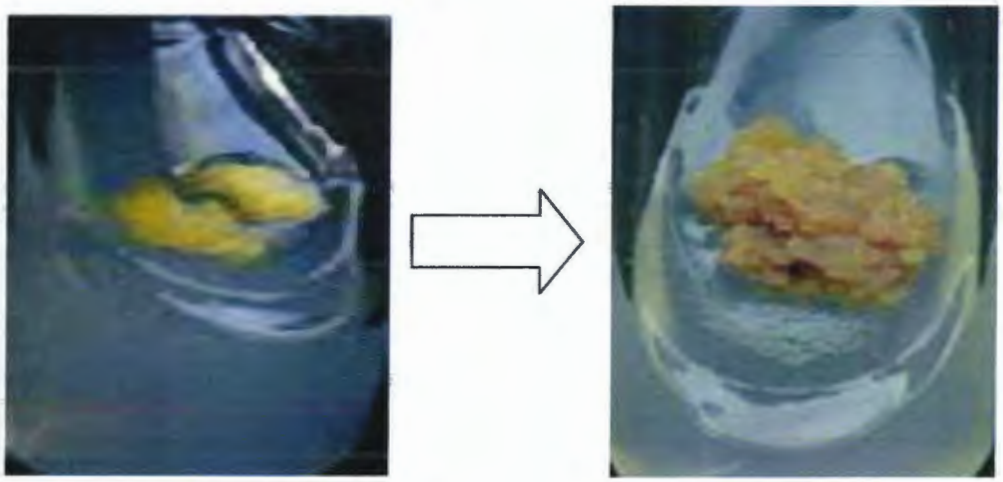
Table :1.6 Size of Callus induction frequency at various concentrations of BAP and IAA

Explants	MS , + BAP and IAA			Control
Hormonal concentration	CI-I 4.5mg/L+0.5mg/L	CI-II 3.0mg/L+ 1.0mg/L	CI-III 2.0mg/L+1.5mg/ L	
Leaves	1.0333 a	1.0000a	1.0000a	0.2933b
Media Mean	0.3300A	0.2700A	0.2133B	0.0600C

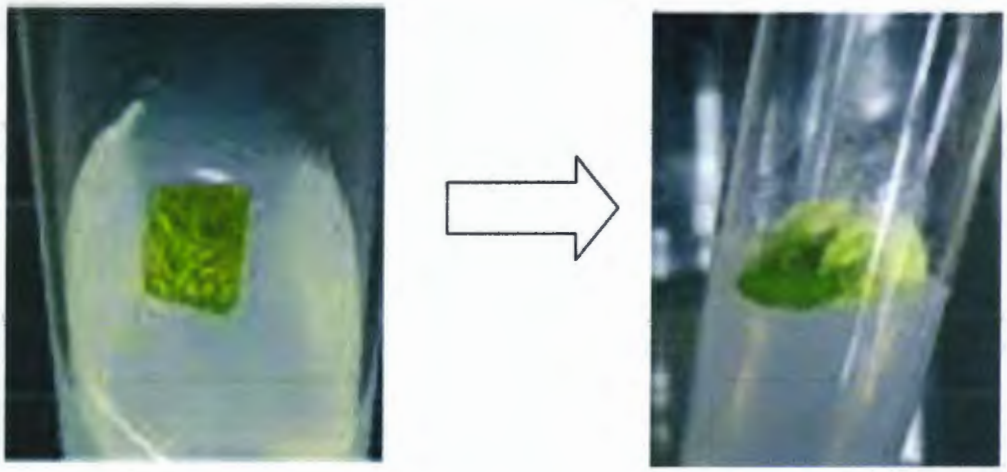
LSD for media =0.0837

LSD for explants =0.0946

(Data followed by Capital alphabets is designated to the explants mean and small Alphabets is designated to the media mean.Each replicates consisted of 10 treatments).



Callus induction from stem



(a) (b)

Callus induction from leaves

Fig :1.5.2 An increase in size of callus from stem and leaves explants on Control MS medium.

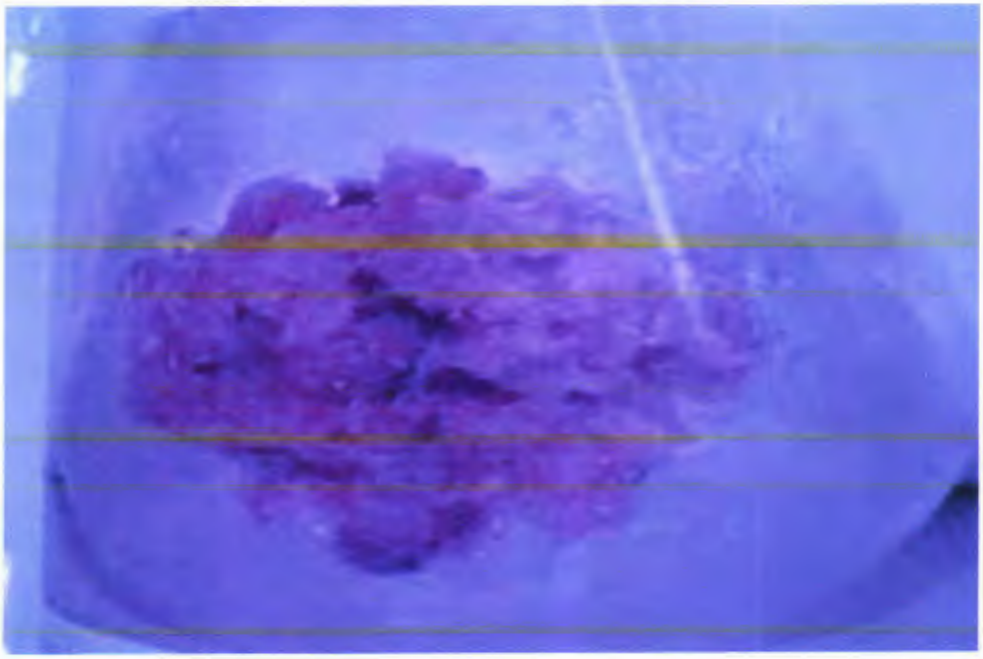
4.2 SHOOT REGENERATION:

Four different kinds of media (SI-I, SI-II, SI-III, SI-IV) were used for shoot regeneration potential. After shifting of calli to regeneration media , calli showed a constant increase in size but exhibiting no growth at all on the regeneration media combinations indicating that this media combination only aiding in increasing size of calli. Reported no organogenesis or shoot production of Malva sylvestris L from the calli.

Table:1.7Regeneration frequency of explants on different media combinations

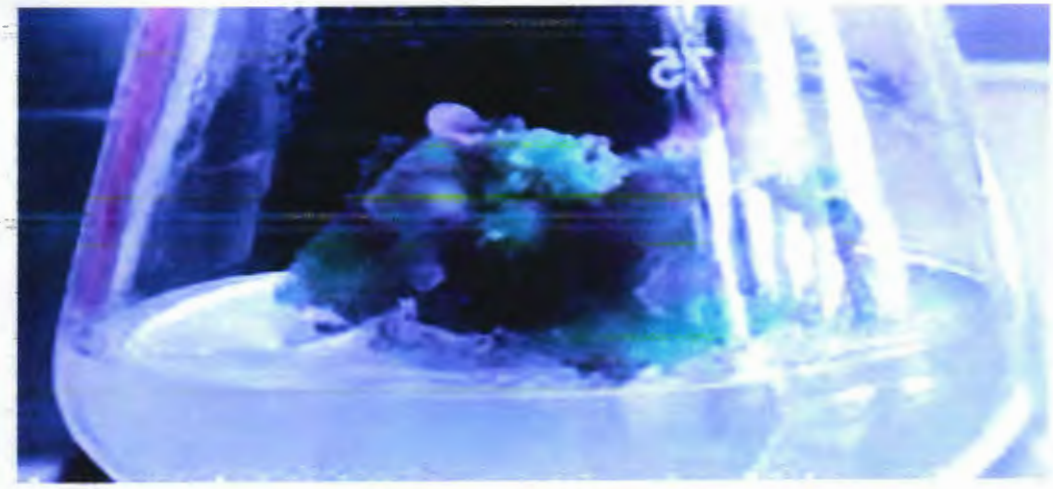
Hormonal Conc	MS + BAP and Kinetin (mg/L)			
	SI-I BAP + Kn (1+0.5mg/L)	SI-II BAP + Kn (2 + 0.5mg/L)	SI-III BAP + Kn (3+ 1.0mg/L)	SI-IV BAP + Kn (4+ 1.5mg/L)
Leaves	Little SI	Little SI	Little SI	Little SI
Hypocotyls	Increase CI	Increase CI	Increase CI	Increase CI
Results	No SI	No SI	No SI	No SI

SI= Shoot initiation ,CI =Callus Induction



(a)

Fig :1.5.3 No regeneration by stem calli



(b)

Fig : 1.5.4 Little Shoot regeneration by leaves calli

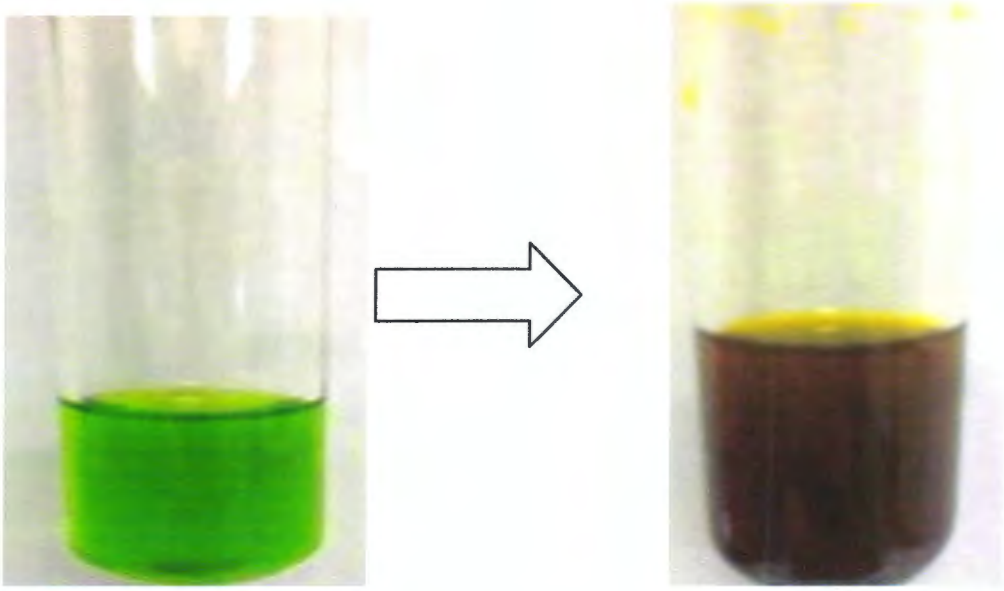
4.3 PHYTOCHEMICAL SCREENING

The results suggested that ethanolic and aqueous extracts of leaves and calli of the Malva sylvestris L. showed the presence and absence of the phytochemical compounds.

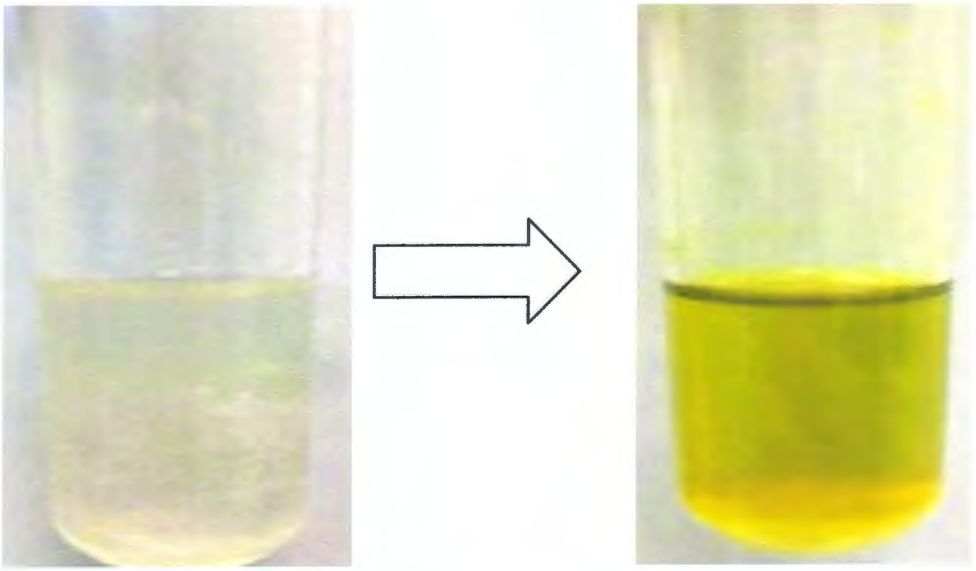
Table :1.8 Qualitative Screening of plant Phytochemicals

Sr No.	Phytochemicals	Ethanolic and Aqueous Extracts	
		Leaves	Callus
1	Alkaloid	+	–
2	Flavonoids	+	–
3	Saponins	+	+
4	Sterol and Steroid	+	–
5	Tanins	+	+

Key (+) present (-) absent

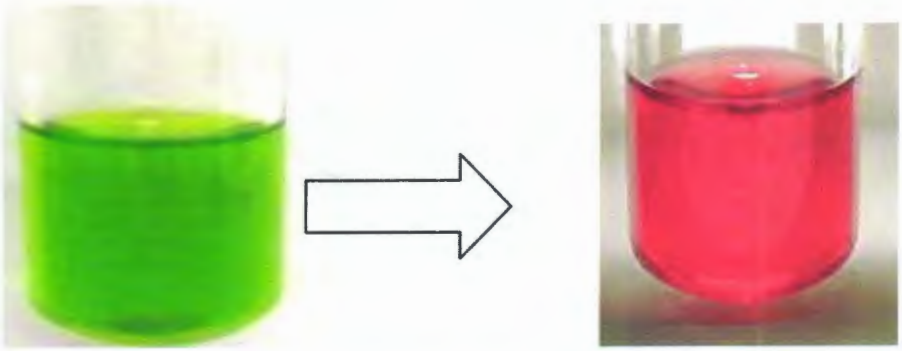


Test for Alkaloids by using Leaves Extract

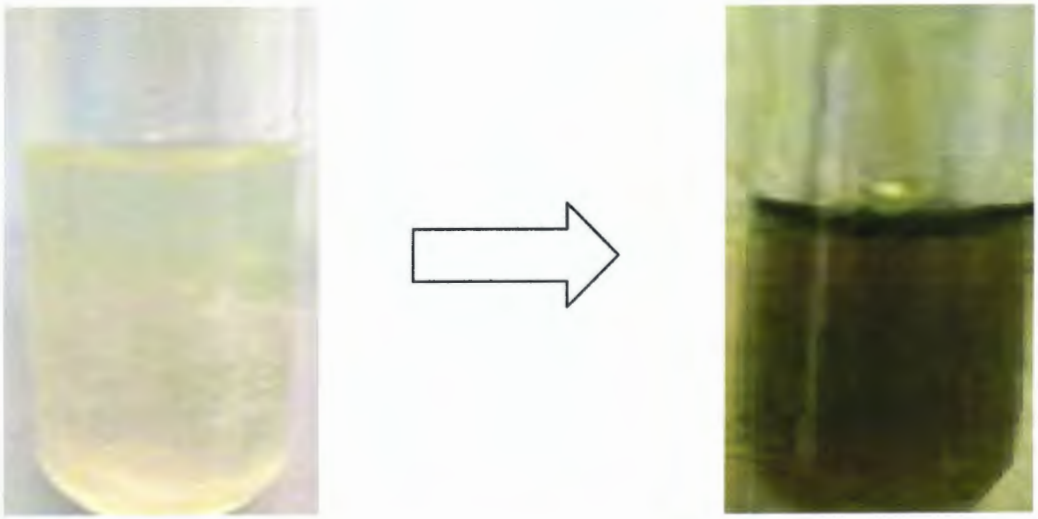


Test for Alkaloids by using Callus Extract

(a)

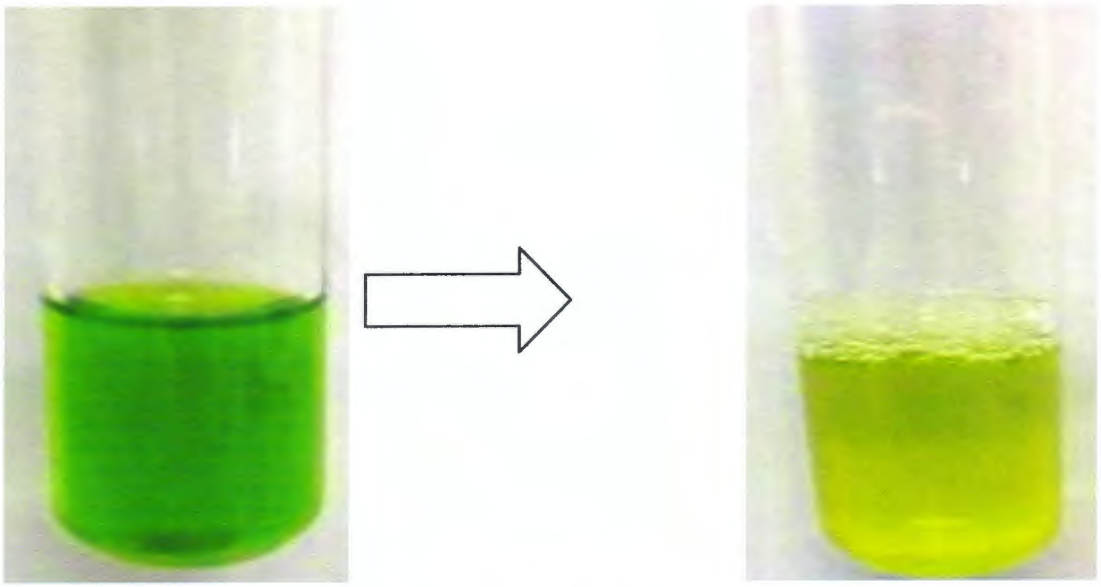


Test for Flavonoids by using Leaves Extract

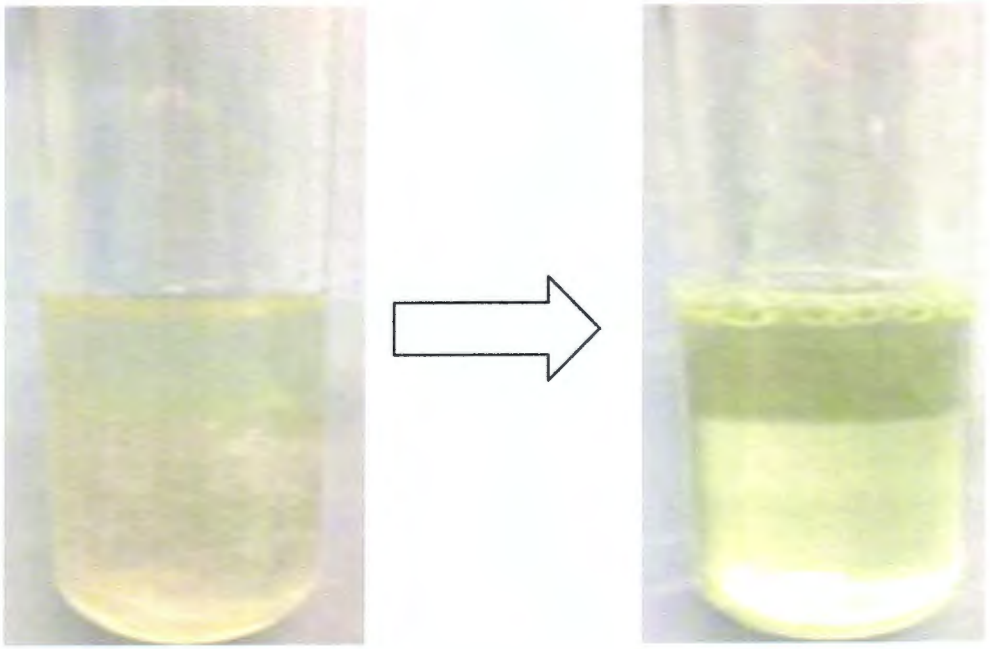


Test for Flavonoids by using Callus Extract

(b)

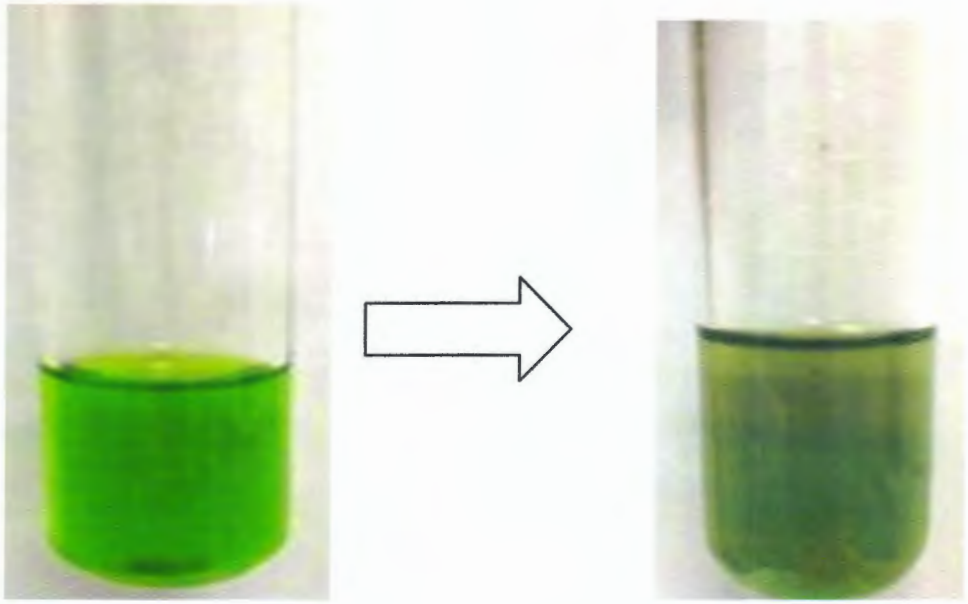


Test for Saponins by using Leaves Extract

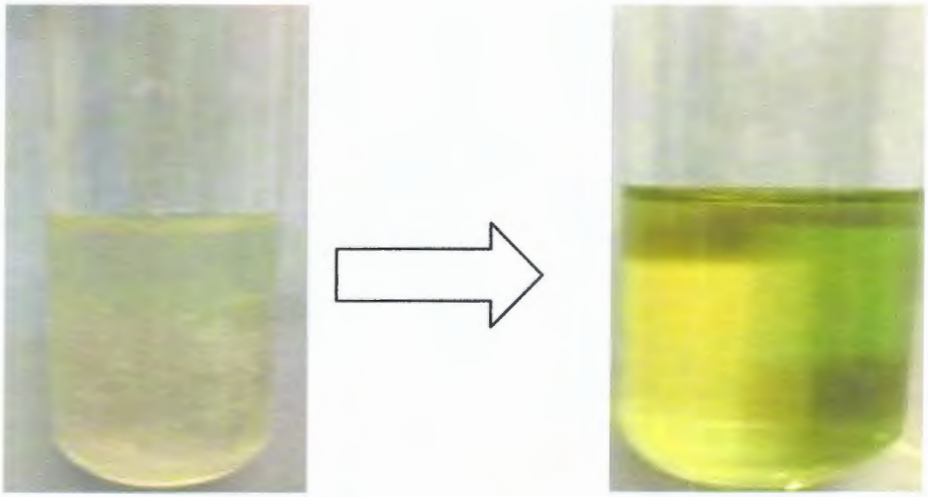


Test for Saponins by using Callus Extract

(c)

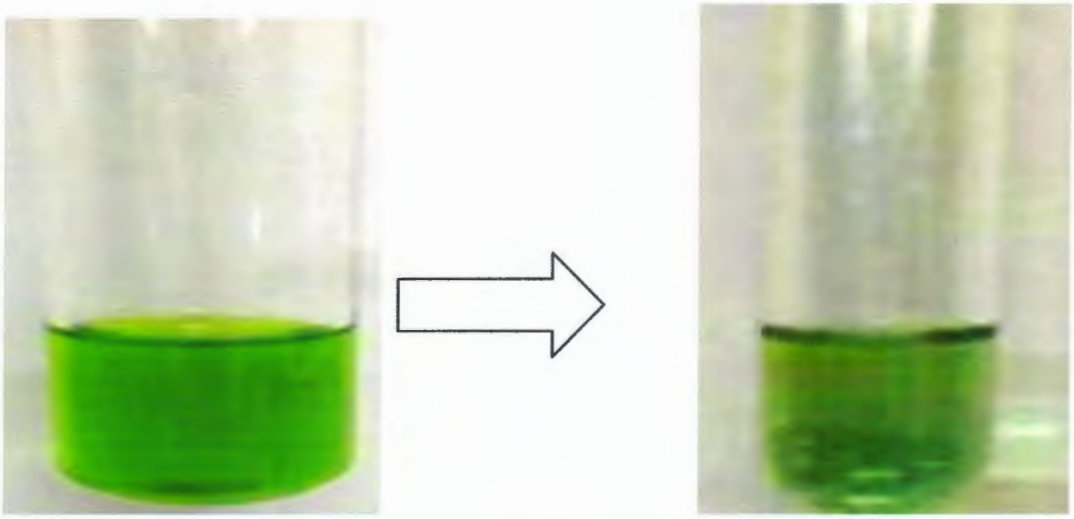


Test for Sterol and Steroid by using Leaves Extract

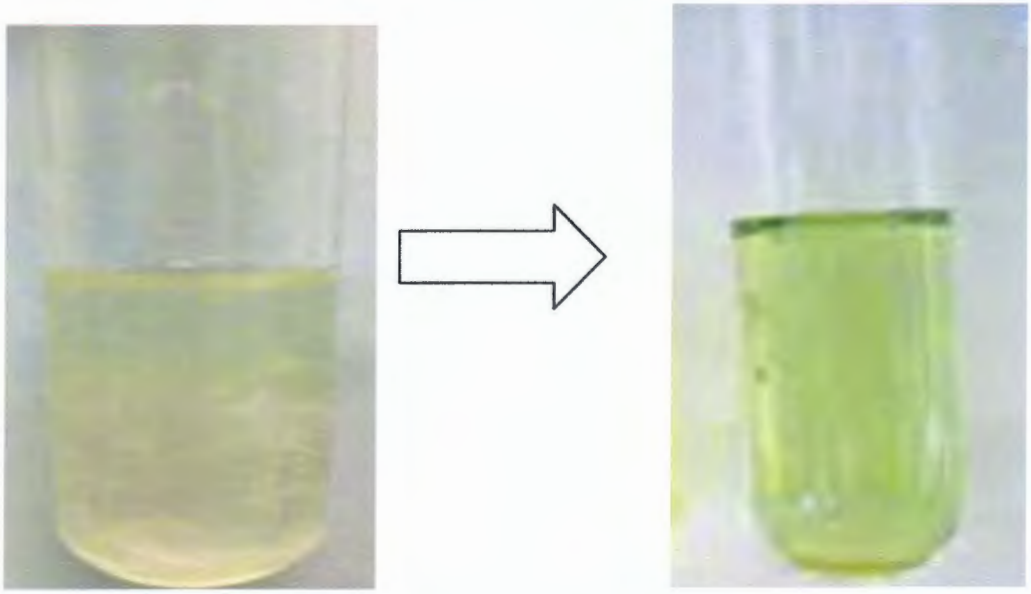


Test for Sterol and Steroid by using Leaves Extract

(d)



Test for Tanins by using Leaves Extract



Test for Tanins by using Leaves Extract

(e)

Fig:1.6 Phytochemical screening of Malva sylvestris Callus and leaves extracts

Alkaloids test for leaves and callus extract showing the presence

Flavonoid test for leaves shows presence and callus extract showing the absence

Saponins test for leaves and callus extract showing the presence

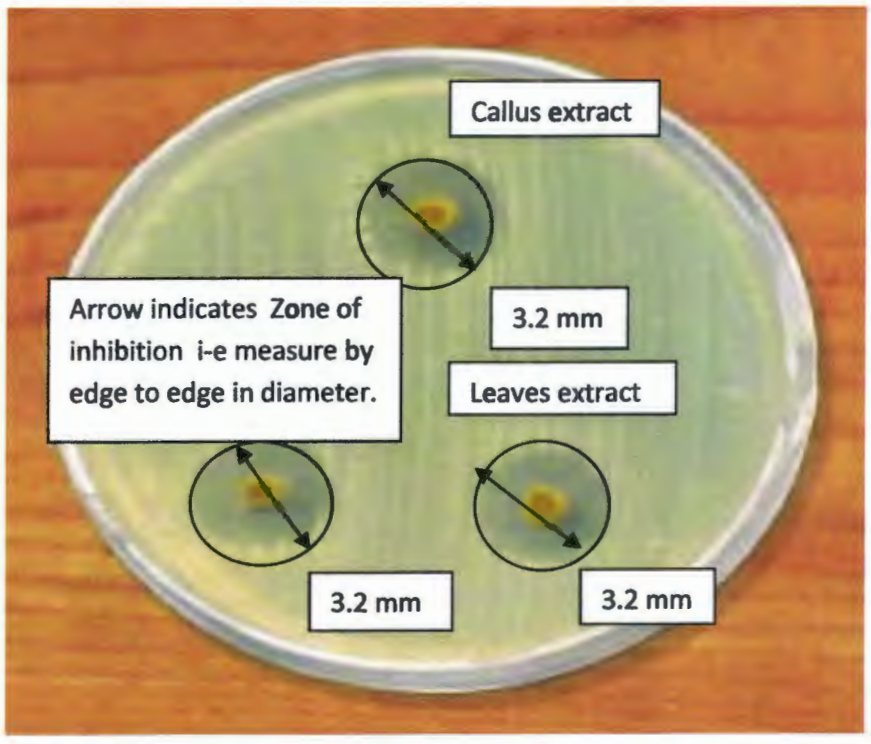
Sterol and Steroid test for leaves showing presence and callus extract showing absence.

Starch test for leaves showing presence and callus extract showing absence

4.4 EVALUATION OF ANTIBIOTICS ACTIVITY :

4.4.1 AGAINST Escherichia coli :

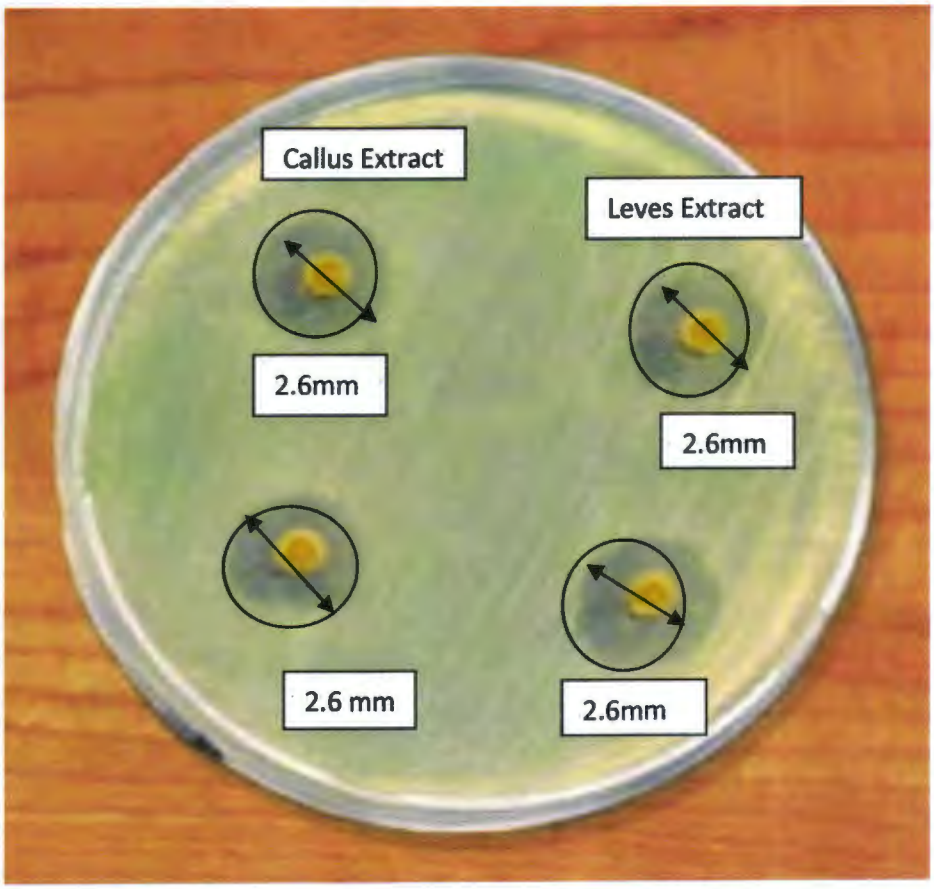
By using disc plate method the effectiveness of Malva Sylvestris extracts were determined against E. coli. (Fig). It was showed the highest inhibition zone against E. coli (3.2 mm).



(a)

4.4.2 AGAINST Staphylococcus aureus:

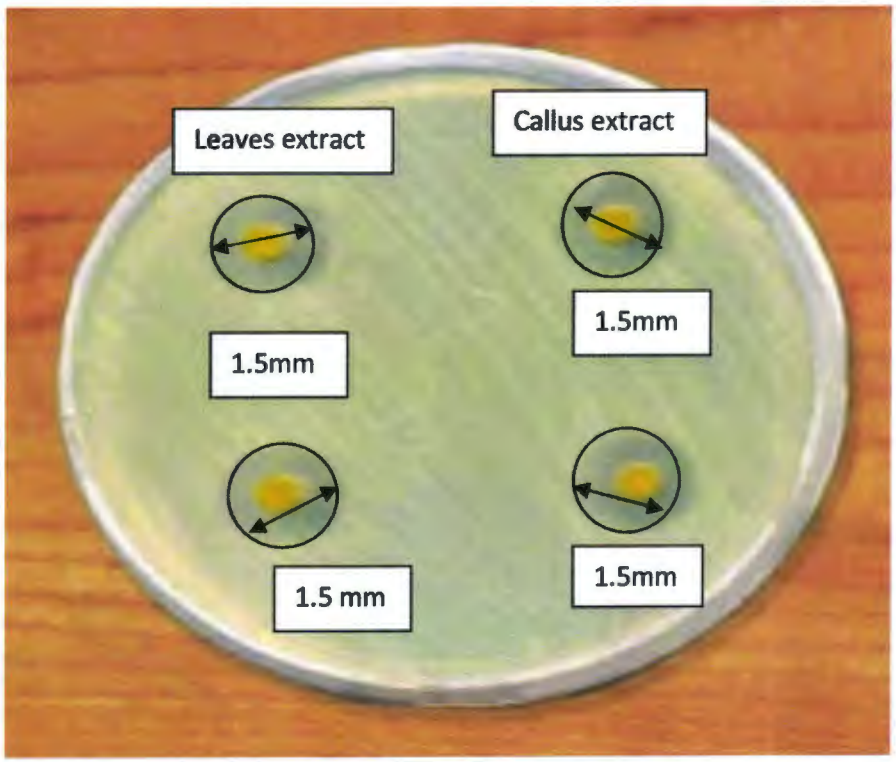
Malva sylvestris L. extracts shows highest inhibition zone (2.6 mm) against S. aureus.



(b)

4.2.3 AGAINST Klebsiella pneumonia:

Malva sylvestris L extracts shows inhibition zone of (1.5mm) against K .pneumonia.



(c)

Fig: 1.7 Aniti bacterial Activity of Malva sylvestris L. Callus and leaves extracts

AGAINST Escherichia coli

AGAINST Staphylococcus aureus

AGAINST Klebsiella pneumonia

5 DISCUSSION:

Medicinal plants are important and were traditionally used for the treatment of numerious diseases in the worldwide since the beginning of mankind civilization. Approximately three-quarters of the world population relies onthe medicine taken from plants for health care.(Rasool Hassan ; 2012)

Every country in the world constitutes various medicinal important plants. Pakistan is also rich country for producing various medicinal plant and one of them is Malva sylvestris L. It is an annual herbaceous medicinal plant (Barros et al., 2010) Malva sylvestris L young leaves should be eaten as raw in salads and also consumed in soups and as boiled in Mediterranean countries, and also in Pakistan. It's essential oil is considered to have antimicrobial anti fungal activity and also showed dermatological effects. (Husain et al., 2008). Its folk use has been documented as anti inflammatory properties, some other pharmacological and clinical effects such as diuretic, laxative, antiseptic, antiacne, bronchodilator activities (Barros et al., 2010; Zare et al ., 2012). In order to get maximum benefit of its medicinal importance we need of the plant in controlled conditions through in –vitro regeneration and tissue culture.

According to the literature survey only two studies on in vitro regeneration of Malva sylvestris L have been conducted on the callus induction and in vitro proliferation of Malva sylvestris up till now (Bernáth et al., 2013) and no work have been done on the in vitro propagation of this plant in Pakistan yet. Phytochemical analysis and antimicrobial activity were also be performed.

For the surface sterilization of explants to eliminate the microorganisms, NaOCl has normally employed for the tissue culture techniques(Sauer and burroughs, 1986). In the current experimental study 10% and 50% Clorox (commercial bleach) was used for the surface sterilization of explants. 50% Clorox concentration was found significant in eradicating the microbial growth . Our results coincides with Oyebanji et al ., (2009) and Yildiz and Er ,(2002) who also achieved a better surface sterilization at 40% and 50% Clorox concentration.

Effects of plant growth regulators on callus induction and regeneration efficiency differs greatly depending on the types and concentration of plant growth regulators and substances added to culture media (Somers et al.,2003). In the present study, leaf and stem explants of Malva sylvestris L were used for calli production on different concentration of BAP and IAA. The concentration use for stems are BAP and IAA 4.5mg/L , 3.0mg/L ,2.0mg/L and the concentration used for leaves explants are 5.0mg/L 4.0mg/L 2.0mg/L.

Our results revealed that for callus induction of Malva sylvestris L all three concentrations of BAP and IAA were found to be adequate , which was deviated from Bras et al 2014. Who reported that for callus induction of Malva sylvestris L. 2.0mg/L of BAP in combination of 0.5 mg/L NAA was found to be sufficient.

For the regeneration of Malva sylvestris L different regeneration media SI ,SII , SIII, SIV [(1+0.5 mg/L) (2 + 0.5mg/L) (3+ 1.0mg/L) (4+ 1.5mg/L)] were examined. The calli shifted to the regeneration media failed to regenerate, similar results were showed by (J .Konstas,2003) who also reported no organogenesis from the calli of Malva sylvestris L .

MS medium supplemented with a Silver Nitrate was also tested to increase the proliferation and regeneration. Plant growth and development is influenced by a ubiquitous plant hormone, ethylene (Liberman .,1979). Ethylene is believed to suppress shoot regeneration frequency in vitro where AgNO3 is a strong inhibitor of ethylene action .(Kumar et al ., 2009) Therefore the activity of AgNO3 was tested which showed an increase in shoot regeneration frequency which was caused by the disturbance of an ethylene signal transduction pathway.(zhang et al.,2001)

In the present study phyto chemical analysis of Malva sylvestris L revealed the presence of Phytosterol ,tannins ,alkaloids, flavonoids, saponins ,starch sterol and steroids in leaves extracts with comparison to callus flavonoids is absent. which coincides with the study of (Sabri Fatima Zohra et al ., 2012)

The anti bacterial results revealed that both leaves and callus of Malva sylvestris L exhibited strong anti bacterial effects against klebsiella pneumonia ,staphylococcus aureus, Escherichia coli ,respectively.

5.1 CONCLUSION:

The in vitro multiplication techniques provide large scale production of pathogen free plants in short time and often used for commercial production of plants as well as for plant research. In vitro callogenesis and direct shoot regeneration of an annual medicinal plant Malva sylvestris L using nodal explants has been established. Through in vitro micro propagation they can be produce in such areas where the production of Malva sylvestris is not otherwise possible.

5.2 FUTURE RECOMMENDATION:

An optimized protocol for in vitro callogenesis and of Malva sylvestris is successfully established which can further be used for the large scale production of plants These plants can be supplied to pharmacological industries and can be used for gene transmission as well. Biological activities of the plant and callus extracts of Malva sylvestris L can also be studied . Most of the Malva species are difficult to shoot and root in vitro and to improve this further awareness of rhizogenesis procedures are required. Therefore further studies can be conducted specially emphasizing on its in vitro shoot regeneration rhizogenesis.

CHAPTER NO 6

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APPENDIX

Callus induction by using stem explant 1

Completely Randomized AOV for fifteen

Source	DF	SS	MS	F	P
Mediia	3	1.12891	0.37630	11.6	0.0028
Error	8	0.26042	0.03255		
Total	11	1.38932			

Grand Mean 0.7604 CV 23.73

Completely Randomized AOV for thirty

Source	DF	SS	MS	F	P
Mediia	3	2.00391	0.66797	2.66	0.1196
Error	8	2.01042	0.25130		
Total	11	4.01432			

Grand Mean 0.9271 CV 54.07

LSD All-Pairwise Comparisons Test of thirty by Mediia

MediiaMean	Homogeneous Groups
1 1.0000	A
2 1.0000	A
3 0.7917	A
4 0.2500	B

Alpha 0.01 Standard Error for Comparison 0.1473
Critical T Value 3.355 Critical Value for Comparison 0.4943
There are 2 groups (A and B) in which the means
are not significantly different from one another.

LSD All-Pairwise Comparisons Test of thirty by Mediia

Explants Mean	Homogeneous Groups
1 1.3333	A
3 1.1250	A
2 1.0000	A
4 0.2500	A

Alpha 0.01 Standard Error for Comparison 0.4093
Critical T Value 3.355 Critical Value for Comparison 1.3734
There are no significant pairwise differences among the means.

Callus induction by using stem explant 2

Statistix 8.1 25-Aug-16, 10:57:55
AM

Completely Randomized AOV for thirty

Source	DF	SS	MS	F	P
Mediia	3	1.39974	0.46658	13.8	0.0016
Error	8	0.27083	0.03385		
Total	11	1.67057			

Grand Mean 0.7396 CV 24.88

Completely Randomized AOV for thirty

Source	DF	SS	MS	F	P
Mediia	3	1.39974	0.46658	13.8	0.0016
Error	8	0.27083	0.03385		
Total	11	1.67057			

Grand Mean 0.7396 CV 24.88

Statistix 8.1 25-Aug-16, 10:58:52
AM

LSD All-Pairwise Comparisons Test of thirty by Mediia

MediiaMean		Homogeneous Groups
1	1.0000	A
2	1.0000	A
3	0.7917	A
4	0.1667	B

Alpha 0.01 Standard Error for Comparison 0.1502
Critical T Value 2.306 Critical Value for Comparison 0.3464
There are 2 groups (A and B) in which the means
are not significantly different from one another.

LSD All-Pairwise Comparisons Test of thirty by Mediia

Explants Mean		Homogeneous Groups
1	1.1000	A
2	1.0200	A
3	0.7917	A
4	0.1667	B

Alpha 0.01 Standard Error for Comparison 0.1502
Critical T Value 2.306 Critical Value for Comparison 1.389
There are 2 groups (A and B) in which the means
are not significantly different from one another.

Size of Callus on Different Concentration of Hormones 2

Statistix 8.1 25-Aug-16, 11:05:30
AM

Completely Randomized AOV for thirty

Source	DF	SS	MS	F	P
MEDIA	3	0.04376	0.01459	9.95	0.0045
Error	8	0.01173	0.00147		
Total	11	0.05549			

Grand Mean 0.1408 CV 27.19

Completely Randomized AOV for thirty

Source	DF	SS	MS	F	P
MEDIA	3	1.20970	0.40323	16.3	0.0009
Error	8	0.19767	0.02471		
Total	11	1.40737			

Grand Mean 0.7617 CV 20.64

Statistix 8.1 25-Aug-16, 11:05:59
AM

LSD All-Pairwise Comparisons Test of thirty by MEDIA

MEDIA	Mean	Homogeneous Groups
1	0.2000	A
2	0.1867	A
3	0.1300	AB
4	0.0467	B

Alpha 0.01 Standard Error for Comparison 0.0313
Critical T Value 3.355 Critical Value for Comparison 0.1049
There are 2 groups (A and B) in which the means
are not significantly different from one another.

LSD All-Pairwise Comparisons Test of thirty by MEDIA

Explants MEDIA	Mean	Homogeneous Groups
1	1.0000	A
2	1.0000	A
3	0.8200	A
4	0.2267	B

Alpha 0.01 Standard Error for Comparison 0.1283
Critical T Value 3.355 Critical Value for Comparison 0.4306
There are 2 groups (A and B) in which the means
are not significantly different from one another.