PHYTOCHEMICAL SCREENING OF BIOACTIVE COMPOUNDS UNDER BIOTIC ELICITATION OF TULSI (*Ocimum tenuiflorum*)

DISSERTATION

Submitted in partial fulfilment of the requirement for the award of degree of

Master of Technology

(Biotechnology)

Submitted by

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DECLARATION

I hereby declare that the Dissertation entitled **"Phytochemical screening of bioactive compounds under biotic elicitation of tulsi (*Ocimum tenuiflorum*)"** submitted for the B.Tech M.Tech in Biotechnology degree is entirely my original work carried out at **Lovely Professional University, Phagwara** under the guidance of **Dr. Arvind Kumar** and all ideas and references have been duly acknowledged.

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CERTIFICATE

This is to certify that Nisha kumari has completed B.tech M.tech Dissertatio titled **"Phytochemical screening of bioactive compounds under biotic elicitation of tulsi (*Ocimum tenuiflorum*)"** under my guidance and supervision. To the best of my knowledge, the present work is the result of their original investigation and study. No part of the Dissertation has ever been submitted for any other degree or diploma at any university.

The dissertation is fit for the submission and the partial fulfilment of the conditions for the award of B.Tech M.Tech in Biotechnology.

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LIST OF ABBREVIATIONS

JA	Jasmonic acid
SA	Salicyclic acid
WTP	Wastewater Treatment Plant
BC	Before Christ
MS	Murashige and Skoog
L-DOPA	L-3,4-dihydroxyphenylalanine
ABA	Abscisic acid
GSH	Glutathione
GSL	Glucosinolates
UV	Ultra violet
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
PR	Plant pathogenesis-related
SAR	Systemic adquired response
PAL	Phenylalanine ammonia lyase
FW	Fresh weight
SD	Standard deviation
NMR	Nuclear magnetic resonance
HPLC	High performance liquid chromatography
MS	Mass spectrometry

ABSTRACT

Many secondary compounds are produced through plant as natural antioxidant. *Ocimum tenuiflorum* is known for its vast application in the pharmaceutical industries and the food processing industries. The aim of this study is to enhance secondary metabolites present in *O. tenuiflorum* L. through biotic elicitation (chitosan). Various concentration of chitosan (0, 50, 100, 150, 200 and 300) milligram per litre were used. The maximum increase in secondary metabolite was noticed at 150 mg per litre chitosan. Flavonoid content and phenolic content were studied by spectrophotometrically. In invivo, Ocimum leaves showed high flavonoid content and low phenolic content. Phytochemical test for tannins, flavonoids, alkaloids, saponins, anthraquinones and reducing sugar were tested +ve for Ocimum leaf. In the present research higher flavonoid content and the phenolic content showed natural antioxidant nature of Ocimum that signifies their medicinal uses. *Ocimum tenuiflorum* L. are widely grown in India. Many secondary metabolite from the plant like phenolics and flavonoids are known to be occur in this plant. The present research was conducted with an aim of qualitatively and quantitatively analysis of phytochemicals in *Ocimum tenuiflorum*. Quantitation of total flavanoids content and phenolics content was completed with the help of spectrophotometrically while qualitative analysis was perfomed by phytochemical tests. Phytochemical screening of distinct metabolite such as tannins, flavonoid, saponin, anthraquinone, alkaloid revealed that plant extract were very rich source of secondary metabolite and thus tested +ve for above tests. From the above study, conclude that Ocimum are very rich source of natural antioxidant that's why it is important source in pharmaceutical industry.

Keywords: Antioxidants, Flavonoids, Ocimum, Phenolics, Phytochemicals, Qualitative analysis

CHAPTER-1

INTRODUCTION

Plants are made from plants constituents and they are the individual chemicals from which they made. They are organic in nature. By the activity of individual cells they synthesized in plants. Plant constituent are complex organic chemical constituents by utilizing enzymes and simple substances formed by the process called biosynthesis (Harborne, 1988). The chemistry of secondary metabolites are made up from organic compounds. For the normal metabolism of the organisms, they are not directly responsible. That's why, these organic compounds are known as secondary-metabolite. The secondary metabolite is complex organic molecule. The secondary metabolites are biosynthesized through the primary plant metabolites in the plant cells. They are usually posses therapeutic activity, which is neither necessary for plants life nor contain more energy bonds. But it is probably synthesized to defensive goals (Hadecek, 2002).

Researchers are intensively studying the constituents of plants and their various nutritional importance from decades. Metabolism is the process by which plants produce various products and intermediates known as the 'metabolites' of plants. Primary metabolites such as carbohydrates, lipids and amino acids are produced. In addition to this, higher plants also synthesize various low molecular weight compounds called secondary metabolites that establish the defense mechanism in them (Wang and Wu, 2013).

Secondary metabolite is organic molecule. It has no role in the normal growth. Also no role in the development of organisms. But primary metabolite has a vital function in growth of the species. They plays an effective role in respiration and also in photosynthesis in the plants. When secondary metabolite is absent, then the plant is not turn to actual death. However, the result is long period destruction of survivability of the organisms, generally they are playing vital role in defense of the plant. These compound is an acutely distinct class of natural products which is synthesize by plants, animals, algae, fungi, and bacteria. The secondary metabolites like alkaloids, phenolic compound and terpenes are divided on the bases on origin of their biosynthesis. Distinct divisions of the compounds are mostly combined with narrow set of species within a phylogenetic group and constitute the photochemical in the various colorant, spice plants, aromatic and functional foods (Roze *et al*., 2011).

Whereas plant secondary metabolite is primarily combined with plant defense feedback across/against herbivore and pathogen and these different compound can be

involve in a large cluster of the ecological function (Bertin *et al*., 2003). Secondary compound is released by plants into their environment that change chemistry of soil. So they increases uptake of nutrients and defending against the metal toxicity (Dakora and Philips, 2002). Chemical signal is necessary for resolving interaction among non pathogenic bacteria of soil and roots of plant, along with di nitrogen (N_2), S fixing bacterial symbionts (Marx, 2004; Dakora and Philips, 2002).

Diversity of mechanisms are used by the plants to discharge secondary compound as volatile, such as compounds which generate scent correlated with a pungent leaves and flowers, whereas roots also can actively release volatile (Steeghs *et al*., 2004). Plant use up to 30 percent of their photosynthetic activity in the production of root exudates. And it affects local soil environment which is term as rhizosphere. All of the processes may be discharge chemical that are mediating allelopathic interaction among the plants (Bertin *et al*., 2003). The chemical constituent that are present in plant and that are not posses definite therapeutic value are called inactive constituents. Whereas, active constituent is involve in development, reproduction and growth of plants. The formation of inactive and the active constituents of plant involve distinct metabolic pathways. Almost the active plant constituents are called as primary plant metabolites since inactive plant constituent is term as plant secondary metabolite (Stamp and Nancy, 2003). Unlike primary metabolite of the plant, existence of secondary metabolite is generally playing an vital role in plant defense system (Osbourn *et al*., 2003).

Also, *Ocimum tenuiflorum* is called as *Ocimum sanctum*, tulsi and holy basil. Tulsi is an aromatic plant. It belongs to Lamiaceae family which is native to the Indian subcontinent and widespread as a cultivated plant throughout the Southeast Asian tropics (Staples *et al*., 1999). It is many branched sub shrub, erect, thirty–sixty centi meter tall with hairy stems and simple purple or green leaf that are strongly scented (Warrier *et al*., 1995). The medicinal properties of Tulsi are mainly because of the presence of a variety of phenolic acids such as rosmarinic acid, caffeic acid flavonoids, and essential oils (Zgórka and Glowniak, 2001).

Tulsi are of three varieties. These are :

1) Light or Rama Tulsi (*Ocimum sanctum*)
2) Dark Tulsi or Shyama *(Ocimum sanctum*)
3) Vana Tulsi (*Ocimum gratissimum*)

Compound which stimulates all type of physiological abnormality of plants is called Elicitors (Radman *et al*., 2003). This explanation of elicitor includes both compound which is

released from the plant by action of the pathogen called endogenous elicitor. And substance of pathogen origin is called exogenous elicitor. Elicitors can be used to raise secondary metabolite of plant synthesis. They can act vital role in biosynthetic pathway to increases the production of commercially necessary compound. The secondary metabolite is discharged because of the defense responses that activated and caused through elicitor, signal compounds of plant defense response (Radman *et al*., 2003).

According to Radman *et al*., (2003) elicitors are classified into two classess that is physical and chemical. Elicitor can also be classified into two types on the basis of their nature : Abiotic and biotic elocitors. The elicitors that have biological origin are called biotic. They are derive from plant itself or from pathogen. And abiotic elicitor has not a biological origin. They are classified into chemical compounds and physical factor. The very first biotic elicitor invented in 1968. It may be divided into general elicitors and race specific on the basis of plant elicitor interaction (Staskawicz *et al*., 1991). There are distinct plant hormones that are acting as Elicitor. Most common plant hormone such as JA and Salicyclic acid (SA). Both are crucial signal for defense gene expression. SA is regulating resistance to pathogen such as fungal, viral or bacterial. While JA regulate the protein production through the octadecanoid pathway. JA and SA both are synthetic mimic. They coulds be applied externally to activate same metabolic change which regulate resistance against pathogen. The biochemical pathway of JA and SA both helpful in plant elicitation process of the plant (Angelova *et al*., 2006).

Polysaccharide chitosan is an example of biotic elicitor which is used in this research. Basically, chitosan is an important derivative of chitin. Chitosan is a polymer of β-1,4-D-glucosamine which is derived from crab-shell chitin. The chitosan use are limited due to its high viscosity, insolubility in water and at high pH it has tendency to coagulate with proteins. It is nontoxic, inexpensive and possesses reactive amino groups. Chitosan is proved to be beneficial in many various areas as a potential elicitor of plant defense responses, as an antimicrobial compound in agriculture, as a flocculating agent in WTP, as a hydrating agent in cosmetics, as an additive in the food industry and more recently it is used as pharmaceutical agent in biomedicine (Rabea, 2003).

CHAPTER-2

REVIEW OF LITERATURE

The approach of secondary metabolite from the plant was first time reported approximately in 300BC by Roman and Greek writer. Hans Molisch, he gave word "allelopathy" to specify the plant plant interactions (Willis, 2004; Rice, 1984). In the modern literature, the word allelopathy could explain all indirect or direct effect of plant chemical compound on the microbe or plant. Whereas they are most generally used to refer to chemical mediated -ve interference among plants (Willis, 2004). Allelopathy is a biological process by which an organism produce one or more biochemical which affects the survival, growth and reproduction of other organisms. These biochemicals are termed as allelochemical. They can have detrimental (-ve allelopathy) and beneficial (+ve allelopathy) effect on the target organism (Stamp and Nancy, 2003).

2.1 Plant used in research

2.2 Classification of secondary metabolite

2.3 Plant Tissue Culture as source of Secondary Metabolite Production

2.4 Production of plant secondary metabolites from medicinal plant through plant tissue culture

2.5 Elicitors use to enhance the production of Secondary Metabolite

2.6 Mechanism of elicitation in plants

2.7 Mechanism of chitosan in enhancing secondary metabolite

2.1 Plant used in research : Tulsi (*Ocimum tenuiflorum*)

Many secondary compound are produced through plant as natural antioxidant. *Ocimum tenuiflorum* is rich source of these secondary metabolites. *Ocimum tenuiflorum* is known for its vast application in the pharmaceutical industries and the food process industries.

Ocimum tenuiflorum is also called as "the elixir of life" since it promotes longevity. Distinct parts of plant are used in siddha systems and ayurveda of medicine for prevention and cure of many illnesses like headache, common cold, wound, insomnia, arthritis, digestive disorders, night blindness, diarrhoea and influenza. The leaves of tulsi are very good for nerves and to enhance memory. Chewing of *Ocimum tenuiflorum* leaves is also treats the ulcers and infections of mouth (Prajapati *et al*., 2003).

Ecological niche:

Kingdom : Plantae

Division : Magnoliophyta

Class : Magnoliopsida

Order : Lamiales

Family : Lamiaceae

Genus : Ocimum

Species : *O.tenuiflorum*

Botanical Name : *Ocimum Tenuiflorum*

Figure 2.1 Represents *Ocimum tenuiflorum* plant

2.1.1 Phytochemical constituents

Tulsi consist of a variety of active compounds in its stem and leaves, including saponins, flavonoids, alkaloids, triterpenoids, and tannins (Jaggi and Madaan, 2003). Phenolic substances have also been reported, which carry anti-oxidant and anti-inflammatory properties. Two water-soluble flavonoids (Uma *et.al.*, 2000) orientin and vicenin are found that provide protection blood lymphocytes of humans from radiation induced by damage of any chromosome.

2.2 Classification of secondary metabolite

Secondary metabolite from the plant could be classified into 3 chemically various group that is Phenolic, Terpenes and N and S containing compound.

2.2.1 Terpene

Terpene are biggest class of the secondary metabolite. They are concerted through their simple biosynthetic origin from glycolytic intermediates or acetyl coenzyme A (Grayson, 1998; Gerhenzon *et al*., 1991; Fraga, 1988). A big majority of various terpene structure produced through the plant as secondary metabolites i.e. assumed to be involves in

defense as toxins or feeding deterrent to huge number of plant feeding mammals and insects (Gershenzon *et al*., 1991).

The fragrance in the plants is due to the presence of essential oils. Some plants use the scent for the protection from herbivores and dangerous pathogens. Humans use essential oils for aromatherapy to enhance mood. It also helps in the functioning of mental and is thought to have various other benefits like oral bacteria potency, wide use in mouthwashes that are antiseptic, for skin problems and respiratory diseases.

Another type is taxol, which have great importance in the medical field to treat breast and ovarian cancers. The bark of Pacific yew tree produces taxol in very few amounts that its synthesis killed the tree. Therefore, other sources were found like a fungus on tree and needles in European yew.

Rubber is the last type which is also the largest of terpenoids because it contains 400 isoprene units. Rubbers have a large number of applications since decades. Today, rubbers are used in shoes, tires, erasers, gloves and spandex.

2.2.1.1 Monoterpene (C10)

Number of derivative are vital agent of the insect toxicity. In case of pyrethroid (monoterpene ester) appear in flower and leaf of the Chrysanthemum species which showed the insecticidal response to insects such as moths, bees etc. and very famous additive in commercial insecticide due to decrease mammalian toxicity and low persistence in the environment (Turlings *et al*., 1995). In Gymnosperm like Pine monoterpene assemble in resin duct found in twings, needle and trunk specially as β-pinene, α-pinene, myrecene and limonene. All are toxic to diverse insect including bark beetles (Turlings *et al*., 1995).

2.2.1.2 Sesquiterpene (C15)

Till now, many sesquiterpene has described for their important role in plant defense like costunolide is anti herbivore agent of family complex characterizes by 5 member lactone ring and big feeding repellence to most herbivorous mammals and insects (Picman, 1986). Also, Abscisic acid is sesquiterpene, playing mainly regulatory roles in maintenance and initiation of the seed, bud dormancy (Van steveninck, 1983) and acting as transcriptional activator (McCarty *et al*., 1991). In addition, it enhances the concentration of cytosolic calcium and cause cytosol alkalinisation (Irving *et al*., 1992). The level of Ultra Violet-B absorbing quercetin, kaempferol and flavonols significantly were enhanced when Abscisic acid (ABA) applied. The concentration of two hydroxyl cinnamic acid, ferulic acid and caeffic acid were also enhanced through abscisic acid. All of the change, in protective compound, anti-oxidant enzymatic activities and sterol corresponded with decrease

membrane damage through UltraViolet-B. Hence, defense system of plants against UltraViolet-B, stimulated in which abscisic acid acting as downstream in signaling pathways (Berli *et al*., 2010).

2.2.1.3 Diterpene (C20)

Abietic acid, example of diterpene. It is found leguminous and pine trees. They are occurs in resin canals of the tree trunk. When, canal broken through feeding the insect, the discharge be of resin may be substantially block the feeding and deliver as a chemical deterrent to continued predation. Another example is phorbol is found in plants of Euphorbiaceae. It works as internal toxins to mammals and skin irritants. Furthermore, phytol is a more hydrophobic twenty carbon alcohol found in the chlorophyll as a side chain helping to anchor some molecule in membrane. That's why, enhances efficiency of the chlorophyll at time of the photosynthesis (Knoff, 1991), biomass production and the strategy for the highest carbon dioxide fixation (Jagendorf, 1967).

2.2.1.4 Triterpene (C30)

Steroid alcohols or sterols are vital components of cell membrane in plants, specially in the plasma membrane they act as a regulatory channel. By reducing the motion of the fatty acid chain, they controls the permeability to smallers molecules. The milk weeds produces distict good tasting glucosides or sterols that protects them against herb ivory (Lewis and Elvin-Lewis, 1977).

2.2.1.5 Polyterpene (C5)n

Certain higher molecular weight polyterpene appear in the plant. Largest terpene include polyterpene and tetraterpene. The main tetra terpenes belongs to carotenoid family of pigment. Example of polyterpene is rubber. Rubber is a polymer. It contains 1500-15000 isopentenyl unit (Eisner *et al*., 1995).

2.2.2 Phenolic compound

Plant produces huge diversity of the secondary product or metabolite, accommodate phenol group. Phenol group is a hydroxyl functional group on aromatic ring. Phenol group is chemical heterogeneous group. Phenol is vital component of plant defense system against disorder like root parasitic nematodes and pest (Saviranta *et al*., 2010).

2.2.2.1 Coumarin

Coumarin is simple phenolic compound. Their function is to act in different plant defense mechanism against fungi, insects and herbivores. Coumarin is derived from shikimic acid pathway. Common in fungi, plants and bacteria, but not present in the animal. Coumarin

is higher active class of molecule with the broad range of antimicrobial activity against bacteria and fungi (Brooker *et al*., 2008).

2.2.2.2 Furano-coumarin

It is a category of coumarin with appropriate interest of phytostoxicity. They are not toxic. Untill, they stimulated through UltraViolet-A light. They causes few furano- coumarin to become stimulated to highly energy electronic state. They are inserted into the double helix of the Deoxyribonucleic acid. And binds to the pyramidine base. Hence, blocks transcription and finally leading to the cell death (Rice, 1987).

2.2.2.3 Lignin

Lignin is highly branched polymer of phenyl propanoid group. It is formed by 3 distinct alcohol that is coumaryl, sinapyl and coniferyl. That are oxidized to free radical through an ubiquitous plant enzymes for example peroxidise enzyme. This enzyme acts together and random to form lignin (Lewis and Yamamoto, 1990). Lignification blocks growth of the pathogen. They gave frequent response to wounding or infection (Gould, 1983).

2.2.2.4 Flavonoid

Flavonoid is one of the biggest class of plant phenolic compound. They performs very distinct function in the plant system includes defense and pigmentation (Kondo *et al*., 1992). 2 another big group of flavonoid occur in flower. These group are flavonols and flavones. Their action is to save cell from the UltraViolet-B radiations (Lake *et al*., 2009). Exposure of plant to enhanced UltaViolet-B light is determined to enhance the synthesis of the flavonols and flavones suggesting that flavonoid may be attempt measure of the protection through screening out dangerous UltaViolet-B radiations (Saviranta *et al*., 2010 ; Caldwell *et al*., 1983).

2.2.3 Sulphur containing secondary metabolite

Sulphur containing secondary metabolite includes GSL, phytoalexins, GSH, allinin and thionin that is combined indirectly or direct with defense of the plants against microbe pathogen (Crawford *et al*., 2000; Hell, 1997) .

2.2.3.1 Glutathione (GSH)

In the soluble fraction of the plant, Glutathione is one of the biggest class of the organic sulphur. In the regulation of plant growth and in the development, it is playing vital role as mobile pool of shortened sulphur (Kang *et al*., 2007).

2.2.3.2 Glucosinolates (GSL)

GSL is a class of light molecular mass nitrogen and sulphur contains plant glucoside which is formed through higher plants to enhance their resistance against unfavourable effects of competitor, parasite and predators due to their break down product is discharge as volatile defensive substance exhibiting toxic or repellent effects for example, mustard oil allyl cys sulfoxides in allium and glucosides in cruciferae (Leustek, 2002).

2.2.4 Nitrogen containing secondary metabolite

Nitrogen containing secondary metabolite are cyanogenic glucoside, alkaloids and non protein amino acid. Mostly, they are biosynthesize by common amino acid. All are important due to their act in anti herbivore defense and toxicity to human.

2.2.4.1 Alkaloid

Alkaloid is larger family of nitrogen containing secondary metabolite. They are found in 20 percent of species of the vascular plant (Hegnauer *et al*., 1988). Broadly, they includes the pyrrolizidine alkaloids. They are toxic. They appears to serve primarily in defense against microbial infections and herbivoral attacks. These amino acid are tryptophan, tyrosine, aspartic acid and lysine (Pearce *et al*., 1991). Now mostly, alkaloid is believed to act as defensive component against predator (Robinson, 1980).

Morphine was the first alkaloid. Morphine can be found in plant *Papaver sonniferum*, or opium poppy. It is used to treat pain and suppress cough.

Cocaine is another compound. It may be dangerous if taken in large amounts. It can be used as an anesthetic. Its derivatives are dangerous when consumed on regular basis and prove to be deadly.

Caffeine is a known compound. It protect its source plant that is tea, coffee and cocoa. Humans use it to stay alert but their high concentration is very toxic. It protect the plant by inhibiting germination of other plants in that area and in humans, caffeine is thought to minimize diabetes and heart problems.

2.2.4.2 Cyanogenic glucoside

They made group of nitrogen containing defensive compound other than alkaloid, releases poison HCN. Mostly occurs in member of family like Graminae and Rosaceae (Seigler, 1981). Cyanogenic glucoside are not toxic. Although they are break down to give off volatile poisonous substance like H2S and HCN. Presence of these blocks feeding by insect and other herbivore like slug and snails (Taiz and Zeiger, 1995).

2.3 Plant Tissue Culture as a source for the Secondary Metabolites production

Tissue culture and plant cell can establishes generally under the sterile circumstances from the explant like stem, plant leaf, roots etc. for both approaches for the extraction and multiplication of secondary metabolite. Earlier, to treat health disorders in human and infection and illness plant with secondary metabolite is used. In the previous hundred years, natural products replaced by synthetic drug. Mostly, higher plant is main source of useful secondary metabolite, used in medicine industry, aroma and flavour industries (Philipson, 1990). Biotech approach for example plant tissue culture is playing vital function in research for substitutes to the production of desirable medicinal compound from the plant (Ravishankar *et al*., 1988). Medicinal plant is mainly used as crude drug and extract on a global scale. Various compounds including many alkaloid like codeine (antitussive), morphine (act as pain killer), ephedrine (stimulant), papaverine (phosphor- diesterase inhibitor), ajmaline (antirrhythmic), reserpine (antihy- pertensive), quinine (antimalarial), scopolamine (travel sickness), galanthamine (acetylecholine esterase inhi- bitor), caffeine (stimulant), berberine (psoria- sis).

2.4 Production of secondary metabolite from medicinal plant through plant tissue culture

2.4.1 Taxol

Plaxitaxol or Taxol is complex di-terpene alkaloid. Taxol occurs in bark of the Taxus tree. Taxol, most s important anti-cancer agent. It is known for its various way of action at micro tubular cell system (Jordan and Wilson, 1995). In this time, production of the taxol through distinct Taxus species cell in the culture is one of the most broadly research sectors of plant cell culture in previous years because of their excessive industrial value of taxol, lack of the Taxus tree, and expensive synthetic process (Suffness, 1995). In 1989, first time the production of placlitaxel (taxol) through Taxus cell cultures reported.

2.4.2 Morphine and Codeine

Papaver somniferum latex is important source of the morphine and codeine. Production of codeine and morphine in morphologically undifferentiated culture is reported (Yoshikawa and Furuya, 1985; Tam *et al*., 1980).

2.4.3 Ginsenoside

The roots of Panax ginseng is called ginseng. It has been generally using as high prized medicine (Tang and Eisenbrand, 1992). Ginseng is re-cognized as promoter of longevity and health. The essential photochemical of ginseng was identified as ginsenoside that is class of triterpenoid saponins..

2.4.4 L-DOPA

L-3,4-dihydroxyphenylalanine (L-DOPA) is the vital medium of the secondary metabolite metabolism in the higher plant. It is called as precursor of the betalain, melanine and alkaloid isolated from the Vinca faba, Baptisia and Lupinus. Also, it is a messenger of catecholamine in animal. For Parkinson's disease this is used as potent drug. (Guggenheim, 1913),

2.4.5 Berberine

It is isoquinoline alkaloid. It is occur in cortex of *Phellondendron amurense* and roots of *Coptis japonica*. These are antibacterial alkaloid. These are identified from number of cell culture for example *Coptis japonica* (Sato and Yamada, 1984), *Berberis* species and *Thalictrum* species. The productivity of the berberine was enhanced in the cell culture through enhancing the levels of phytohormones and nutrient in growth medium (Sato and Yamada, 1984).

2.5 Elicitor to increase the production of Secondary Metabolite

Compound that stimulates each type of physiological abnormality of the plant is called Elicitors (Radman *et al*., 2003). This explanation of elicitor includes both the compound that are discharged from the plant through the action of the pathogen, these are called endogenous elicitor. And substance of pathogen origin is called exogenous elicitor. Elicitor can be used to raise secondary metabolites synthesis in the plant. They can play vital act in biosynthetic pathway to increase the production of the commercial vital compound. The secondary metabolite of plant are discharged due to defense response that activated and triggered through elicitor, the signal compound of defense response in the plant(Radman *et al*., 2003)..

2.5.1 Classification of Elicitors

Elicitor is divided into 2 class that is physical and chemical according to Radman *et al*., (2003). Elicitor is divided into 2 class on the basis of their nature : Abiotic and biotic elicitor. The elicitors that have biological origin are called biotic. They are derive through the plant itself or from the pathogen. And abiotic elicitor are not have biological origin. They are classified into chemical compounds and physical factor. The very first biotic elicitor was invented in 1968. It may be classified into general elicitor and race specific on the basis of plant elicitors interaction (Staskawicz *et al*., 1991). There are distinct plant hormones that are acting as elicitor. The most familiar plant hormone such as JA and SA. Both are crucial indicator for the defense gene expression. In this SA is regulating resistance to pathogen such as fungal, viral and bacterial. While JA regulate the protein production through the

octadecanoid pathway. JA and SA both are synthetic mimics. They can be applied from the outside to activate same metabolic change which regulates resistance against pathogen. The biochemicals pathway of JA and SA both are helpful in process of elicitation in the plant (Angelova *et al*., 2006).

Table 2.1shows the classification of Elicitor.

ELICITORS	
BIOTIC ELICITOR	ABIOTIC ELICITOR
1. POLYSACCHARIDE (CHITOSAN, PECTIN,CHITIN)	1. PHYSICAL (UV RADIATION, DROUGHT, THERMAL STRESS)
2. YEAST EXTRACT	2. CHEMICAL (HEAVY METALS)
3. FUNGUS EXTRACT (*Aspergillus niger*)	3. HARMONAL (SA,JA)

Table 2.1 Shows the classification of Elicitor

2.5.1.1 Abiotic Elicitor

. Abiotic elicitor are not having a biological origin. They are classified in chemical compounds and physical factor.

For example : Metal ions (silver,europium, lanthanum, Cadmium, calcium), oxalate etc.

2.5.1.2 Biotic Elicitor

Biotic elicitors, they are having biological origin. They derive through the pathogen or by the plant itself. The first biotic elicitor was invented in 1968.

For example :Carbohydrates, fungus extract, yeast extract

- Polysaccharide : Alginate, Pectin, Chitosan.
- Oligosaccharide: Guluronate, Mannan, Galacturonides Mannuronate.
- Peptides: Glutathione.

2.5.2 Plant hormone as Elicitor

There are distinct plant hormones that are acting as elicitor. The most familiar plant hormone such as JA and SA. Both are crucial indicator for the defense gene expression. In this SA is regulating resistance to pathogen such as fungal, viral and bacterial. While JA regulate the protein production through the octadecanoid pathway. JA and SA both are synthetic mimics. They can be applied from the outside to activate same metabolic change which regulates resistance against pathogen. The biochemicals pathway of JA and SA both are helpful in process of elicitation in the plant (Angelova *et al*., 2006).

2.5.3 Elicitors from Carbohydrates

There are various carbohydrates which are advantageous in the over production of secondary metabolite in the plant tissue culture. In cell culture of the tobacco, carbohydrate elicitor is activating the signal transfer with the help of H2O2 production and calcium influx (Negeral *et al*., 1995).

2.5.4 Use of Elicitors

(1) ecological safety, because this process is explained by the inauguration of endemic immune potential of gene.

(2) elicitor protective effect is prolonged.

(3) presence of many defense system in induced resistance.

(4) inauguration of non specific resistance to many of bacteria, viruses etc.

2.5.5 Distinct Features of Elicitors

2.5.5.1 Elicitor concentration

Concentration of elicitor is playing a vital function in the elicitation process of plant. Namdeo *et al*., Explained more aggregation of ajmalicine in *C. roseus* culture when it is treat with various concentration of elicitor extracts of *Aspergillus niger, F. Moniliforme* and *T. virid,*. Ajmalicine aggregation is more in the cell elicited by more concentration (five percent) of the elicitor extract as comparing to less concentration (0.5 percent). So, enhancing the concentration further upto 10 percent negatively damaged the aggregation of ajmalicine (Roewer *et al*., 1992).

2.5.5.2 Period of elicitor exposure

Cells of *C. roseus* exposed with elicitor extract of the *Aspergillus niger, T. viride* and *F. moniliforme* for 96hour, 72hour,48hour and 24hour. Approximately three-fold enhance in the ajmalicine production through *C. roseus* cell elicited with the extract of *T. viride* for exposure time 48 h, however 2 fold enhancement observed in the cell which is elicited with the *F. moniliforme* and *A. niger* (Namdeo *et al*., 2002). Further enhancing exposure time results in reduction in ajmalicine content.

2.5.5.3 Age of culture

This is vital component in the bioactive compound production through elicitation. In previous study, through elicitation cells of *C. roseus* of twenty day old culture was showing more yield of the ajmalicine (Namdeo *et al*., 2002).

2.5.5.4 Nutrient composition

For elicitation process in plant, medium selection or composition of medium is also playing an important function. Accumulation in case of ajmalicine has been observed large in

the Zenk's medium. In Murashige and Skoog medium the accumulation is less. So we can say that elicitation process is depend upon media selection. The elicitation process efficiency is depend upon presence of growth regulator in the media, nutrient composition of the medium, environmental condition,s elicitor specificity etc.

Out of these, method of elicitation is high economical beneficial in the plant tissue culture (Manorma *et al*., 2011). Biochemistry and phytochemistry of elicitor are very helpful to increase the potential of plant cell for the production of the secondary metabolite of plant like flavonoids volatile oils, alkaloids, resins tannins etc.(Namdeo *et al*., 2004).

2.6 Mechanism of Elicitation in plants

Every cell has obtained the ability to react to the pathogens, to develop a defense reaction and natural worries in plant guard frameworks. Plant reaction is resolved through different variables. It is fundamentally relies on their physiological state and hereditary attributes. By and large, plant imperviousness to infections is hereditarily controlled through plant resistance gene (R) and pathogen avirulence gene (Avr) (idea of quality connection) (García *et al*., 2006). Nonetheless, activating resistance is not generally in light of particular avirulence quality (Avr) items that enacts guard reactions in cultivars having the coordinating plant resistance gene (R) in any case, rather, continues from the activity of general elicitor, ready to initiate barriers in various cultivars of one or numerous animal varieties (García *et al*., 2006). The initial phase in the reaction of plant against elicitors is the boost observation through receptors that is confined in the plasma film of plant cell **(Figure 2.2),** such as protein kinases, that is a standout amongst the most essential in pathogen discernment for various parasitic elicitors (Montesano *et al*., 2003), or could be situated inside the phone to start flagging procedures that actuates plant defense system.

The elicitor flag transduction is a key subject of examination. In this sense, different creators portrayed that plants react to elicitors by enacting a variety of guard systems on the surface of plasma layer **(Figure 2.2),** includes acceptance of pathogenesis related protein and chemicals of oxidative anxiety assurance, hypertensive reactions, described by quick cell demise in the prompt region of the purpose of introduction to the pathogen (García *et al*., 2006), the generation of ROS and RNS, the initiation of resistance related gene, changes in the capability of plasma film cell and upgraded particle fluxes (Cl^- and K^+ efflux and Ca^{2+} convergence), fast changes in protein phosphorylation, lipid oxidation and the basic protective hindrances, for example, support and lignification statement motel cell divider, and so forth and the actuation and the all over again

biosynthesis of translation components, which straightforwardly control the declaration of qualities required in auxiliary metabolites generation (Ferrari, 2010) (Smetanska, 2005) (Zhao et al., 2005) (**Figure 2.2).**

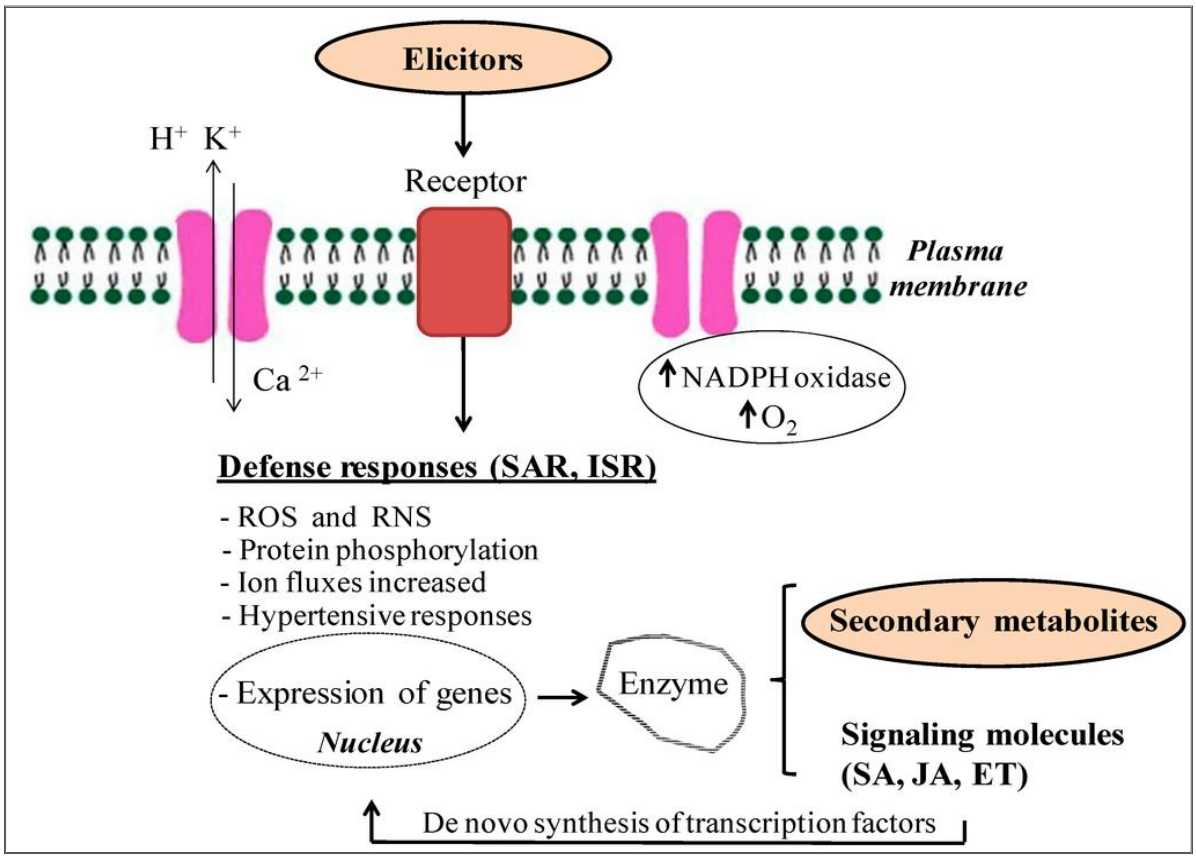


Figure 2.2 Represents Mechanism of elicitation in plants

2.7 Mechanism of chitosan in enhancing secondary metabolite

Chitosan improves phytoalexin optional metabolites in germinating nut (Cuero *et al*., 1991) and furthermore activate the development of phytoalexins in plants and legumes (Cote and Hahn 1994). In this manner, chitosan may included in the singnaling pathway for phenolic compounds biosynthesis and then activate chitinase and chitosanase, these are individuals from a family of plant pathogenesis-related (PR) proteins (van Loon *et al*., 1994). These PR proteins corrupt the cell dividers of some phytopathogens. Thusly, may assume a part in host plant resistance frameworks (Graham and Sticklen, 1994). In addition, chitosan may likewise initiate plant immune systems (Systemic adquired response), which is long lasting and regularly presents expansive based imperviousness to the diverse pathogens. SAR creates in uninfected parts of the plant; therefore, the whole plant is more impervious to an optional contamination, for example, β-1,3 glucanases, antifungal chitinases, PR-5 and PR-1 (Sathiyaba and Balasubramanian, 1998). Further study has shown that chitosan induces the expression of distinct gene required in plant

defense reactions, for example, a quality encoding PAL and protease inhibitors (Vander *et al*., 1998). Despite the fact that chitosan has been proved to elicit defense gene in a few species types, for example, slash pine, rice (Mason and Davis, 1997) and tomato (Ben-Shalom *et al*., 2000). The above genetic reviews supported that chitosan can enhance JA pathways, since transcription activation of genes encoding PAL and protease inhibitor was induced through both chitosan and JA (Doares et al. 1995). Crude plant extract of Arabidopsis with chitin under the appropriate conditions created N-acetylglucosamine and secondary metabolites (Mitchell-Olds and Pedersen 1998).

CHAPTER-3

OBJECTIVES

Secondary metabolite is organic molecule. It has no role in the normal growth. Also no role in the development of organisms. But primary metabolite has a vital function in growth of the species. They plays an effective role in respiration and also in photosynthesis in the plants. When secondary metabolite is absent, then the plant is not turn to actual death. However, the result is long period destruction of survivability of the organisms, generally they are playing vital role in defense of the plant. The objectives are :

1. Detection of various bioactive compounds present in *O. tenuiflorum* through phytochemical screening.
2. Quantitative analysis of various bioactive compounds that are present in *Ocimum tenuiflorum*.
3. Enhancement in the quantity of bioactive compounds in *Ocimum tenuiflorum* in plant tissue culture by the addition of biotic elicitor.

CHAPTER-4

MATERIALS AND METHODS

4.1 Sample collection

Fresh sample of *Ocimum tenuiflorum* belonging to the family Lamiaceae were collected from Herbal garden of Lovely Professional University, Phagwara (Punjab).

4.2 Extraction of sample

Plant material (leaves) was sun dried for 2 weeks and put into methanol for overnight. After the drying, convert it into powered form by Mortar and Pestle as shown in figure 4.1. This powder of leaf was used for the various phytochemical tests.

Figure 4.1 Represent mortar and pestle

4.3 Qualitative test

4.3.1 Phytochemical screening

Active constituents in the plant extract of *O. tenuiflorum* were identified and detected by doing chemical tests. Phytochemicals like phobatannins, tannins, saponin, terpenoids such as flavonoids and alkaloids were detected based on standard tests.

4.3.1.1 Tannins (Edeoga, 2005)

The procedure for Tannins test was taken from Edeoga,2005. Figure 4.2 shows the complete procedure for tannins test.

Chemicals used : 0.1percent ferric chloride.

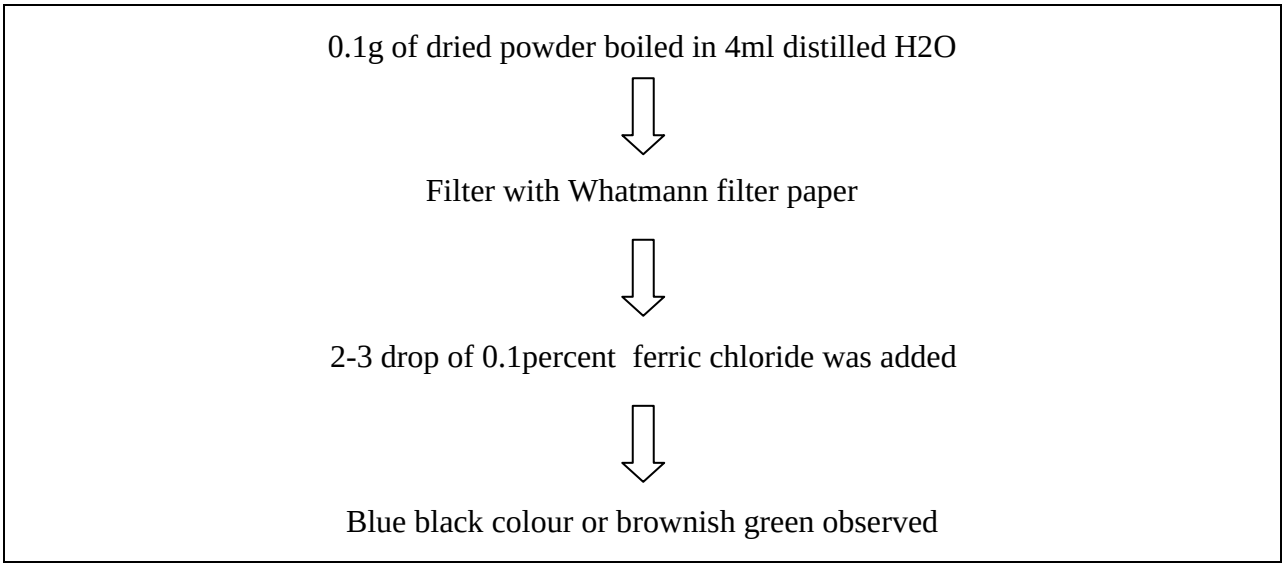


Figure 4.2 Flowchart representation for tannins test

4.3.1.2 Phlobatannins (Edeoga, 2005)

The procedure for this test was taken from Edeoga, 2005. Figure 4.3 shows complete procedure for this test which was performed in the laboratory.

Chemicals used : 1 percent aqueous HCL.

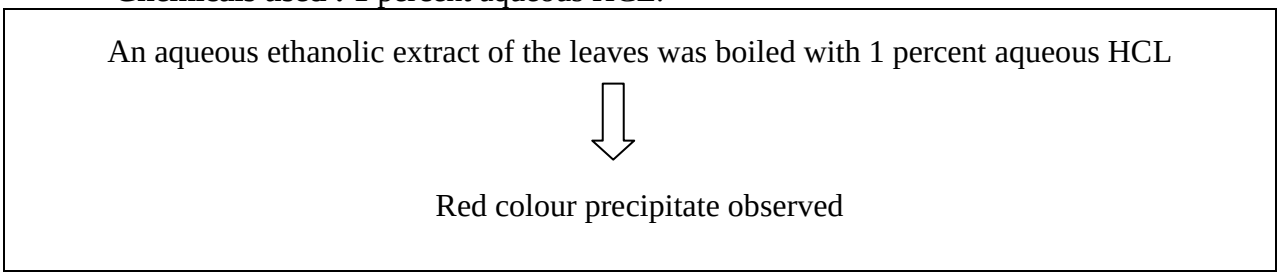


Figure 4.3 Flowchart representation for phlobatannins test

4.3.1.3 Saponins (Edeoga, 2005)

The procedure for saponins test was taken from Edeoga, 2005. In this test saturated oil (ghee) is used. There is a formation of emulsion occurs. The flowchart of the procedure was show in figure 4.4.

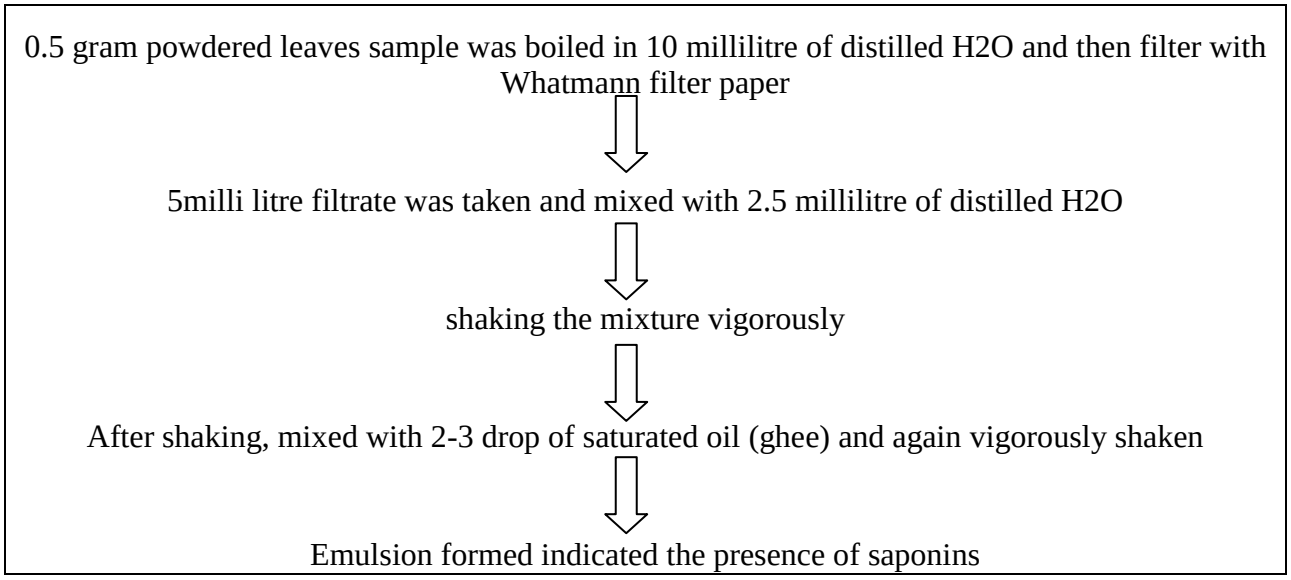


Figure 4.4 Flowchart representation for saponins test

4.3.1.4 Flavonoids (Harborne, 1973 ; Sofowara, 1993)

The procedure for this test was taken from Harborne, 1973 and Sofowara, 1993. Figure 4.5 shows the flowchart for the procedure of flavonoids test.

Chemicals used :

1. 1 millilitre conc. H_2SO_4,
2. 3 millilitre of diluted ammonia.

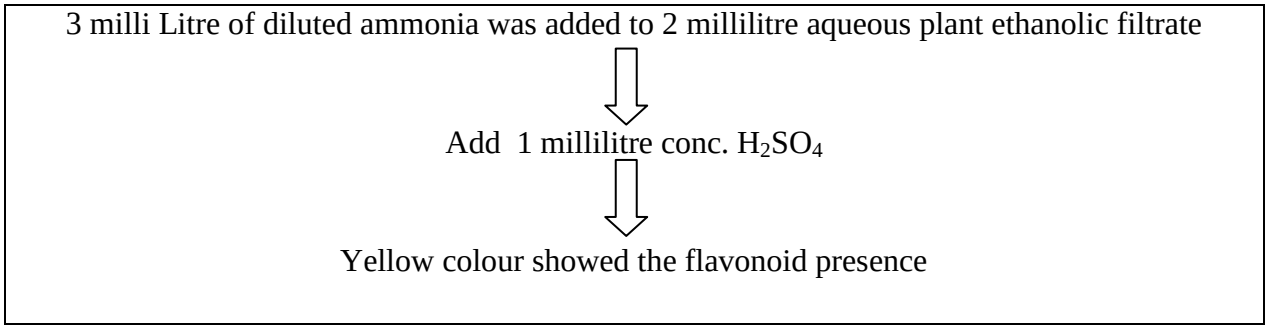


Figure 4.5 Flowchart representation for flavonoids test

4.3.1.5 Terpenoids (Edeoga, 2005)

The procedure for this test was taken from Edeoga, 2005. Figure 4.6 shows the complete flowchart for the procedure of terpenoids test in *Ocimum tenuiflorum*.

Chemicals used :

1. 1 millilitre of chloroform,
2. 1 millilitre of conc. H_2SO_4.

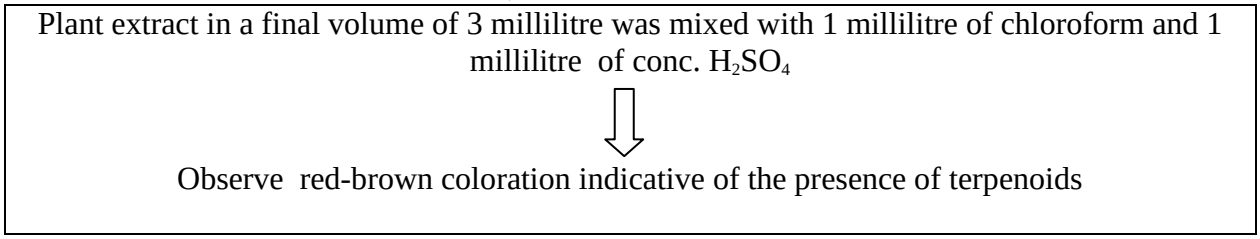


Figure 4.6 Flowchart representation for terpenoids test

4.3.1.6 Reducing sugars (Ayoola, 2008)

The procedure for reducind sugar test was taken from Ayoola, 2008. Figure 4.7 shows procedure for reducing test.

Chemicals used :

1. 1 millilitre ethanol,
2. One millilitre of Fehling's solution A ands Fehling's solution B.

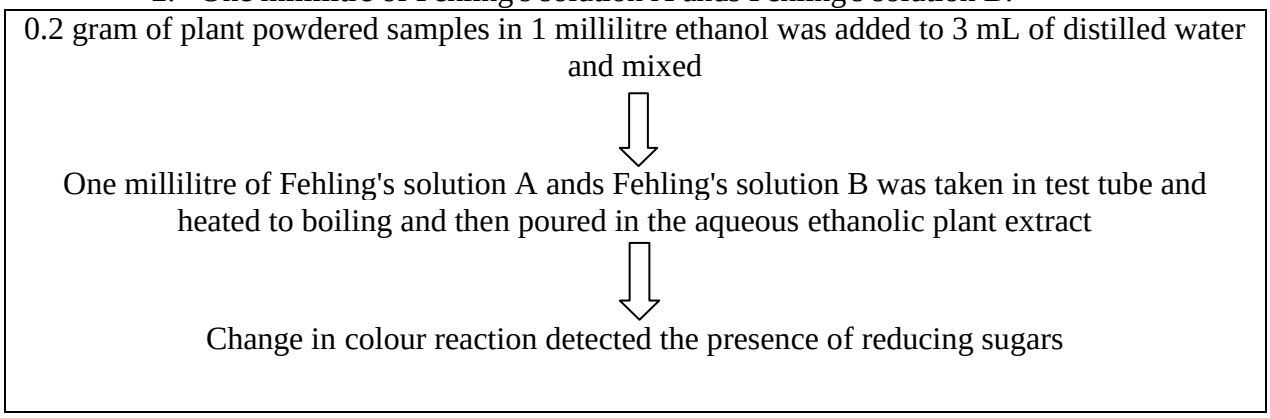


Figure 4.7 Flowchart representation for reducing sugar test

4.3.1.7 Alkaloids (Harborne, 1973 ; Sofowara, 1993)

The procedure for alkaloids test was taken from Harborne, 1973 and Sofowara, 1993. Figure 4.8 shows procedure for alkaloids test.

Chemicals used :

1. 2 mL of hexane,
2. 3 mL 2% HCL, picric acid.

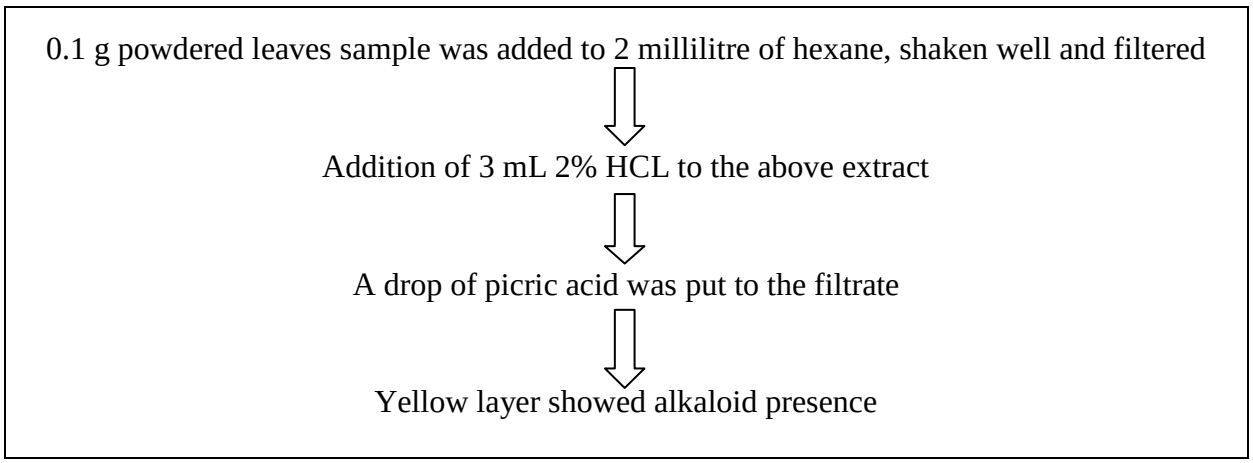


Figure 4.8 Flowchart representation for alkaloids test

4.3.1.8 Anthraquinones (Ayoola, 2008)

The procedure was taken from Ayoola, 2005. Figure 4.9 shows flowchart for the test of anthraquinones.

Chemicals used :

1. 4 mL concentrated H_2S0_4
2. 3 millilitres of chloroform
3. 1 millilitre of diluted ammonia.

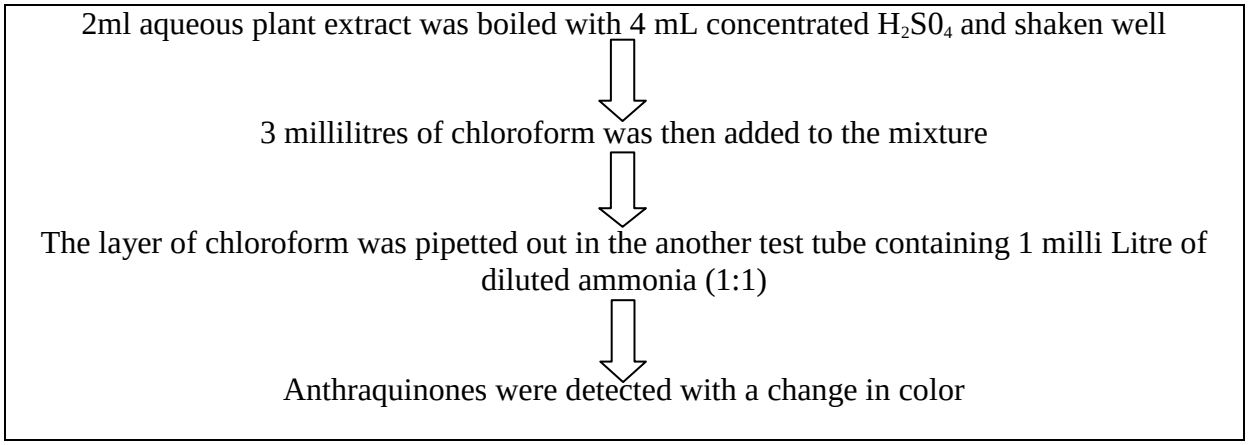


Figure 4.9 Flowchart representation for anthraquinones test

4.4 Quantitative test

4.4.1 Quantitation of total phenolic and flavonoid content

Plant material (500 gram) was sun dried and place it overnight in 1 Litre of methanol. The extract was then filter through Whatman filter paper. This methanolic plant extract is used for further quantification tests.

4.4.1.1 Total phenolics content (Mallick and Singh, 1980)

Total content of phenolics was determined by Folin-Ciocalteu (FC) reagent. Figure 4.10 shows complete procedure for the quantitative test of phenolic content. Gallic acid was used as standard for this test.

Chemicals used :

1. 0.5 milli litre of FC reagent
2. 2 mL of 20% Na_2CO_3.

Equipment used : Spectrophotometer

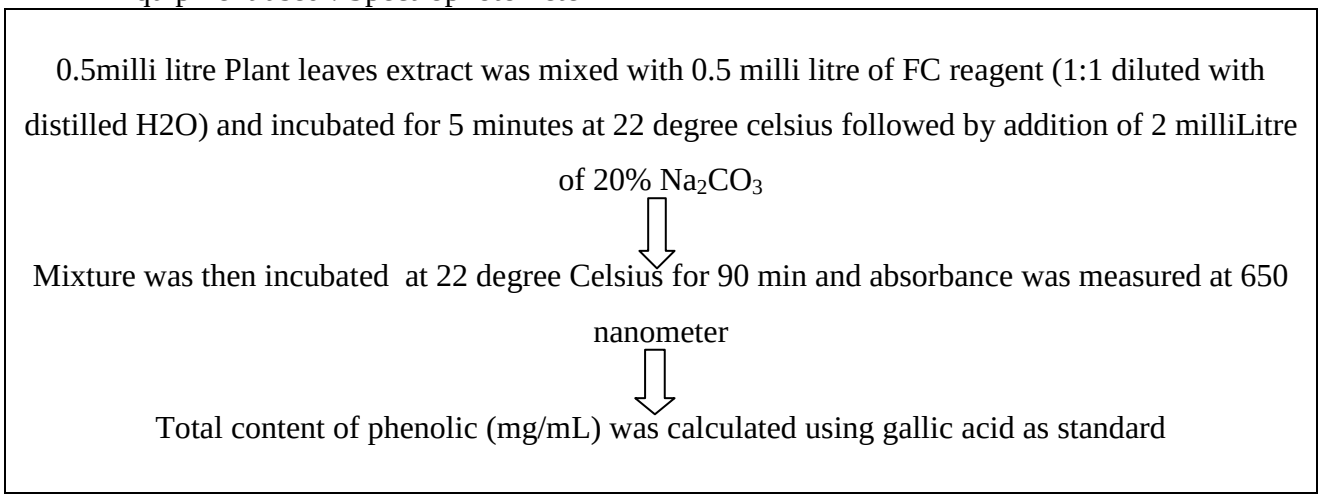


Figure 4.10 Flowchart representation for total phenolics content

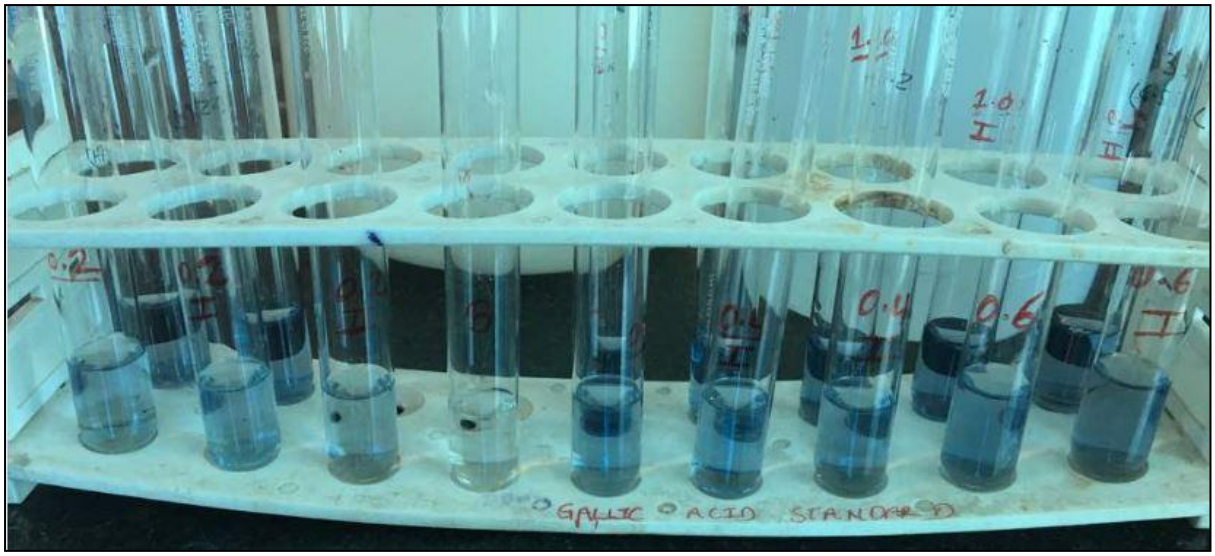


Figure 4.11 Represent Gallic acid standard for Total Phenolic content for *Ocimum tenuiflorum*

4.4.1.2 Total flavonoids content (Kariyone, 1953; Naghski, 1951)

The total content of flavonoid (mg/mL) was determined by the help of aluminum chloride ($AlCl_3$) method. Figure 4.11 shows the procedure for quantitative test for flavonoids content. Quercetin was used as a standard for this test.

Chemicals used :

1. 0.3 millilitre of 5 percent $NaNO_2$
2. 0.3 millilitre of 10% $AlCl_3$
3. 2 millilitre of 1 M sodium hydroxide (NaOH).

Equipment used : Spectrophotometer

Mixture consisting of 0.5 mL of the plant extract, 0.5 millilitre distilled H2O, and 0.3 milli Litre of 5 percent $NaNO_2$ was incubated for 5 min at 25°Celsius

⇩

add 0.3 millilitre of 10% $AlCl_3$ immediately

⇩

2 millilitre of 1 M sodium hydroxide (NaOH) was then added to the mixture

⇩

Absorbance was measured at 510 nano meter

⇩

Quercetin was used as a standard

Figure 4.12 Flowchart representation for total flavonoids content

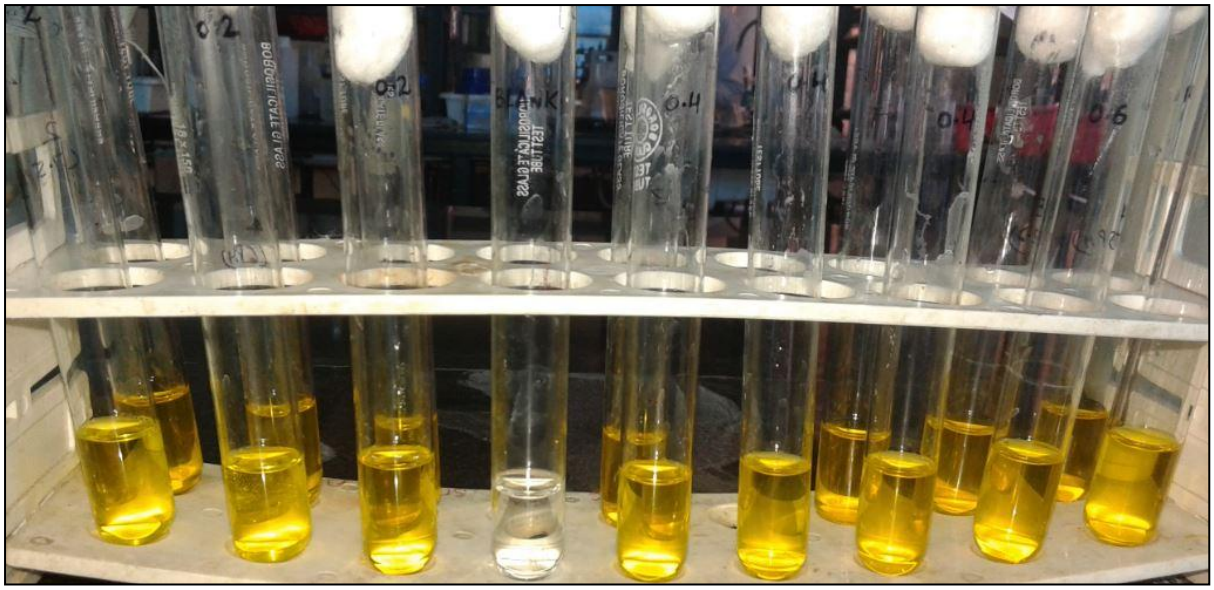


Figure 4.13 Represent Quercetin standard for Total Flavonoid test for *Ocimum tenuiflorum*

4.5 Plant tissue culture

4.5.1 Media Preparation

Murashige and Skoog (MS) is most commonly used media for plant tissue culture. The most vital character of the Murashige and Skoog medium that made it common in plant tissue culture is its potassium high concentration, ammonia and nitrate (Murashige and Skoog, 1962). Figure 4.14 shows complete procedure for MS media preparation.

4.5.1.1 Media Preparation for Control (Murashige and Skoog, 1962)

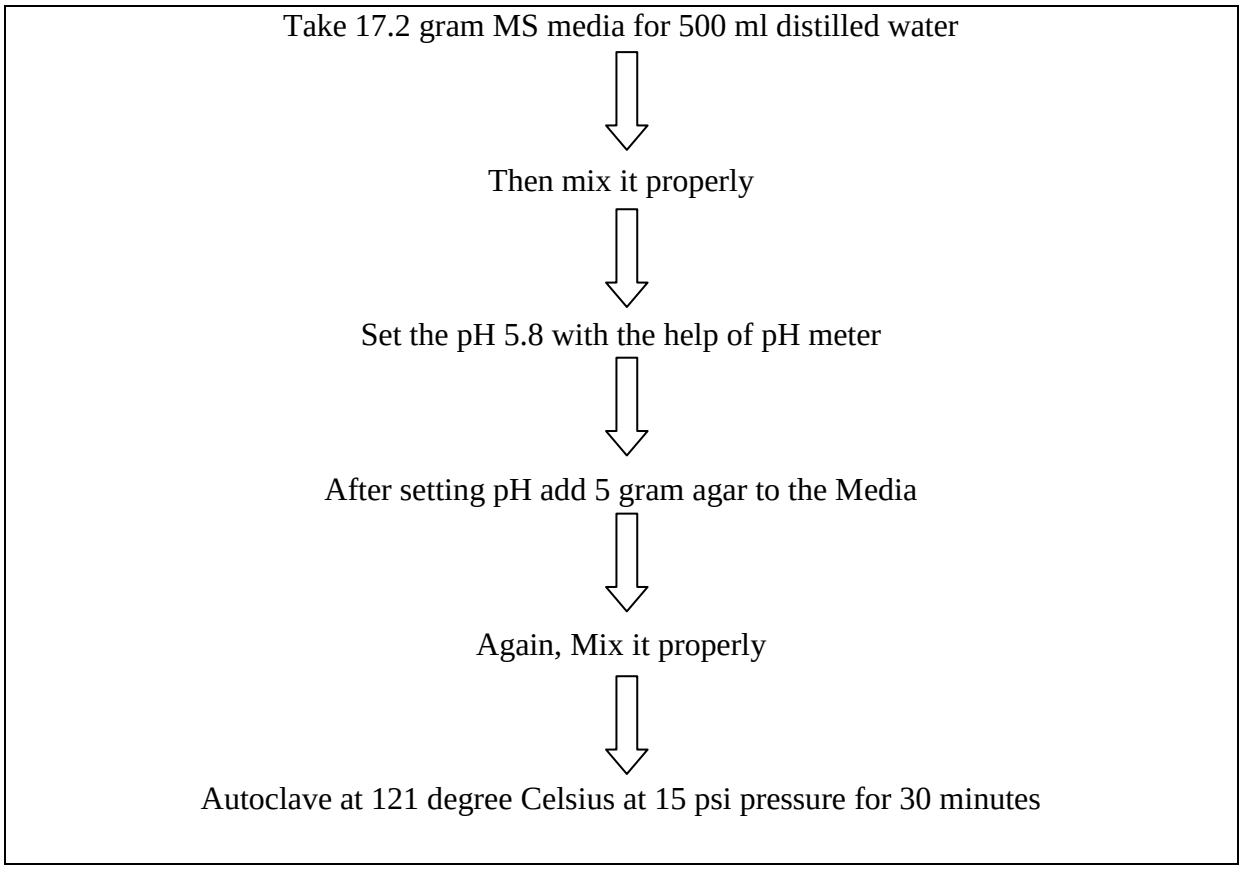


Figure 4.14 Flowchart representation for MS media preparation

4.5.1.2 Elicitation solution Preparation of Polysaccharide Chitosan (Sonja *et al*., 2014)

Chitosan solution was made of five different concentration (0, 50, 100, 150, 200 and 300) mg per litre (Lingyun *et al*., 2016, Asghari *et al*., 2009). The chitosan use are limited due to its high viscosity, insolubility in water and at high pH it has tendency to coagulate with proteins. So, glacial acetic acid is used to dissolve chitosan with continuous stirring at 60 degree Celsius (Sonja *et al*., 2014).

Figure 4.15 shows complete procedure for the preparation of 50 mg per litre chitosan solution. Same procedure was followed to make other treatment solutions. Chitosan is a polysaccharide, it will degrade at high temprature that is why it is sterilised with the help of membrane filter. Figure 4.16 shows the filter sterilisation of the chitosan solution. The chitosan treatment solution and MS media prepared separately.

5mg Chitosan was dissolve in 1 percent Glacial acetic acid by Continous Stirring on Heating Mantle

↓

After complete mixing, add distilled water to make volume 50ml

↓

pH 5.8 with 1M Sodium Hydroxide

↓

Filter Sterilised with the help of membrane filter (0.22 micron)

↓

Then, 50ml Chitosan elicitation solution is added to the 50ml MS media

Figure 4.15 Flowchart representation for Chitosan elicitation solution preparation

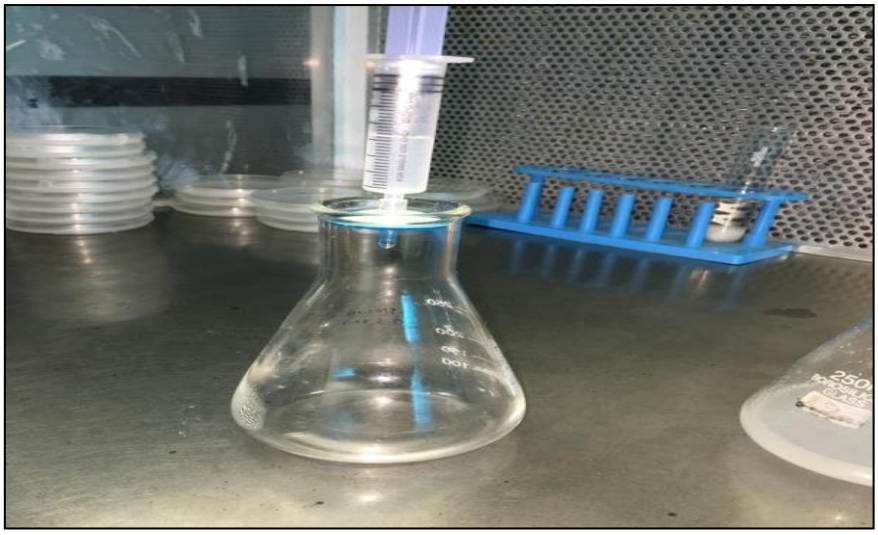


Figure 4.16 Represent filter sterilisation of chitosan solution

4.5.2 Pouring

Pouring of media is done inside the laminar air flow. The control contains only MS media and another petri plates contains chitosan media with MS media in which different concentration of chitosan were used. The range of chitosan is (0, 50, 100, 150, 200, 300)mg per litre (Lingyun *et al*., 2016). Figure 4.17 shows the pouring of all chitosan treatment solution with MS media.

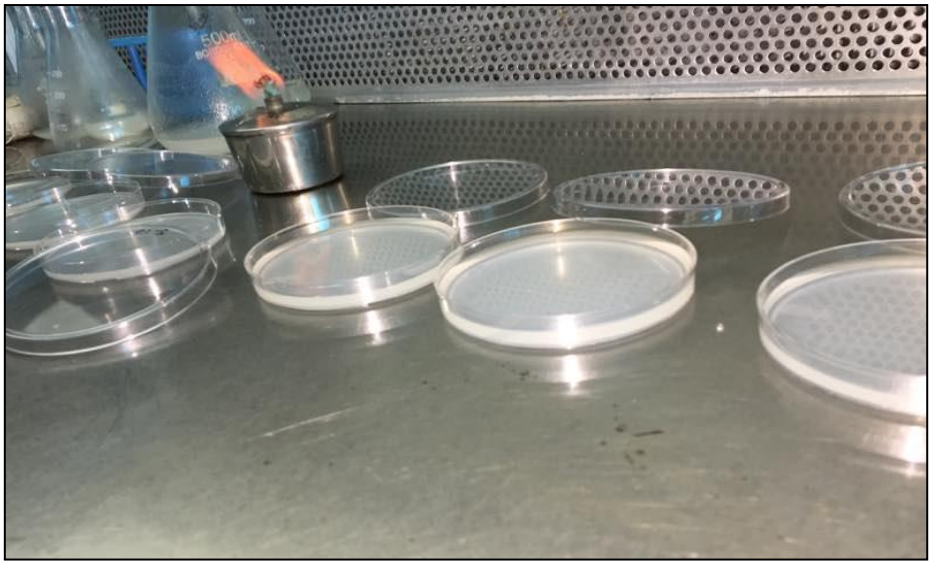


Figure 4.17 Represent media pouring

4.5.3 Seed collection

Seeds of *Ocimum tenuiflorum* species were collected from Ebay.com. *O. tenuiflorum* seeds are used in this research because seeds are easily grown in MS media than explants and takes less time and there is less chances of contamination.

Figure 4.18 Represent seeds of *Ocimum tenuiflorum*

4.5.4 Surface sterilisation of seeds (Sauer and Burroughs, 1986)

The surface sterilisation of seeds is done inside the laminar air flow under aseptic conditions. Surface sterilisation is a technique to remove the contamination of the seeds. It prevents bacterial, fungal and viral contamination. Figure 4.19 shows complete procedure of surface sterilisation of *O. tenuiflorum* seeds this procedure is followed in this research. Figure 4.20 shows surface sterilisation of seeds with Tween 20, 70% ethanol and sodium hypochloride.

Chemicals used :

- Tween 20
- 70% ethanol
- Sodium Hypochlorite (disinfectant)

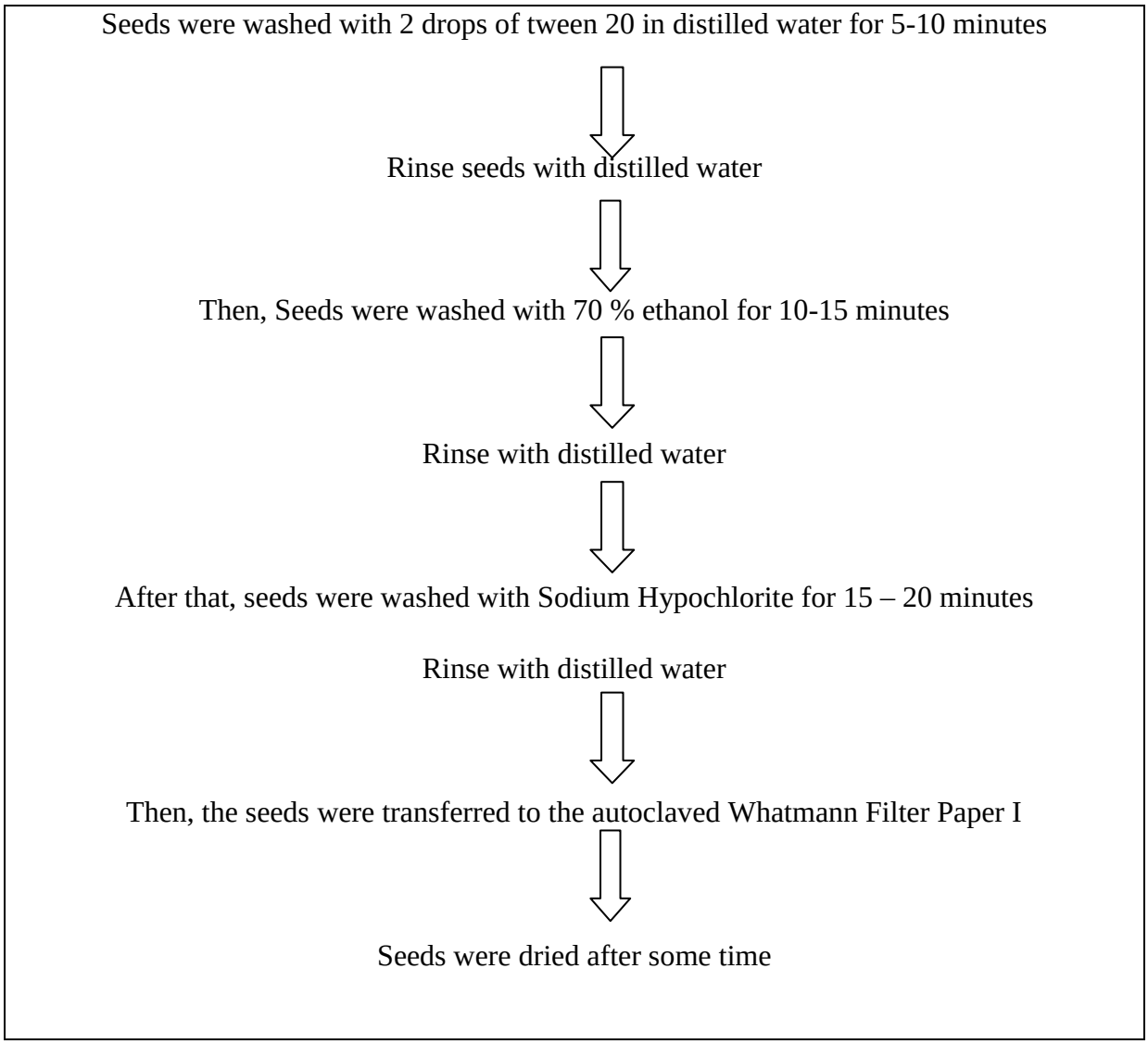


Figure 4.19 Flowchart representation for surface sterilisation of seeds

Figure 4.20 Represent surface sterilisation of seeds

4.5.5 Inoculation

Seeds were inoculated in the laminar air flow. Figure 4.21 shows inoculation of *O. tenuiflorum* seeds in media containing petri plates with chitosan treatment. Disposable plastic petri plates were used in this research because in these petri plates there is less chances of contamination.

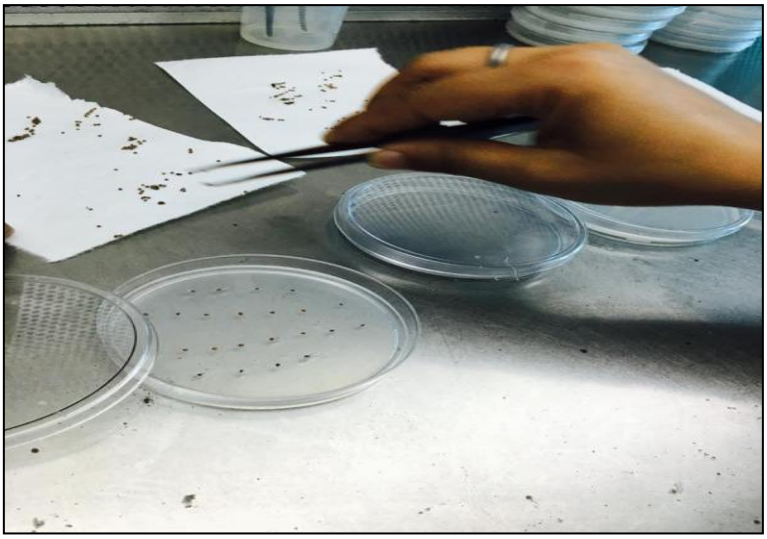


Figure 4.21 Represent inoculation of seeds

4.5.6 Incubation

The inoculated petri plates were incubated for three days in dark room. And after, three days petri plates were transferred to the culture room. Figure 4.22, shows incubation of inoculated petri plates in dark. After 3 days, inoculated petri plates were transferred to the culture room. Figure 4.23, shows the incubation of these plates in culture room where temperature is maintained at 25-27 degree Celsius.

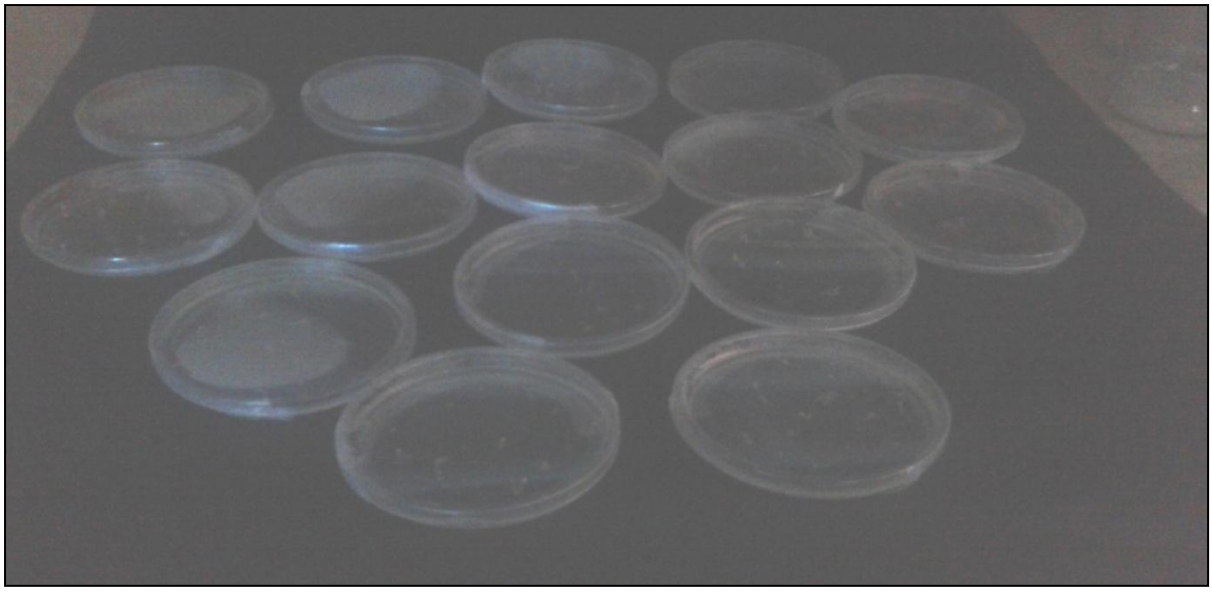


Figure 4.22 Represent incubation in dark room

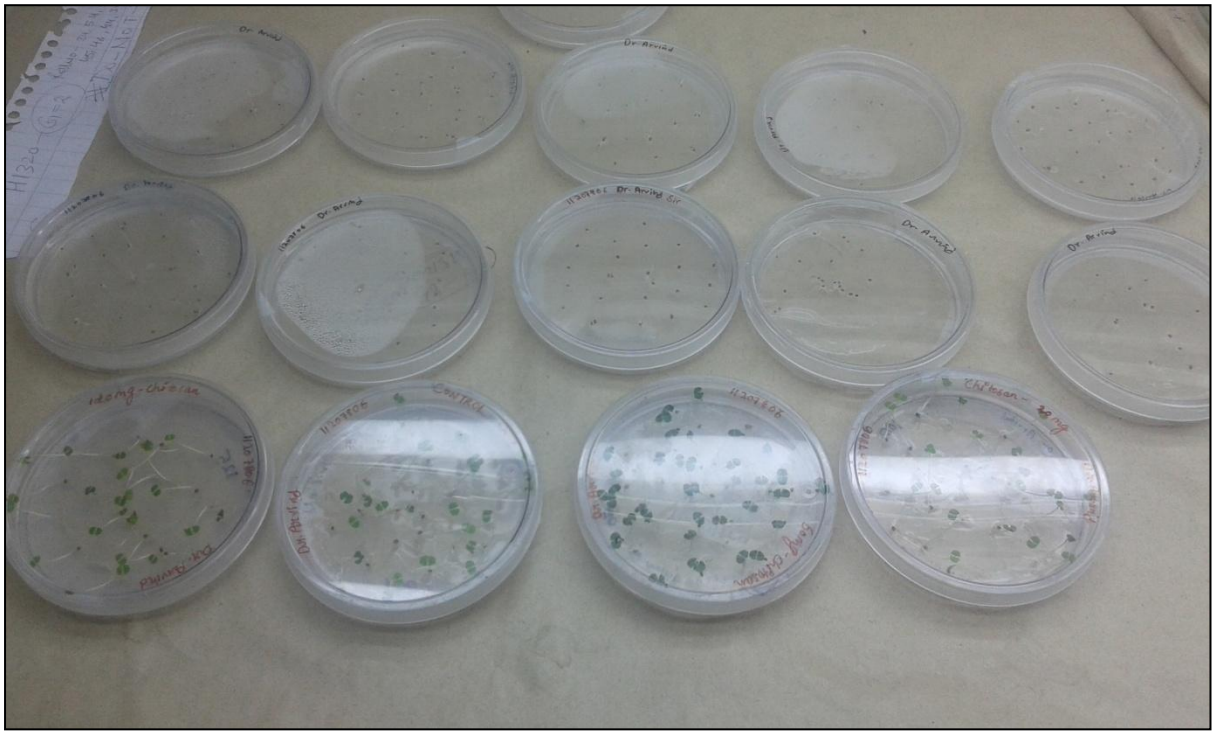


Figure 4.23 Represent incubation of petri plates in light

4.5.8 Tissue collection

Tissue collection is done inside the laminar air flow. And stored in the refrigerator at -20 degree Celsius for further phytochemical screening. These tissue were used for the quantitative test.

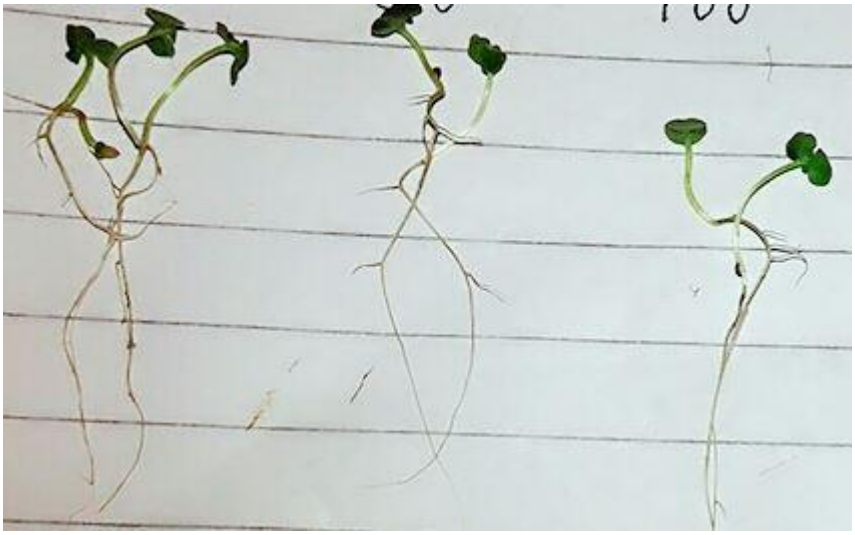


Figure 4.24 Represent tissue collection from inoculated petri plates

CHAPTER-5

RESULTS AND DISCUSSION

5.1 Phytochemical tests results

Results with different phytochemical tests are shown in **Table 5.1**. The results showed that Ocimum are rich in tannin, saponins, flavonoids, alkaloids, anthraquinone, and reducing sugars. Terpenoids were absent in Ocimum plant extract. Phobatannins test showed negative results for Ocimum plant extract.

Sr. No.	**Phytochemical test**	***Ocimum tenuiflorum* (leaf)**
1.	Tannin	Positive
2.	Phlobatannin	Negative
3.	Saponin	Positive
4.	Flavonoid	Positive
5.	Terpenoid	Negative
6.	Reducing sugar	Positive
7.	Alkaloid	Positive
8.	Anthraquinone	Positive

Table 5.1 Results of phytochemical test.

5.1.1 Test for tannins

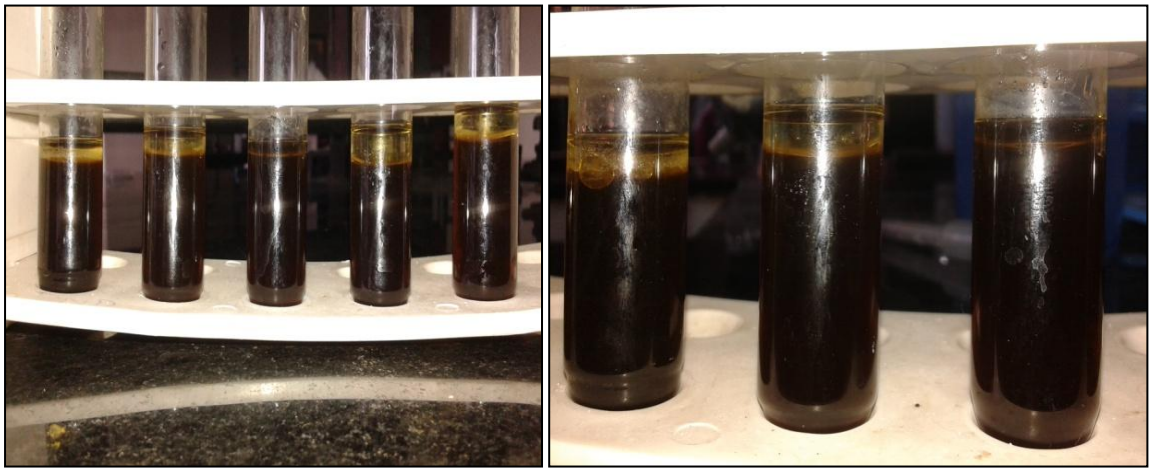
In tannins test, after the addition of ferric chloride change in colour was observed, and the colour was blue-black or brownish green colour which showed tannin presence in plant extract sample. Edeoga, 2005 reported same result for tannins test in *Ocimum tenuiflorum*.

(a) **(b)**

Figure 5.1 (a) (b) Represents positive tannins test for *Ocimum tenuiflorum*

5.1.2 Test for saponins

In this test, after the addition of saturated oil, emulsion formed which indicates the presence of saponins. Edeoga, 2005 reported same result for saponinss test in *Ocimum tenuiflorum*.



(a) **(b)**

Figure 5.2 (a) (b) Represents positive saponins test for *Ocimum tenuiflorum*

5.1.3 Test for flavonoids

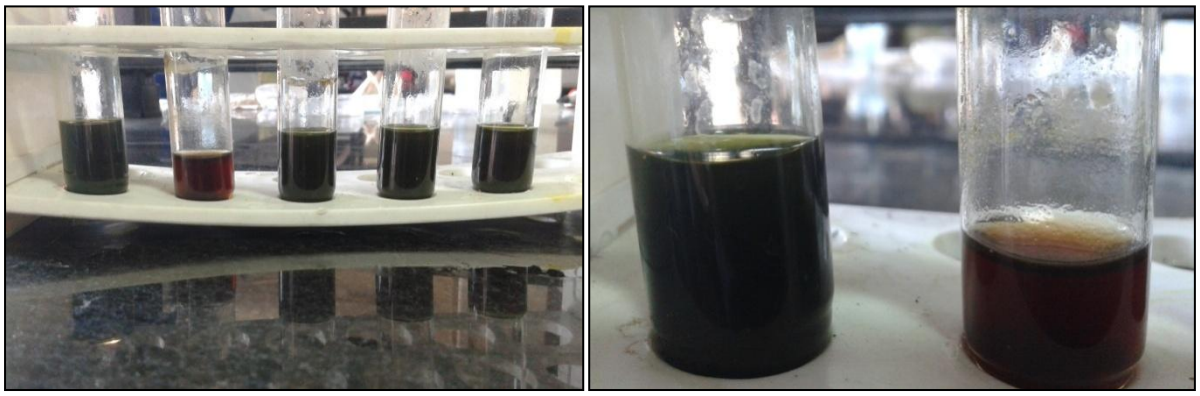
When add 1 milli Litre conc. H_2SO_4. Yellow colour appears which showed the presence of flavonoids in *Ocimum tenuiflorum*. Harborne, 1973 and Sofowara, 1993 reported same result for flavonoids that flavonoid is present in *Ocimum tenuiflorum*.

(a) **(b)**

Figure 5.3 (a) (b) Represents positive flavonoids test for *Ocimum tenuiflorum*

5.1.4 Test for reducing sugar

1ml of Fehling's solution A and B was taken in the test tube and heated to boiling and then poured in the aqueous ethanolic plant extract. Change in colour reaction showed the presence of reducing sugars. Ayoola, 2008 reported same result for reducing sugar that reducing sugar is present in *Ocimum tenuiflorum*.

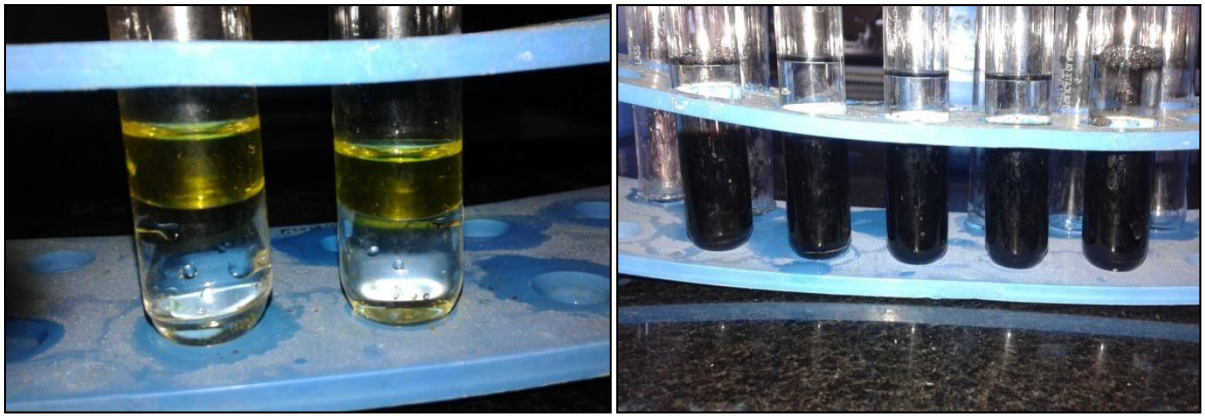


(a) **(b)**

Figure 5.4 (a) (b) Represents positive reducing sugar test for *Ocimum tenuiflorum*

5.1.5 Test for alkaloids

Positive alkaloids test result shown in figure 5.5 (a). Harborne, 1973 ; Sofowara, 1993 reported same result for alkaloids that alkaloids is present in *Ocimum tenuiflorum*. So, alkaloids is present in Tulsi and it is its main component.



(a) **(b)**

Figure 5.5 (a) Represents positive alkaloids test for *Ocimum tenuiflorum* (b) Represents positive anthraquinones test for *Ocimum tenuiflorum*

5.1.6 Test for anthraquinones

Positive anthraquinones test result shown in figure 5.6 (b). Ayoola, 2008 was reported same results for anthraquinones that it is present in *Ocimum tenuiflorum*.

5.2 Result of quantitative test

The formula which is used in the spectrophotometer to find the amount of secondary metabolites in *O. tenuiflorum* is

$$\text{Formula used} = \frac{\text{OD of test}}{\text{OD of standard}} \times \text{concentration of standard} \times \frac{\text{volume made}}{\text{volume taken}} \times \frac{1}{\text{weight}}$$

5.2.1 Total phenolic content

Total phenolic content in a leaf of the *O. tenuiflorum* L. was 0.58 mg/mL.

Mallick and Singh, 1980 was reported same result for total phenolic content in *Ocimum tenuiflorum*. Phenolic content is less in *Ocimum tenuiflorum* as compare to Flavonoid content.

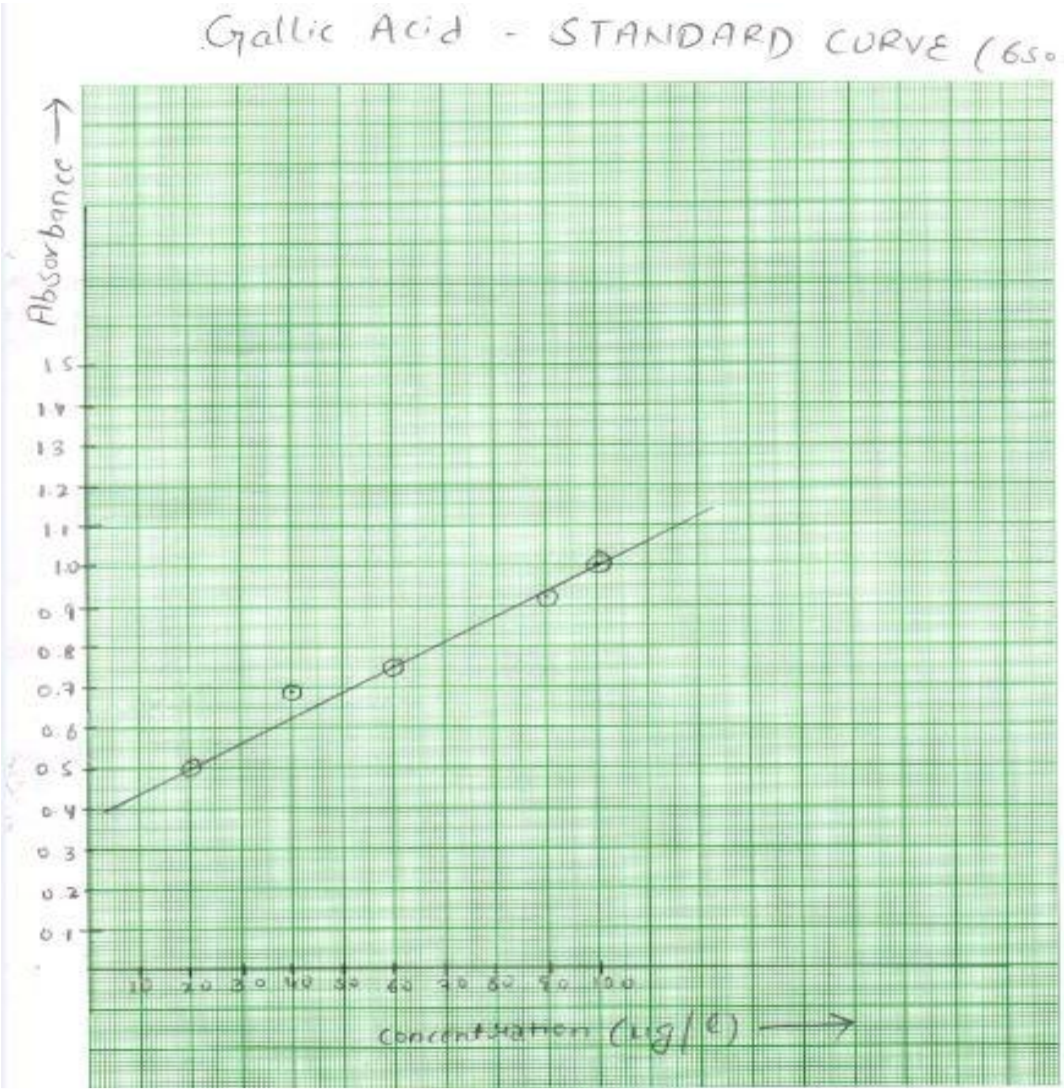


Figure 5.6 Graphical representation for standard Gallic acid curve

5.2.2 Total flavonoid content

Total flavonoid content in Ocimum leaf was 4.30 mg/mL. Results indicated higher total flavonoid content in Ocimum plant extract.

Kariyone, 1953 and Naghski, 1951 was reported same result for flavonoid content in *Ocimum tenuiflorum.* The flavonoid content in *Ocimum tenuiflorum* is more than phenolic content. So, Flavonoid content was high in leaf extracts of Ocimum.

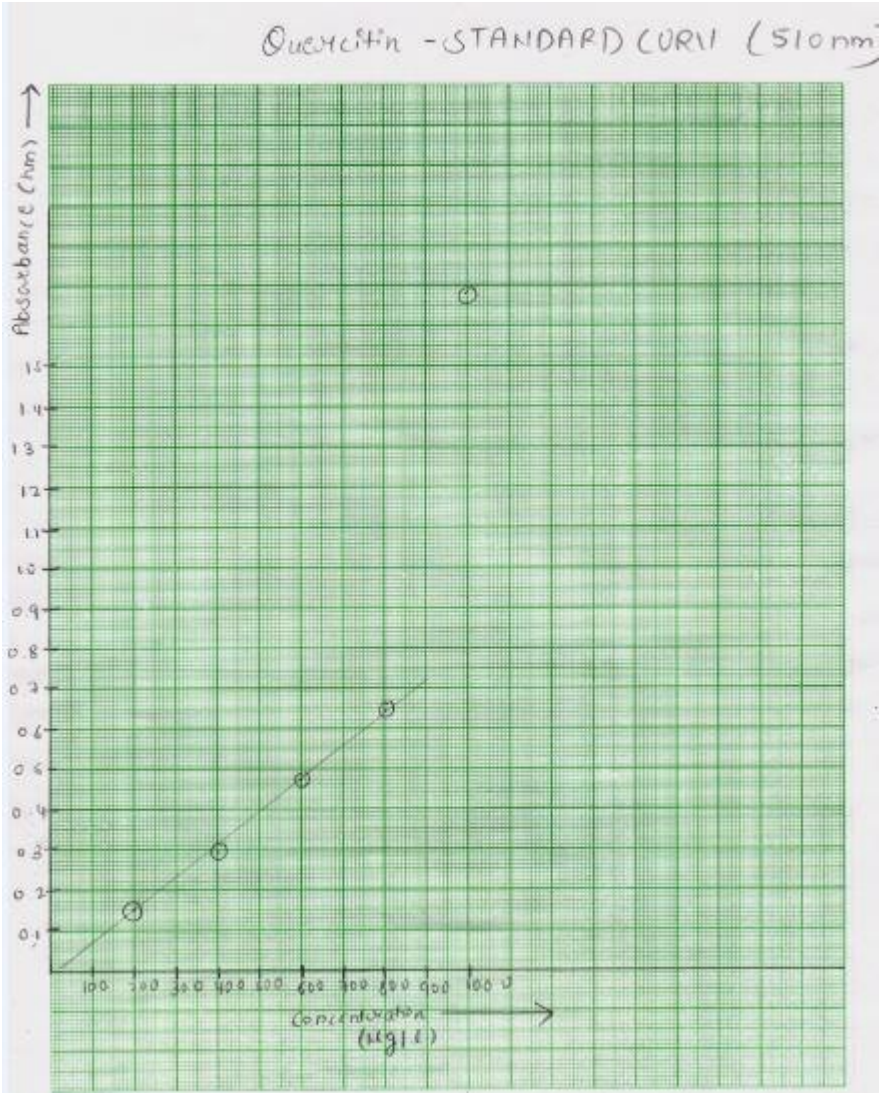


Figure 5.7 Graphical representation for standard Quercetin curve

Table 5.2 shows result for Total flavonoid test and Total phenolic content test in *O. tenuiflorum*.

Plant Species	Total Phenolic content (mg/ml)	Flavonoid content (mg/ml)
Ocimum tenuiflorum	0.58	4.30

Table 5.2 Results of Quantitative test

5.3 Results of Plant tissue culture

5.3.1 Time course growth and effect of biotic elicitor

Time course growth after three days shown in the figure 5.8. The petri plates were kept in the dark for three days and after three days, the results shown in the figure 5.8.

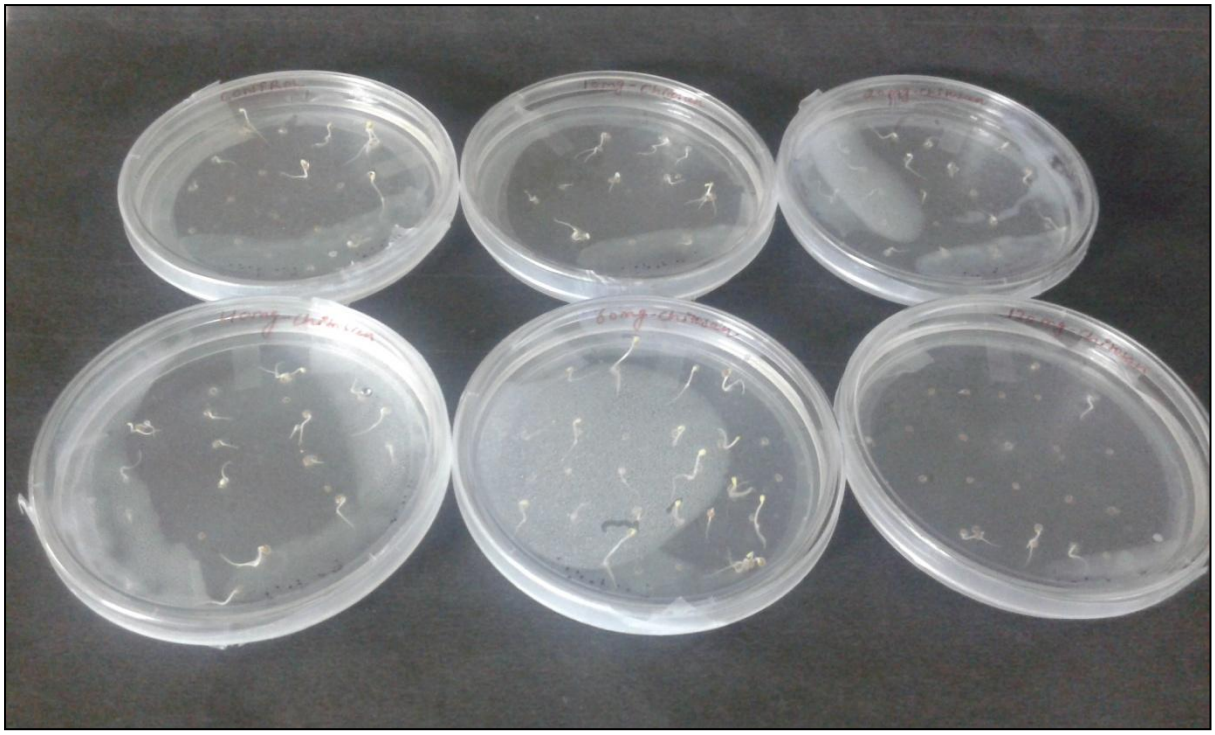


Figure 5.8 Represents growth after three days (after dark)

5.3.2 Effects of various concentration of chitosan on the Growth of *Ocimum tenuiflorum*

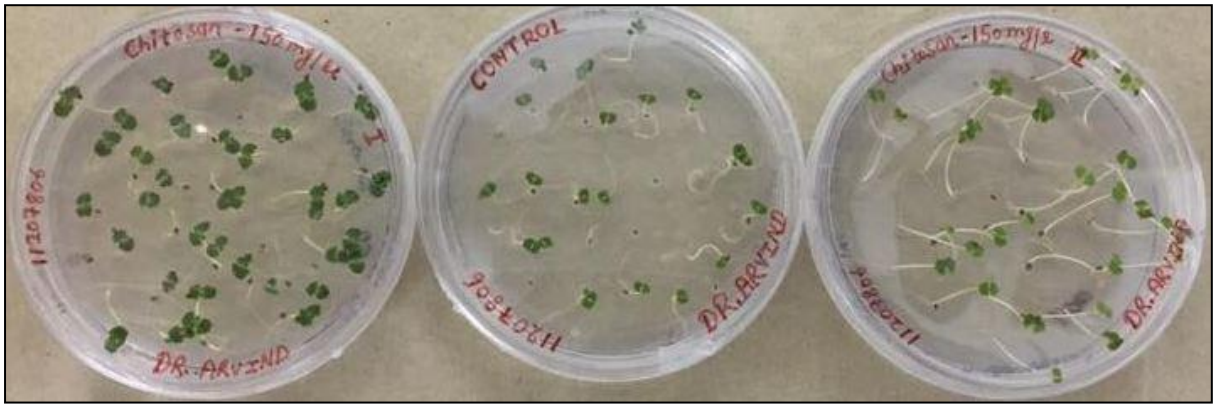
After dark room incubation, the petri plates was transferred to the culture room. After 7-8 days incubation, the effect of different chitosan concentration on the growth of Ocimum tenuiflorum have been seen and shown in the figure 5.9. In Figure (a), the growth at 50 mg per litre chitosan as compare to control is little more. The shoot length is long in treatment than control. In figure (b), the shoot length is little more than 50 mg per litre treatment. In figure (c), petri plates contains 150 mg per litre chitosan has shown maximum growth that is supported by Lingyun *et al*., 2016 and Asghari *et al*., 2009. Asghari *et al*., 2009 gave the chitosan treatment to *Solanum tuberosum* L. And observed maximum growth at 150 milligram per litre chitosan. After that, growth starts decreasing in chitosan treatment 200 mg per litre and 300 mg per litre shown in figure (d) and (e). Minimum growth was observed in 300 mg per litre chitosan.


Chitosan-50mg/l
II
DR.ARVIND
CONTROL
DR.ARVIND
Chitosan-50mg/l
I
DR.ARVIND

(a)


Chitosan-100mg/l
I
DR.ARVIND
CONTROL
DR.ARVIND
Chitosan-100mg/l
II
DR.ARVIND

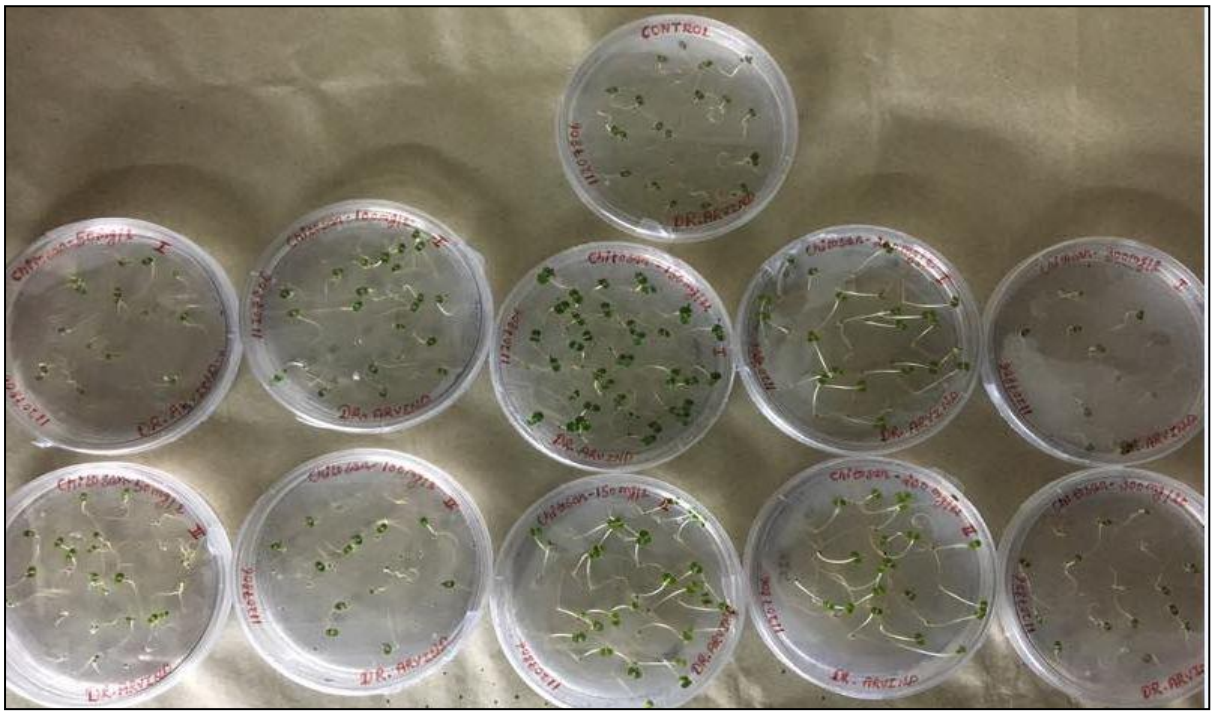
(b)


Chitosan-150mg/l
I
DR.ARVIND
CONTROL
DR.ARVIND
Chitosan-150mg/l
II
DR.ARVIND

(c)

(d)

(e)



(f)

Figure 5.9 Represents (a) growth of *Ocimum tenuiflorum* in Murashige and Skoog media containing 50 milligram per litre chitosan and also represents growth in control that contain only MS media (b) growth of *Ocimum tenuiflorum* in Murashige and Skoog media containing 100 milligram per litre chitosan and growth in control (c) growth of *Ocimum tenuiflorum* in Murashige and Skoog media containing 150 milligram per litre chitosan and growth in control (d) growth of *Ocimum tenuiflorum* in Murashige and Skoog media containing 200 milligram per litre chitosan and growth in control (e) growth of *Ocimum tenuiflorum* in Murashige and Skoog media containing 300 milligram per litre chitosan and growth in control (f) comparison of all treatments with control.

Figure 5.10 represent the shoot length and root length at various chitosan concentration. As increase the concentration of the chitosan shoot length and root length increased but after its 150 mg per litre treatment it decreased. Asghari *et al*., 2009 supported the results. He also got maximum growth at 150mg per litre chitosan. He gave the same treatment to *Solanum tuberosum* L.

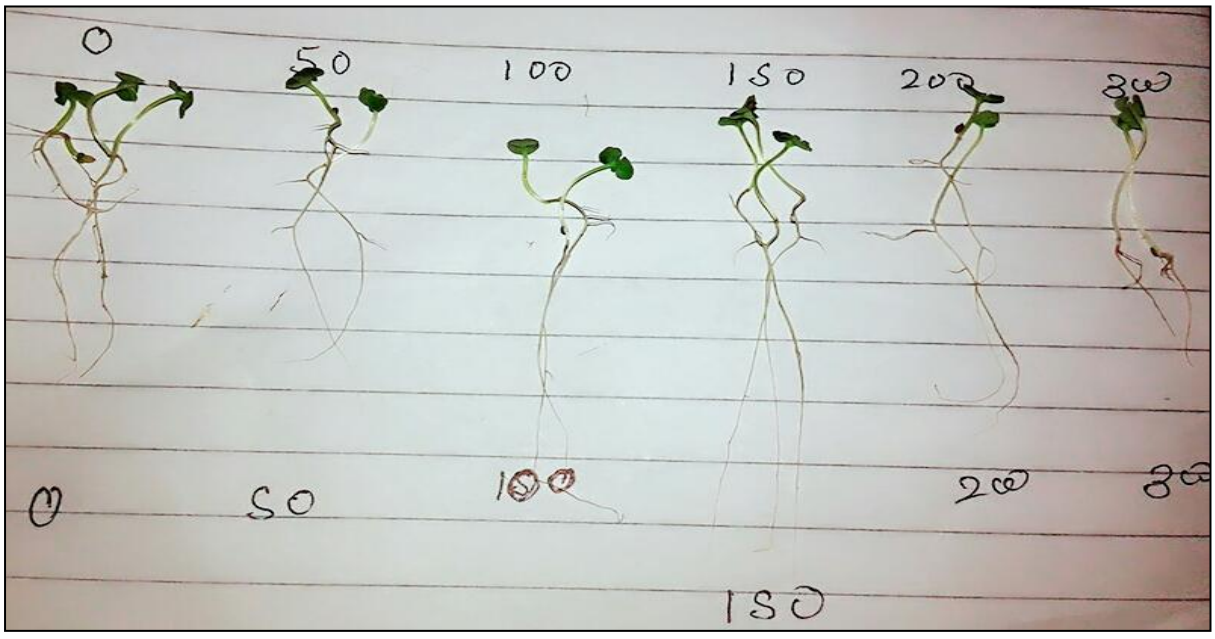


Figure 5.10 Represents difference between shoot length and root length of the different chitosan treatment.

5.3.3 Measurement of Germination Rate, Sprout Biomass and Flavonoid Content of *O. tenuiflorum*

In the end of the culture period, the germination rate of *O. tenuiflorum* seeds was counted. The percentage (%) of seed germination was determined by (G/t) × 100. In this

formula, G is an average number of 2 replicates of germinated seeds and t is an average value of 2 replicates of the total seeds in each test. And for biomass of sprout measurement, sprout were harvested and then rinsed thoroughly with distilled water and after that blotted dry through paper towels to get the fresh weight (fw), and then dried at 40–45 degree celsius in an oven to attain the constant dry weight (dw). This was supported by Lingyun *et al*., 2016.
The germination rate was 100% at 150 milligram per litre chitosan treatment. The germination percentage was decreased after 150 milligram per litre chitosan and at its very high concentration at 300 milligram per litre, the percentage was 68.055% on day 6. Lingyun *et al*., 2016 calculated germination rate with same formula, and they got higher germination rate at 150 milligram per litre polysaccharides concentration. So, they supported these results.

Table 5.3 represent effect of various concentration of chitosan on germination percentage (%) in *O. tenuiflorum*.

Chitosan concentration (mg per litre)	**Day 4 (%)**	**Day 5 (%)**	**Day 6 (%)**
0	79.0±0.707	83.3±1.414	94.4±1.414
50	73.6±2.121	84.7±0.707	90.2±0.707
100	76.3±2.121	83.3±1.41	93.0±0.707
150	84.7±0.707	87.5±0.707	100±0.000
200	79.1±0.707	83.3±1.414	87.5±0.707
300	56.9±0.707	62.5±0.707	68.0±0.707

Values represent mean ± standard deviation (n = 2).

Table 5.3 Shows effect of various concentration of chitosan on germination percentage

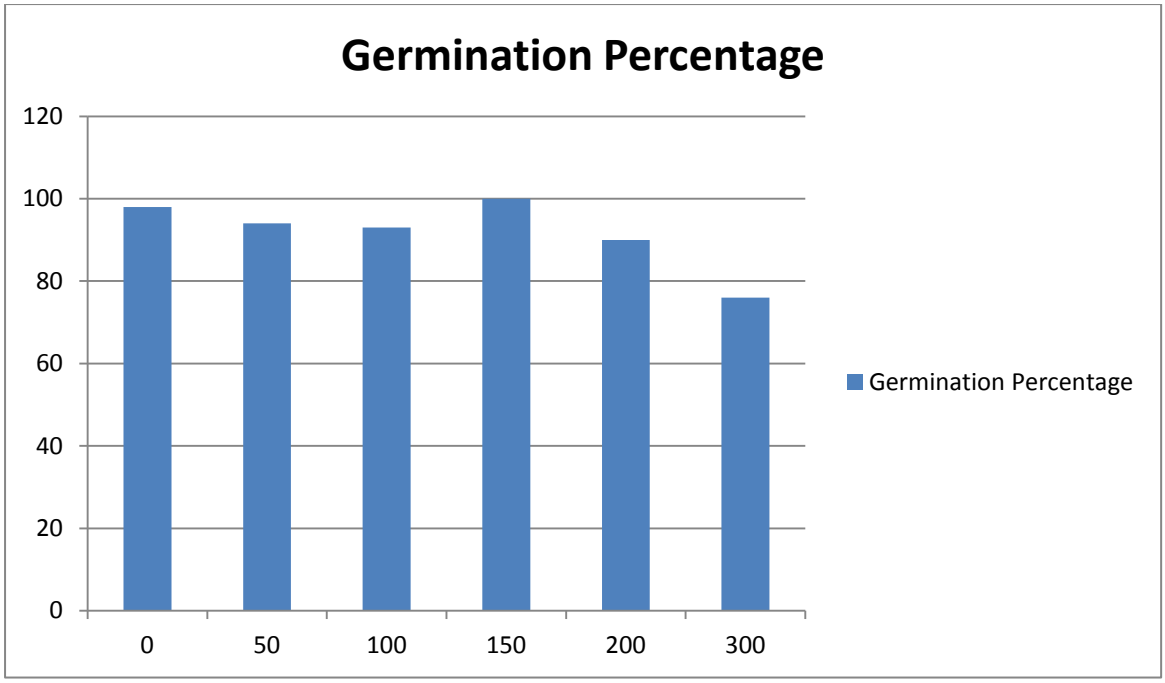


Figure 5.11 Shows effect of various concentration of chitosan (mg per litre) on germination percentage

Table 5.4 Shows various figures of shoot & root length as per chitosan various concentration in *O. tenuiflorum*.

Chitosan concentration (mg/litre)	Shoot length (centimetre)	Root length (centimetre)	Ratio (Root/Shoot)	Total length (centimetre)	Fresh weight(gram)
0	2.55±0.0707	1.40±0.141	0.60	3.95±1.626	2.015±0.007
50	2.70±0.141	1.65±0.070	0.61	4.35±1.484	2.035±0.007
100	2.80±0.141	1.80±0.141	0.62	4.60±1.414	2.135±0.007
150	3.00±0.141	1.90±0.141	0.63	4.90±1.55	2.205±0.007
200	2.50±0.141	1.55±0.070	0.62	4.05±1.343	2.005±0.007
300	2.35±.0.070	1.35±0.070	0.63	3.70±1.414	0.555±0.077

Values represent mean ± standard deviation (n = 2).

Table 5.4 Shows various figures of shoot and root length as per chitosan concentration.

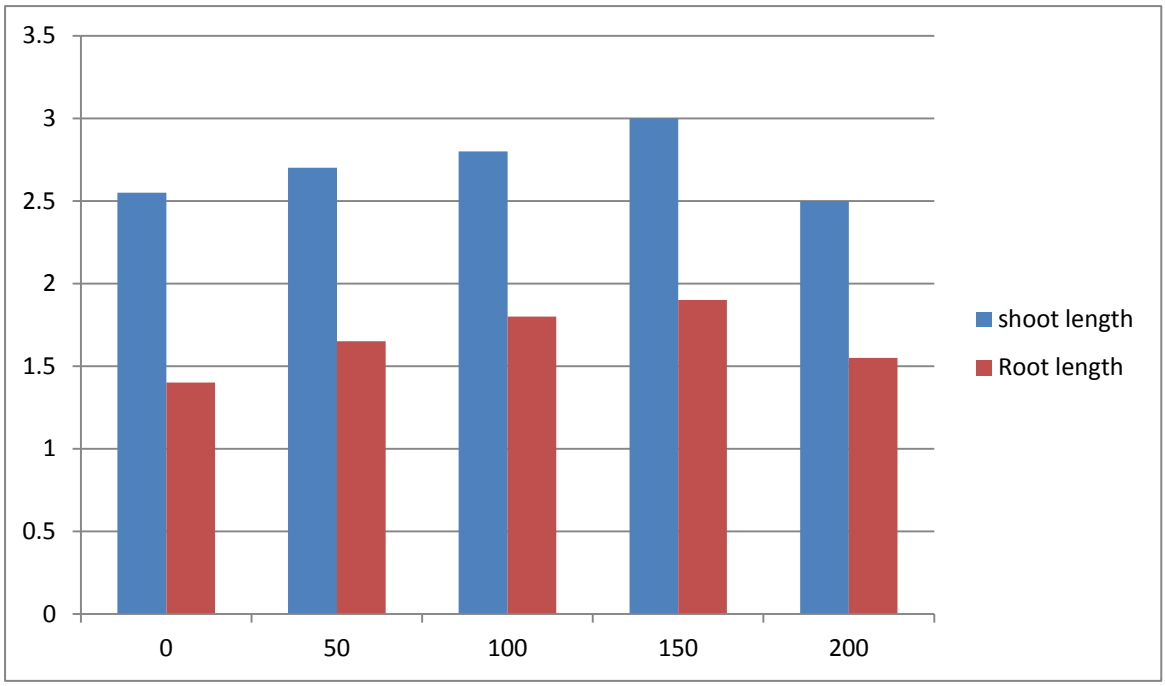


Figure 5.12 Shows shoot length and root length (cm) of *O. tenuiflorum* affected by the various concentrations of the Chitosan (milligram/litre).

5.4 Results of Phytochemical screening (quantitative test) under biotic elicitation

It is observed that phenols and flavonoids accumulation were highly influenced with the addition of chitosan as biotic elicitor in *0. tenuiflorum*. Regarding of reported publications on chitosan affecting the secondary metabolites production in cell suspension cultures of important plant species, attempts were made at quantification of secondary metabolites in the elicited cell cultures of *0. tenuiflorum* with chitosan at different concentrations (0,50,100,150,200 and 300 milligram/litre) and were compared with invivo quantification. Regarding total phenols content it was recorded 0.8775 mg per ml with 150 mg per litre chitosan. And the total flavonoid content was reported 5.39 mg per ml in 150 mg per litre chitosan. The less amount was reported at 300 milligram per litre chitosan concentration. Taha *et al*., 2017 was reported the higher accumulation at 100 milli gram per litre chitosan treatment in total phenolic content. They gave same treatment to *Brassica rapa* L. Their range was (0, 25, 50, 100 and 200 milligram/litre).

Table 5.5 shows the effect of the different chitosan concentration on total Flavonoid content and Phenolic content in *O. tenuiflorum*.

Various Chitosan concentrations(milligram per litre)	Total phenolic content (mg/ml)	Total flavonoid content (mg/ml)
0	0.735±0.007	4.57±0.107
50	0.767±0.003	4.77±0.011
100	0.795±0.007	4.87±0.002
150	0.877±0.003	5.39±0.014
200	0.745±0.007	4.90±0.169
300	0.705±0.007	4.17±0.063

Values represent mean ± standard deviation (n = 2).

Table 5.5 Shows the effect of the different chitosan concentration on total Flavonoid content and Phenolic content in *O. tenuiflorum*.

The total phenolic (mg/ml) content were determined in *O. tenuiflorum* cell cultures which is elicited with different concentrations of chitosan. Illustrated data in Figure 5.13 clearly stated that elicitation of *O. tenuiflorum* cultures with 150 mg per litre of chitosan gave the highest value of total phenolic content (0.8775 mg/ml). However, increasing of chitosan concentration up to 300 milligram/l reduced the accumulation of total phenolic content to 0.700 mg/ml. Regarding determination of Total flavonoid content (mg/ml) in different elicited with various concentrations of chitosan. The highest amount of flavonoid was noticed at 150 milligram per litre is 5.39 mg/ml. These results was supported by Lingyun *et al*., 2016.

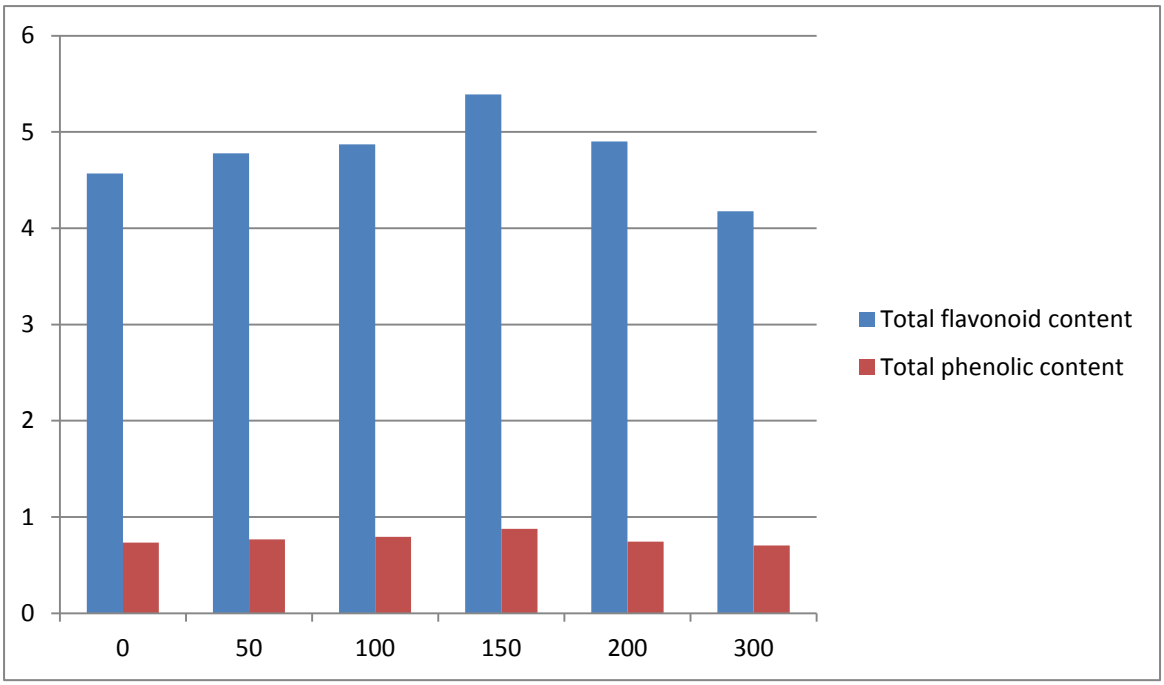


Figure 5.13 Shows the effect of the different chitosan concentration (mg/litre) on total Flavonoid content and Phenolic content (mg/ml) in *O. tenuiflorum*.

5.5 Comaprison between invivo and invitro on the basis of Total phenolic content and total flavonoid content in *O. tenuiflorum*

In invivo, the phytochemicals concentration is low as compared to invitro concentration. And the shoot length and root length grow faster as compare to invivo. The conclusion of this study is that the accumulation of secondary metabolite in invitro is high as comparison to invivo.

Table 5.6 shows comparison between invivo and invitro on the bases of Total flavonoid content and Total Phenolic content.

Phytochemical	**Invivo (mg/ml)**	**Invitro (mg/ml)**
Total Phenolics content	0.58	0.87±0.003
Total Flavonoid content	4.30	5.39±0.014

Values represent mean ± standard deviation (n = 2).

Table 5.6 Comparison between invivo and invitro on the basis of Total flavonoid content and Total Phenolic content

Figure 5.12 compare invivo and invitro on the basis of Total flavonoid content and Total Phenolic content**.** The phytochemicals was reported high in invitro in *O. tenuiflorum*.

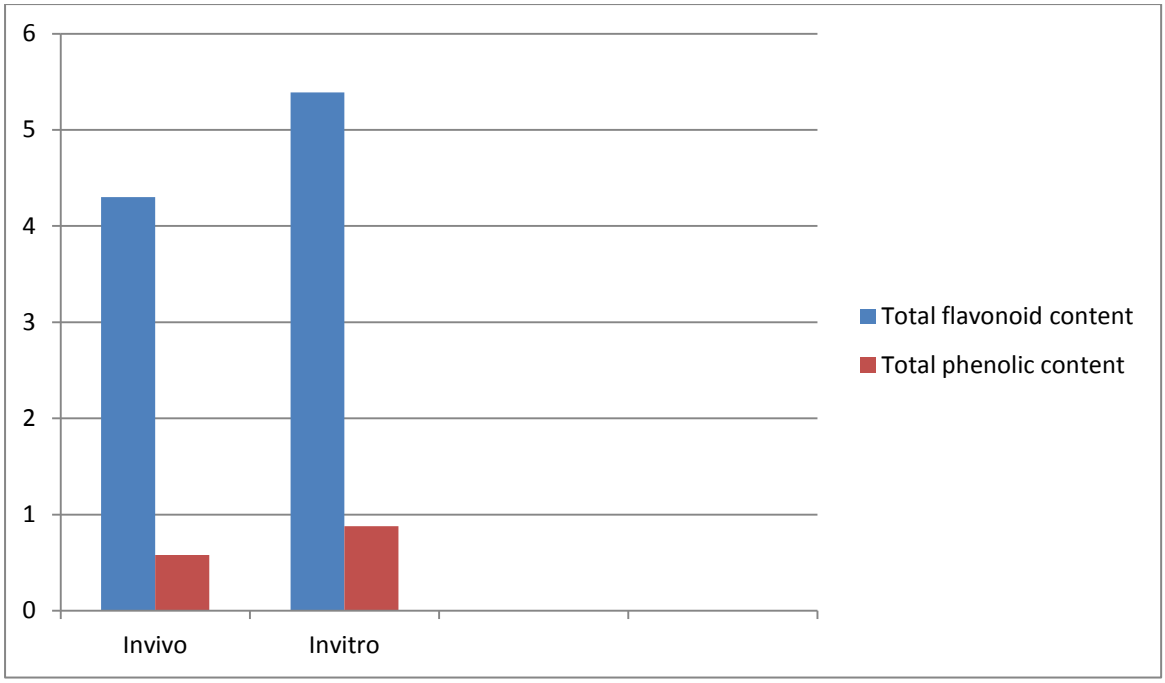


Figure 5.14 Comparison between invivo and invitro on the basis of Total flavonoid content and Total Phenolic content (mg/ml).

5.6 Statistical Analysis

All treatments for elicitation were carried out two times. The results were expressed by the mean values and standard deviations (SD) of 2 replicates in each case.

Discussion

Secondary metabolites are being produced using plant tissue cultures from three decades. We have good plant tissue culture techniques but their production in large scale is still lacking. To improve their production in plant tissue cultures different methods are used. Elicitation is one such strategy that is largely used for *in vitro* production of secondary compounds. Many scientists in several countries have carried out experiments to check responses of plants towards different abiotic elicitors. The exact mechanism of elicitation is not exactly understood till date. Therefore using elicitors as an agent is thought to be huge scope for producing secondary molecules in large scale using *in vitro* plant culture system.

A number of active constituents in the leaf extract of *O. tenuiflorum* were identified and detected by performing various phytochemical tests. The preliminary phytochemical study showed that the plant extract contains flavonoids, alkaloids, saponins, reducing sugars, tannins and anthraquiones. For the test for flavonoids, yellow coloration in each extract showed its presence; for the test for alkaloids, yellow precipitate indicated its presence; for reducing sugars, change in color reaction detected its presence; for the presence of saponins,

formation of emulsion was observed; for the test for tannins, blue-black or brownish green color change was observed; similarly for anthraquiones, change in color was observed

In this study the maximum accumulation of secondary metabolites was reported at 150 milligram per litre chitosan treatment in *O. tenuiflorum*. The amount of flavonoid and phenolic at this concentration was 5.39 milligram per ml and 0.8775 milligram per ml. After this concentration the accumulation of metabolites was decreased and also shoot length and Root length was decreased. Because plant defense can fight wth elicitors at a optimum concentration. After that concentration it will start decrease. Lingyun *et al.*, 2016 was supported these results. They reported the maximum concentration of Polysaccharides elicitor at 200 milligram per litre.

CHAPTER-6

SUMMARY AND CONCLUSION

High antioxidant activity is reported from distinct medicinal plants (Lachance and Nakat, 2001). Flavonoids and Phenolics are very common antioxidants present in plants (Sankhalkar, 2014). The present work was the qualitative and quantitative analysis of medicinal plant *O. tenuiflorum*. Flavonoid content was high in leaf extracts of Ocimum. Genus Ocimum is known to be rich in phenolic compounds. So, it is broadly applied in medicine traditional system (Wang *et al*; 2014). Flavonoids and phenolics have at least one hydroxyl ion substituted with aromatic ring. They can form chelate complexes with the metal ions by getting easily oxidized and are the means for donating electrons to scavenge free radicals. The linear correlation between antioxidant activity and phenolic content has also been reported (Borneo *et al*., 2008).

Variation in antioxidant properties in relation to leaf position have been seen in Ocimum. Reports are available that shows the presence of flavonols, flavones, flavonoid tannins, aglycone, polyphenols etc. in Ocimum species. Flavonoids, they are known for their high antioxidant properties (Sharma, 2014).

Phytochemicals are the chemical compounds present in plants with different physiological action on the human body (Vimala *et al*., 2013). Flavonoids, alkaloids, terpenoids, phenolics and essential oils are few vital bioactive phytochemicals (Anwar *et al*., 2006). A number of reports are available that shows the presence of phytochemicals such as glycosides, quercetin, rutin, tannins, kaempferol glycosides and chlorogenic acids (Kiranmai and Mohammed, 2012). Rosmarinic acid is the predominant phytochemical present in the leaf extracts of *Ocimum basilicum*. In the present study Ocimum, report the presence of phenolics, flavonoids and antioxidants. Like earlier reports, also hypothesize that antioxidant nature of *O. tenuiflorum* L. may be because of increase in flavonoid content and phenolic content. However, further quantitation and identification of the chemical structure of these phytochemicals need to be undertaken by gas chromatography, nuclear magnetic resonance (NMR) spectroscopy, HPLC and MS. Identification of active constituent in medicinal plant is hence highly significant in pharmaceutical and food industry due to its natural antioxidants are least harmful than synthetic antioxidants. However, physicochemical evaluation of the drug is a very essential parameter to study the quality of the plant material for future research applications (Javanmardi *et al*., 2002).

From the present study, conclude that the leaf extract of *O. tenuiflorum* L. exhibit high phytochemical potential and antioxidant potential. The phytochemicals present in *Ocimum tenuiflorum* are tannins, saponins, flavonoids, reducing sugar, alkaloids and anthraquinones. The plant extract contains high amounts of phenolics and flavonoids. The study showed that the plants are a source of significant natural antioxidants. They may be useful in protection against oxidative stresses.

The medicinal properties of *Ocimum tenuiflorum* are mainly because of the presence of a variety of phenolic acids like rosmarinic acid, flavonoids, and essential oils (Zgórka and Glowniak, 2001).

In the present study, the biotic elicitors chitosan added in the medium resulted in higher accumulations of flavonoid and phenol with increased growth of roots and shoots. Elicitors are compounds which increase the plant cell secondary metabolites and they are usually derived from components of plant cell walls or fungal cellwall. The use of elicitors has been proven to be an useful method to enhance secondary metabolites in plant cell cultures. The effect of different concentration of chitosan has been observed. The conclusion of this study is that the accumulation of secondary metabolite in invitro is high as comparison to invivo.

With various medicinal properties of flavonoids and phenol, research is going on to increase the production of flavonoids through biotechnological tools. The concept of elicitation of secondary metabolite using elicitors is therefore, gained high importance because it targets straight to the bioactive compounds. This investigation has shown that the application of Chitosan elicitor are potent enough to enhanced the synthesis of flavonoids and phenol in *O. tenuiflorum* and the elicitation is dependent phenomenon on the type of elicitor, treatment duration and its doses.

CHAPTER-7
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