# PHYTOCHEMICAL SCREENING OF BIOACTIVE COMPOUNDS UNDER ABIOTIC 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

**BIJI A. VARGHESE**

M Tech Biotechnology

Reg. No. 11203017

UNDER THE GUIDANCE OF

**DR. ARVIND KUMAR**

Assistant Professor

**SCHOOL OF BIOENGINEERING AND BIOSCIENCES**

**LOVELY PROFESSIONAL UNIVERSITY**

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**TOPIC APPROVAL PERFORMA**

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| SR.NO. | NAME OF STUDENT | REGISTRATION NO | BATCH | SECTION | CONTACT NUMBER |
|---|---|---|---|---|---|
| 1 | Biji A Varghese | 11203017 | 2012 | B1202 | 7508982970 |

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|---|---|---|
| PAC Member 1 Name: Dr. Ashish Vyas | UID: 12386 | Recommended (Y/N): NA |
| PAC Member 2 Name: Himanshu Singh | UID: 11691 | Recommended (Y/N): NA |
| PAC Member 3 Name: Dr. Joydeep Dutta | UID: 14336 | Recommended (Y/N): Yes |
| PAC Member 4 Name: Dr. Umesh Goutam | UID: 14691 | Recommended (Y/N): NA |
| DAA Nominee Name: Mamta Sharma | UID: 18431 | Recommended (Y/N): NA |

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**PAC CHAIRPERSON Name:** 11840::Dr. Neeta Raj Sharma **Approval Date:** 07 Nov 2016

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# DECLARATION

I hereby declare that the project entitled **"Phytochemical screening of bioactive compounds under abiotic elicitation of Tulsi (*Ocimum tenuiflorum*)"** is an authentic record of my own work carried out at School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, for the partial fulfilment of the award of B.Tech-M.Tech Biotechnology dual degree under the guidance of **Dr. Arvind Kumar**.

This work is my original work and has not been submitted for any degree/diploma in this or any other university. The information furnished in this dissertation is genuine to the best of my knowledge and belief.

Lovely Professional University
Phagwara, Punjab

Biji A.Varghese
(11203017)

# CERTIFICATE

This is to certify that Biji A.Varghese has completed the Dissertation report titled **"Phytochemical screening of bioactive compounds under abiotic elicitation of Tulsi (*Ocimum tenuiflorum*)"** under my guidance and supervision. To the best of my knowledge, the present work is the result of her original investigation and study. No part of the report has ever been submitted for any other degree at any university.

The dissertation is fit for the submission and the partial fulfillment of the award of B Tech-M Tech Biotechnology dual degree.

(Dr. Arvind Kumar)
Assistant Professor

Lovely Professional University
Phagwara, Punjab

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(Biji A. Varghese)

Reg. No. 11203017

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# LIST OF ABBREVIATIONS

RNA: Ribonucleic acid

DNA: Deoxyribonucleic acid

US: Ultrasound

SA: Salicylic acid

JA: Jasmonic acid

MeJA: Methyl jasmonic acid

GA: Gibberellic acid

NaCl: Sodium chloride

GTP: Guanosine triphosphate

MAPK: Mitogen-activated protein kinase

ROS: Reactive oxygen species

MS media: Murashige and Skoog media

FC reagent: Folin-Ciocalteu reagent

TLC: Thin layer chromatography

CC: Column chromatography

HPLC: High-performance liquid chromatography

# ABSTRACT

The secondary metabolites in plants form unique sources for the development of products having medicinal importance so that new drugs can be developed. Thus they form an important source for pharmaceuticals, food additives, flavors and various other industries. The drugs that are sold nowadays are modified compounds obtained from the natural substances. The secondary metabolites in plants have gained commercial importance in these years, that made the secondary metabolism in plants a burning topic of study, specially in altering production of active constituents in plants. The present commercial goal cannot be fulfilled due to low yield and productivity of most secondary metabolites. So, it is necessary to consider strategies for enhancing their production in large scale. Various research has been carried out few years back on the production of these secondary compounds by using elicitors *in vitro*. The present communication briefly describes introduction, classification, recent improvements of production, importance of secondary compounds and the effect of abiotic elicitor specially, saline conditions on plant's productivity from *in vitro* culture. Seeds of *Ocimum tenuiflorum*, estabilished on MS medium, were employed to study the influence of different concentrations of NaCl (0mM, 25mM, 50mM, 100mM, 125mM and 200mM) on the growth and production of phenolics and flavonoids. The phenolic and flavonoid contents in control were 0.82 mg/ml and 4.79 mg/ml respectively. The highest phenol (0.87 ± 0.0007 mg/ml) and flavonoid (5.19 ± 0.002 mg/ml) accumulation was observed in media treated with 50mM NaCl. Also, In vitro results were compared with in vivo results, where in vitro results came out to be higher.



**Keywords: P**lant secondary metabolites, pharmaceuticals, elicitors; abiotic elicitation, in vitro culture, saline condition, phenolics, flavonoids.

# CHAPTER-1

# INTRODUCTION

A major portion of our everyday meal constitute plants. 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).

There are three main groups of secondary metabolites on the basis of their biosynthetic origins. They are terpenoid, flavonoid and phenolic compounds, and nitrogen-containing alkaloid and sulphur-containing compounds (Mahmoud and Croteau, 2002). According to their specific structure, we have glycosides, tannins and saponins as well.

These secondary compounds are important sources for various industries like pharmaceuticals, food additives, flavor and fragrances, dye and pigments, pesticides and industrially important biochemicals (Murthy and Paek, 2014). Most of the drugs that are sold nowdays are modified forms of the natural compounds. This utilization of natural substances has gained much attention over the past decades, particularly in the possibility of altering the production of bioactive plant metabolites (Canter and Ernst, 2005).

Since ancient times these metabolites have played a significant role in the field of medicine. In Ayurveda, Unani and other traditional medical systems, plants were directly used as food or herbs to treat various diseases for many years (Mukherjee *et.al*., 2007; Fabricant and Farnsworth, 2001). The discovery of many bioactive compounds such as Taxol, Morphine, Metformin etc has witnessed plants to be an important source for drug discovery. These discovered molecules have been isolated to treat diseases like obesity, cancer and diabetes (Saklani and kutty, 2008).

The vast applications of these secondary compounds made them important for mass production. It was estimated that the international market value of naturally derived drugs was 18 billion dollars in 2005 and in 2011, it increased to more than 26 billion dollars (Saklani and Kutty, 2008). However, these secondary constituents are produced often very low in the plant itself and their accumulation depends on some particular conditions, or growth stage, or may be

on stress. Also, some of these compounds are specific to a particular plant family or genus or even a species. Most of the important plants of medicinal value can only be cultivated in particular climates like tropic or subtropics regions. There are some plants that are slow in growing and some are even hard to cultivate, thus they are required to be harvested from the wild that can extinct those plant species (Alfermann and Petersen, 1995; Verpoorte *et al*., 2002). For such reasons, an approach that uses cell or tissue cultures of plants is being preffered as an alternative method for the production of these important bioactive compounds from plants.

This project is undertaken to study, the enhancement of secondary metabolites using abiotic elicitation in holy basil (*Ocimum tenuiflorum*) as, not much research has been carried out on the same. These plants are well known for their therapeutic potentials (Prakash *et al*., 2005). *O. tenuiflorum* is an herb with strong aroma cultivated throughout India. In Hindi it is called 'Tulsi' and in English 'Holy Basil'. In traditional medical systems its different parts (leaves, stem, flower, root, seeds and even whole plant) were recommended for the treatment of diseases such as bronchitis, malaria, diarrhea, skin diseases, arthritis, chronic fever and insect bite and many other. *O. tenuiflorum* has also been thought to possess properties such as anticancer, anti-diabetic, anti-microbial, cardioprotective, analgesic etc. Many researchers have reported that different chemical constituents are possessed in all members (even the close ones) of the same genus *Ocimum*. Razdan (2003) has reported that *O. tenuiflorum* can cross-pollinate with different plants of the same genus due to which some plants may not be of true-type. Hence, if some genetic variations are present, there would be different chemical constituents found in the plants. Here is the situation to apply plant *in vitro* culture that will help to solve this problem because the tissue culturing of plants will produce the identical offspring to that of the parent plant.

For multiplication and extraction of secondary metabolite we can estabalish cell and tissue culturing of plants under sterile conditions from seeds or explants (leaves, stems, roots, and meristems). Further, various strategies can be used to increase the production of bioactive compounds like precursor feeding, media optimization, in situ product removal, elicitation, immobilization etc (Verpoorte *et al*., 1999; Zhang and Furusaki, 1997). Among all these strategies, elicitation is proved to be one of the efficient way to enhance the production (Verpoorte *et al*., 1999). *In vitro* culture of plant cells respond to microbial, physical, or chemical factors called "elicitors". This process of induced production of secondary metabolites by plants for their defence is called elicitation. "Elicitors" are termed as the chemicals (biotic or abiotic)

that gets triggered by a stress induced response or stimulate the defense mechanism in plants which therefore accumulates secondary metabolites (Radman *et al*., 2003).

Plants are vulnerable to various environmental stresses that restrict their growth and yield causing a huge loss to agricultural production across worldwide (Shao and Chu, 2008). One such important factors affecting growth of plants and their secondary plant products is salt stress (Nikolova and Ivancheva, 2005). Salinity has adverse effects on plant's physiological and metabolic activities. It results in late germination, stunted growth, poor quality and reduced productivity (Muhammad and Hussain, 2010). Also there is decrease in the rate of photosynthesis and respiration due to salt stress.

In the present investigation, stress response of different concentrations of sodium chloride (NaCl) in *Ocimum tenuiflorum* (tulsi) has been studied to determine the salt stress response on its growth and production of secondary plant compounds. Previous studies have shown that NaCl induces a no. of stress responses from plants including decreased root length (Samantha and Julie, 2012). Also the secondary plant products are found to be higher in plants that are grown under saline conditions than those in normal conditions (Said and Omer, 2011).

The aim of present work is phytochemical screening of bioactive compounds under abiotic elicitation of Tulsi (*Ocimum tenuiflorum*).

The attempts were made as follows :

i. Preliminary phytochemical tests for qualitative analysis of various bioactive compounds in *O. tenuiflorum* leaf extract.
ii. Quantification tests (in vivo) to determine total phenolic and flavonoid contents.
iii. Abiotic elicitation (salt stress by NaCl) through *in vitro* culture of *Ocimum tenuiflorum* to increase the productivity of secondary compounds.
iv. Quantitative analysis (in vitro) of the salt stressed plants.
v. Comparison of in vivo and in vitro results of quantitative metabolite analysis.

# CHAPTER-2

# REVIEW OF LITERATURE

Through a process called 'metabolism', plants can produce different bio-active constituents. Thus they have been considered as the source for many important drugs. Plant cell carries out two major metabolism namely, primary and secondary. The production of proteins, lipids, polysaccharides by utilizing amino acids, fatty acids, nucleotides and sugars is by primary metabolism. Plant secondary metabolism is related with the defense mechanism in plants which activates only during specific growth stages or during stress times, at the time of nutrient deficiency or microbial attacks (Yazaki *et al*., 2008).

2.1 Secondary metabolites of plants

2.2 Plant studied: *Ocimum tenuiflorum*

2.3 *in vitro* production of secondary compounds

2.4 Defense response produced by secondary substances: elicitation concept

2.6 Effect of various abiotic elicitors on the productivity of secondary compound

2.7 Salt stress and its effect on secondary plant products

2.8 Factors which influence elicitation

2.9 Mechanism of elicitation

## 2.1 SECONDARY METABOLITES OF PLANTS

Secondary compounds are the organic compounds that have no role in the life processes of plants that produce them but, they do provide them survival functions. These compounds are an important feature of all plants because these bioactive molecules produce defense mechanism in plants against different microbes ans herbivores (Stamp and Nancy, 2003). The various functions served by them are as follows:

(a) They can be used as transporting agents of metals;

(b) They act as protective agents against insects, microbes and higher animals;

(c) They can act as sexual hormones;

(d) They can also act as differentiation effectors.

Some compounds, including antibiotics helps in formation of spore and stop the germination process. Thus, the secondary compounds can perform functions like:

(a) Slowing down of spore germination till a favorable condition exists

(b) Protecting dormant spores from amoebae; and

(c) Cleansing of competitive microbe environment at the time of germination (Demain and Fang, 2000).

The extraction and purification of these molecules are difficult because they are often specific to species or genus and also produced in very low amounts. They are very high value products and have huge commercial applications in industries like food additives, biopesticides, colors, fragnances, medicines, agrochemicals, etc (Cusido *et al*., 2013).

### 2.1.1 CLASSES OF SECONDARY METABOLITES

The plant secondary products can be classified as terpenoid, alkaloid and phenolic (Verpoorte, 1998). According their specific structure, we have glycosides, tannins and saponins as part of them.

#### 2.1.1.1 TERPENOID

Terpenoid is the largest group of plant secondary metabolites. They are volatile compounds. They are made of condensation of isoprene units (C5). They are classed by five carbon units found in the core structure (Mahmoud and Croteau, 2002). Isoprene is a gas released by the leaves that are produced in the chloroplasts. It protects the plants from heat.

The fragnance in the plants are 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 are 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.

#### 2.1.1.2 PHENOL AND FLAVONOID

Phenolics have one aromatic ring with hydroxyl groups attaced with it. It present throughout the plant kingdom (Strack, 1997). Phenolics perform functions like they prevent block activity of enzyme and division of cells (Shapiro *et al*., 1994).

Flavonoids are the first group of phenols. They are found in vacuoles and are water-soluble. In plants, they perform functions like UV protection, pigmentation, disease resistance (Koes *et.al*., 1994). Flavonoids are further grouped into three classes that are anthocyanin, flavone and flavnol. Anthocyanin ranges from red color to blue and purple color. The pH of environment decides its color. They can be found in grapes and berries. It can be used to treat heart diseases, diabetes and cancer. They are found in skincare products also to minimize process of aging . The other groups are flavones and flavnols that have white or yellow pigments. As a group, the phenols help in pollination of plants.

Salicylic acid is another important compound , that is obtained from bark of willow tree. It is an active ingredient in aspirin. It has been used to effectively in the treatment of aches and fevers and have cosmetic applications.

Lignin is the last compound that is present in plant structure. It provide stiffness or strength to plant cell walls. It do not allow water to enter cell wall and therefore prevent attacks by fungus.

#### 2.1.1.3 NITROGEN CONTAINING COMPOUNDS : ALKALOIDS

Plant secondary metabolites have large amounts of nitrogen in their structure. Alkaloids form part of this category which is known for its anti-herbivore property. They are of great interest because of their medicinal properties. They can also be highly toxic to the humans.

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 PLANT STUDIED: *Ocimum tenuiflorum*

This project is undertaken to increase the productivity of plant secondary compounds using abiotic elicitation in tulsi as not much research have been carried out on the same.

#### 2.2.1 SCIENTIFIC NAME: *Ocimum tenuiflorum*

### 2.2.2 SYNONYMS:

*Geniosporum tenuiflorum, Lumnitzera tenuiflora, Moschosma tenuiflorum, Ocimum anisodorum, Ocimum hirsutum, Ocimum inodorum, Ocimum sanctum, Ocimum tomentosum, Ocimum villosum.*

### 2.2.3 ECOLOGICAL NICHE

**Kingdom:** Plantae

**Division:** Magnoliophyta

**Class:** Angiospermae

**Order:** Lamiales

**Family:** Lamiaceae

**Genus:** Ocimum

**Species:** O. *tenuiflorum*

### 2.2.4 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 observed, which carry anti oxidant and anti inflammatory functions. 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.

## 2.3 *IN VITRO* PRODUCTION OF SECONDARY COMPOUND

The production is often low (> 1 percent dry weight) which is dependent on geo-climatic and plant growth stage (Rao and Ravishankar, 2002). Accumulation of such metabolites is directly dependent on external and internal stresses. As a response to stress factors viz. mechanical injury, minerals in soils, microbial infections, UV radiation, and accumulation of secondary products largely varies in terrestrial plants.

Most of the plants are difficult to cultivate and hence are over-harvested (Rates, 2001). Secondly, the chemical production of these active compounds is not feasible for commercial production. In this situation, *in vitro* culturing of plant cell offers a best option for secondary compound production (Jian *et al*., 2005).

## 2.4 DEFENCE RESPONSE PRODUCED BY SECONDARY SUBSTANCES: ELICITATION CONCEPT

The induced production of metabolites by adding trace amounts of elicitors is called Elicitation. The substances that are introduced in small amounts to generate plant defense mechanism are called elicitors (Klarzynski and Friting, 2001). Elicitation is stated as the most preffered technique for increasing the productivity of desirable secondary substances *in vitro* (Namdeo, 2007).

## 2.5. CLASSIFICATION OF ELICITORS

Elicitors are classified as abiotic and biotic depending on their origin (Radman *et al*., 2003).

### 2.5.1 ABIOTIC ELICITORS

Abiotic elicitors are from non-biological origin (Gorelick and Bernstein., 2014). Salts such as aluminium chloride, calcium chloride, copper chloride, potassium chloride etc. can be used to elicit production in different plant species in culture systems (Verpoorte *et.al*., 2002).

Abiotic elicitors are classified as hormonal ,physical or chemical. Figure 2.1 shows the classification of abiotic elicitor.

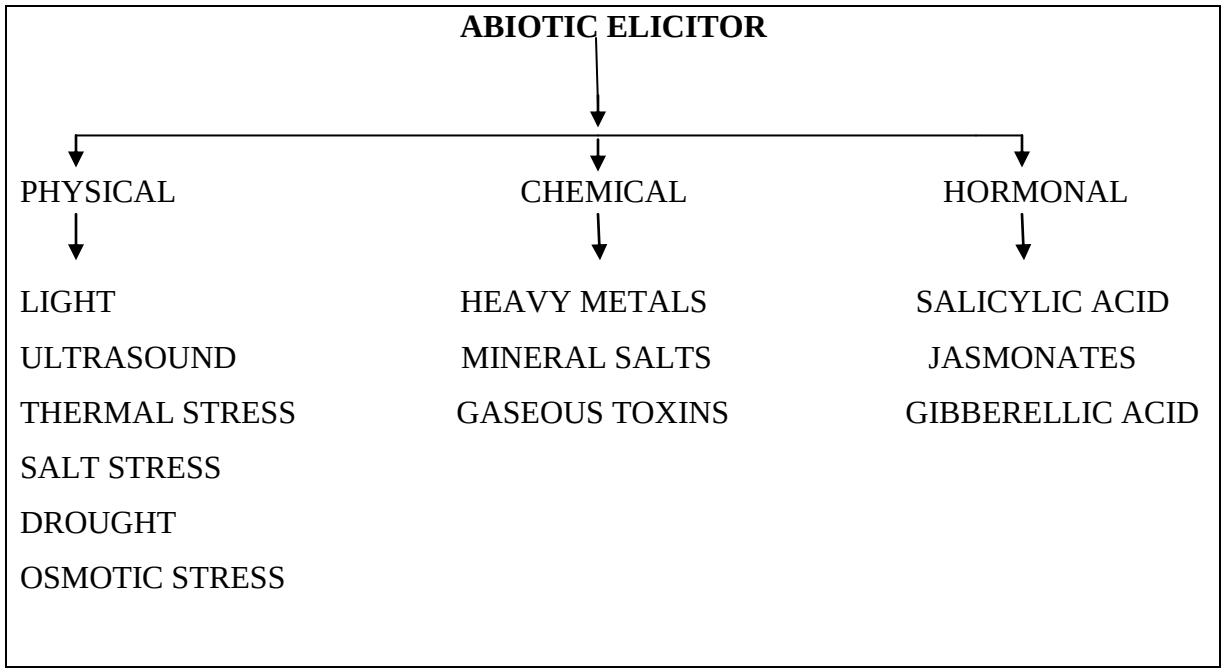



**Figure 2.1 Flowchart representation for classification of abiotic elicitor (Naik and Al-Khayri; 2015)**

### 2.5.2 BIOTIC ELICITORS

These are substances of biological origin. They include polysaccharide originated from cell wall of the plant for example pectin and cellulose and microorganisms for example yeast, fungal extracts and bacterial extracts.

These are molecule of either host or pathogen origin which can generate guard responses in plant tissue. Biotic elicitors are recognized by unique receptors bound to the membrane of the cell. A signal transduction system transfers this stimulus, that produce changes and lead phytoalexins formation (Baenas *et.al*.,2014).

## 2.6 INFLUENCE OF VARIOUS ABIOTIC ELICITORS ON THE PRODUCTION OF SECONDARY PLANT COMPOUNDS

The effects of abiotic elicitors are studied in the following section. Abiotic elicitors were not much used in past years as compared to biotic elicitors (Radman *et al*., 2003). Studies have been done to observe the plant response towards different abiotic elicitors such as heavy metals, light, drought and many others (Rodziewicz *et al*., 2014).

### 2.6.1 PHYSICAL FACTORS

Physical factors such as ultrasound, light, osmotic stress, salinity, drought and thermal stress can be used.

#### 2.6.1.1 ULTRASOUND

US acts as an abiotic elicitor (Yu *et al*., 2015). It was studied that US treatment increased the production of shikonin 2-3 times in *Lithospermum erythrorhizon* cultures (Lin and Wu, 2002). In *Panax ginseng* culture, US increased production of saponins (Lin *et al*., 2001).

#### 2.6.1.2 LIGHT

It was observed that light increased the production of gingerol and zingiberene in culture of *Zingiber officinale* (Anasori and Asghari, 2008). Exposure light to light increased productivity of artemisinin in *Artemisia annua* (Liu *et al*., 2002).

#### 2.6.1.3 OSMOTIC STRESS

It is reported that osmotic stress influence growth of plants and secondary compound formation (Liu and Cheng, 2008). A study showed that in *Capsicum chinensis* cultures, the osmotic stress increased the productivity of capsaicin (kehie *et al*., 2014).

#### 2.6.1.4 SALT STRESS

When plants are given salinity conditions they accumulate secondary compounds like terpene, alkaloid and phenol (Selmar, 2008). It was reported that *Bacopa monnieri* cultures produced bacoside A content in large amounts when given potassium chloride and calcium chloride treatments (Ahire *et al*., 2014). Similarly, when sodium chloride treatment was provided to *Nitraria tangutorum* culture, sitosterol content was increased (Ni *et al*., 2015).

#### 2.6.1.5 DROUGHT STRESS

Drought can limit the growth in plants as well as their reproductive development. All plant species posses different tolerance from drought stress. A study showed that when plants were provided drought conditions, it lead to accumulation of secondary substances (Al-Gabbiesh *et al*., 2015). Polyethylene glycol was treated with date palm callus to induce drought stress. With increase in PEG concentration there was a increase in the content of proline also (Al and Ibraheem, 2014).

#### 2.6.1.6 THERMAL STRESS

Extreme temperature conditions can restrict the growth in plants by affecting their metabolic processes (Xu *et al*., 2013). To induce callus tissues a temperature range of 17-25 degree Celsius is used (Rao and Ravishankar, 2002). A study reported that the anthocyanin yield in the culture of *Perilla frutescens* was maximum at temperature 25 degree Celsius (Zhong and Yoshida, 1993). Similarly, ginsenoside content was increased in *Panax quinquefolius* by 5 degree increase in temperature (Jochum *et al*., 2007).

### 2.6.2 CHEMICAL ELICITORS

#### 2.6.2.1 METALS

Metals can be used to produce secondary substances in plants (Nasim and Dhir, 2010). It is thought to be an abiotic stress for plants due to their wide use in industries, agro-technology (Cai *et al*., 2013). A study reported that metals such as nickel, silver, iron and cobalt can elicit the secondary compound production in many plants (Wang and Wu, 2013). Cobalt was used in the culture of *Vitis vinifera* to increase production of phenolic acid (Cai *et al*., 2013).

### 2.6.3 HORMONAL ELICITORS

#### 2.6.3.1 SALICYLIC ACID

SA is an important hormonal elicitor. A study reported that in culture of *V. vinifera*, SA induced production of stilbene (Xu *et al*., 2015). Another study showed that SA increased production of tanshinones in *S. miltiorrhiza* hairy root culture (Hao *et*., 2015).

#### 2.6.3.2 JASMONIC ACID

JA is an important compound of pathway that can be used for elicitation (Jeandet *et al*., 2002). When the callus of *V. vinifera* was treated with mannitol and JA, maximum accumulation of resveratrol was observed (Raluca *et al*., 2011). MeJA is the active derivative of JA. Both can be used to increase the productivity of secondary compounds like terpenoid, alkaloid and rosmarinic acid in different cultures (Krzyzanowska *et al*., 2012).

#### 2.6.3.3 GIBBERELLIC ACID

Another a well known effective elicitor is Gibberellin (GA), a phytohormone that can also be used for producing plant secondary compounds (Liang *et al*., 2013).

### 2.7 SALT STRESS AND ITS EFFECT ON SECONDARY PLANT PRODUCTS

The saline conditions affects the plant growth, productivity and quality of the produce. Thus, salt stress is thought to be a major threat to the agriculture across worldwide (Zhu, 2007; Pattanagul and Thitisaksakul, 2008). The effects of salt stress vary depending on the different stages of plant development. Salinity has adverse effects on plant's physiological and biochemical processes that further leads to a decrease in biomass production (Ratnakar and Rai, 2013). Many scientists studied the plant responses to abiotic stress on various plants after an exposure to different concentrations of various salts.

Root length and the shoot length are the most imperative parameters for analyzing salt stress conditions as roots are in direct contact with soil. Roots ingest water from soil which is then translocated by shoots to rest of the plant (Assadi, 2009). Root plays an critical part in the development of the shoot under saline conditions as it is the main organ presented to saltiness (Lopez and Satti, 1996). In *Trigonella foenum-graecum*, the root length as well as the shoot length diminished with expanding levels of salt (Assadi, 2009). Jaleel *et al*.,(2008) likewise detailed a reduction in root length in *Catharanthus roseus* under saltiness. As indicated by Nawaz *et al*.,(2010), salt anxiety diminishes the capacity of plants to retain water which prompts decrease in development. In any case, Heidari *et al*., (2011), on the premise of their examinations

on *Helianthus annuus* recommended that lessening in plant development is because of diminishing turgor pressure in the cells under saline condition.

### 2.7.1 EFFECT OF SALT STRESS ON SECONDARY PLANT PRODUCTS

The growing conditions, especially the stress conditions have a great influence on the concentrations of various secondary plant products.

#### 2.7.1.1 ALKALOIDS

A study reported that the concentration of ricinine alkaloids in *Ricinus communis* was significantly reduced due to salt stress in its roots, but was increased in its shoots (Ali *et al*., 2008). Also, it was observed that there was a considerable increase in the alkaloid level of *Solanum nigrum* (solasodine) (Bhat *et al*., 2008), *Catharanthus roseus* (indole alkaloids) (Jaleel *et al*., 2008), and *Achillea fragratissima* (Abd EL-Azim and Ahmed, 2009) when exposed to salt stress. A study published that different concentrations of NaCl, Na2SO4, AlCl3 and CaCl2 stimulated the accumulation of tropane alkaloids (Ajungla *et al*., 2009).

#### 2.7.1.2 PHENOLIC COMPOUNDS

A study reported that in spearmint (Al-Amier and Craker, 2007) and *Matricaria chamomilla* (Cik *et al*., 2009), the phenolic acid concentration was increased when exposed to salt stressed conditions. Also, it was observed that there was an increase in the phenol content of *Nigella sativa* (Bourgou, 2010) and *Mentha pulegium* (Queslati, 2010) when exposed to saline treatment.

#### 2.7.1.3 TANNINS

A study published that in *Achillea fragratissima*, the tannin level was increased significantly with increasing saline conditions (Abd EL-Azim and Ahmed, 2009).

#### 2.7.1.4 ESSENTIAL OIL

It was reported that the essential oil yield was decreased due to salt stress in some medicinal plants, e.g. *Mentha piperita* (Tabatabaie and Nazari, 2007); basil (Said-Al and Mahmoud, 2010); apple mint; peppermint (Aziz and Al-Amier, 2008); *Trachyspermum ammi* (Ashraf and Orooj, 2006). Whereas, it was observed that in *Matricaria recutita*, the main essential oil constituents were increased under salt treatment (Baghalian *et al*., 2008).

It was shown that in *Satureja hortensis* (Baher *et al*., 2002) and *Salvia officinalis* (Hendawy and Khalid, 2005), the essential oil yield increased under lower levels of saline conditions. Also, in coriander leaves it was found that there was an increase in essential oil

content under low and moderate salt stress, while it decreased at high salt stress (Neffati and Marzouk, 2008). Essential oil yield was increased considerably with increasing NaCl concentrations in the case of coriander roots (Neffati and Marzouk, 2009; Petropoulos *et al*., 2009).

**2.7.1.5 CARDENOLIDES**

It was observed that in the leaves and roots of *Digitalis purpurea*, the total cardenolide content was higher in 100mM NaCl concentration than those in 200mM NaCl concentration and control (Morales *et al*., 1993).

**2.8 FACTORS WHICH INFLUENCE ELICITATION**

Some of the important factors that can affect the elicitor-plant cell interaction and hence the elicitation response are as follows:

**2.7.1 ELICITOR SPECIFICITY**

Elicitors are specific to each plant culture. If we treat a particular culture with different elicitors also we will observe the accumulation of same compounds only. The induction kinetics or level of accumulation may vary with different elicitors but, the metabolite class depends on the plant species. A study reported that elicitation by biotic or abiotic compounds in *Brugmansia candida* hairy root culture showed differences in induction kinetics and release of hyoscyamine and scopolamine levels (Pitta-Alvarez *et al.*, 2000).

**2.7.2 CONCENTRATION OF ELICITOR**

The concentration of elicitor strongly affects the response intensity and dose effectiveness. The elicitor concentration may vary according to the plant species. A study reported that at a concentration of 0.5 percent sodium chloride productivity of ginseng saponin content increased 1.13 times the control value (Jeong and Park, 2007). Another report showed that yield of glycyrrhizic acid in *Taverniera cuneifolia* root cultures increased to 2.5 times when treated with MeJA of 100 microM concentration (Awad *et al* ., 2014).

**2.7.3 TREATMENT INTERVAL**

Each plant species require different time interval for maximum metabolite accumulation. It is generally preceded by an increased metabolic enzymatic activity. A study described that the anthraquinone content was doubled when *Pythium aphanidermatum* was used to treat *Rubia tinctorum* cell cultures which was preceded by an increase in the activity of isochorismate synthase (Van Tegelen, 1999).

### 2.7.4 GROWTH STAGE

The elicitor should be added during the log phase of growth when the activity of enzyme is maximum (Vasconsuelo *et al*.; 2003). Hence, an efficient response can be achieved.

### 2.7.5 COMPOSITION OF MEDIUM

The growth factors in the medium also affect the elicitation. A study showed that if auxin is not provided in the medium, the carrot cells in the culture do not respond to elicitation (Kurosaki *et al*., 1985).

### 2.7.6 LIGHT CONDITIONS

The light conditions of culture also plays a major role. A study reported the production of hypericin in *Hypericum perforatum* L. cells showed higher cell growth under light than when incubated in the dark (Walker *et al*., 2002).

## 2.8 MECHANISM OF ELICITATION

Escalated inquire about has been committed to build up the component of elicitation in plants. While getting elicitor signals, plant receptors are initiated, and afterward thus enact their effectors, for example, particle channels, GTP restricting proteins (G-proteins), and protein kinases. Enacted effectors exchange the elicitor signs to second dispatchers, which additionally intensify the elicitor flag to other downstream responses (Ebel and Mithoefer, 1998; Blume et al., 2000) takes after impression of elicitor by receptor, reversible phosphorylation and dephosphorylation of plasma layer proteins and cytosolic proteins, cytosolic [Ca2+] cyt spiking, plasma film depolarization, Cl-and K+ efflux/H+ inundation, extracellular alkalinization and cytoplasmic fermentation, mitogen-actuated protein kinase (MAPK) initiation, NADPH oxidase enactment and receptive oxygen species (ROS) creation, early guard quality expression, ethylene and jasmonate generation, late protection reaction quality expression, and optional metabolite collection.

### 2.8.1 MECHANISM OF PLANT SALT RESPONSE

The unfavorable impacts of salt anxiety have been accounted for at cell, organ and entire plant level. It is seen at osmotic stage (early/short term response) and ionic stage (late/long term response) (Muchate et al., 2016). The review distributed that a negative effect is applied by high saline conditions on plant procedures, for example, cell osmolarity, ionic balance, photosynthesis, vitality digestion, protein union and lipid digestion.

Plants have developed some physiological and biochemical systems to adjust and endure salt anxiety. These instruments are coordinated by various bio-synthetic pathways of osmotic tweak, particle homeostasis, ROS searching and hormonal adjust. With regards to the sub-atomic level, adjustment is managed by enactment of falls of quality regulations and union of 'defence metabolites'.

# CHAPTER-3

# OBJECTIVES

The aim of the present work is "Phytochemical screening of bioactive compounds under abiotic elicitation of Tulsi (*Ocimum tenuiflorum*)".

The outline of the work is presented below:

- Preliminary Phytochemical tests for qualitative analysis of various bioactive compounds in *O. tenuiflorum* leaf extract.
- Quantification tests to determine amount of phenolic and flavonoid contents in the leaf extract.
- *In vitro* culture of *O. tenuiflorum.*
- Introducing Abiotic elicitation (Salt stress by NaCl) in *in vitro* culture of *O. tenuiflorum* to enhance the production of secondary metabolites.
- Quantitative analysis of salt stressed plants.
- Comparison of in vivo and in vitro results of quantitative metabolite analysis.

# CHAPTER-4

# METHODOLOGY

## 4.1 SAMPLE COLLECTION

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

## 4.2 EXTRACTION OF THE SAMPLE

Plant material (leaves) was sun dried for 2 weeks and homogenized using mortar and pestle as shown in **figure 4.1**. The powdered extract was used for the analysis of bioactive compounds in *O. tenuiflorum*.

**Figure 4.1 Represents powdered leaf extract of *O. tenuiflorum.***

## 4.3 QUALITATIVE ANALYSIS OF THE SAMPLE

Qualitative tests are performed to detect the various active constituents in the leaf extract.

### 4.3.1 FLAVONOIDS (Sofowara and Nigeria, 1993; Harborne, 1973)

**Reagents:**

3millilitre of dilute ammonia

1millilitre of conc. Sulphuric acid

**Procedure:**

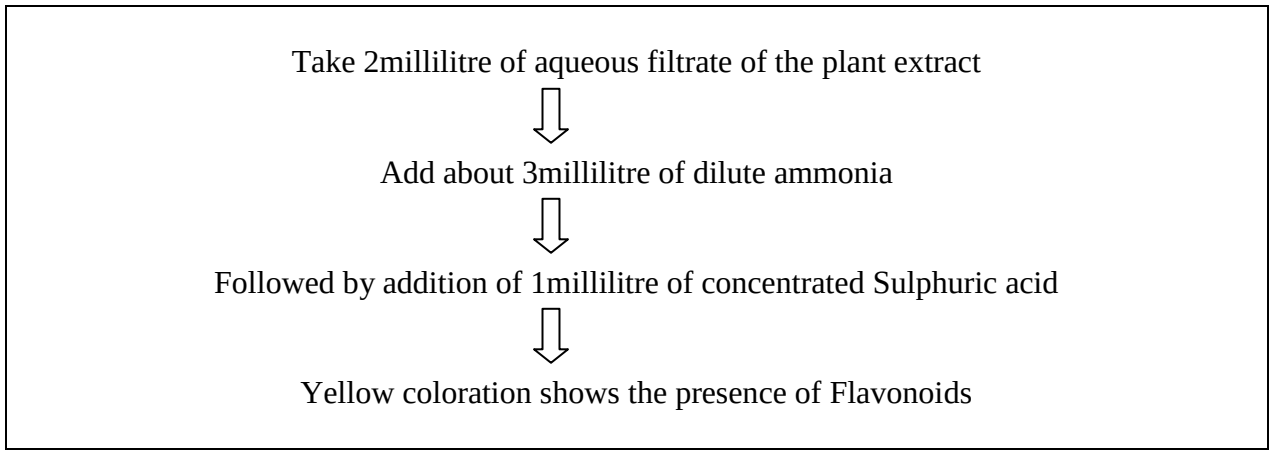



**Figure 4.2 Flowchart representation for Flavonoids test**

**4.3.2 ALKALOIDS (Sofowara and Nigeria, 1993; Harborne, 1973)**

**Reagents:**

2millilitre of hexane

3millilitre 2% HCl

a drop of picric acid

**Procedure:**

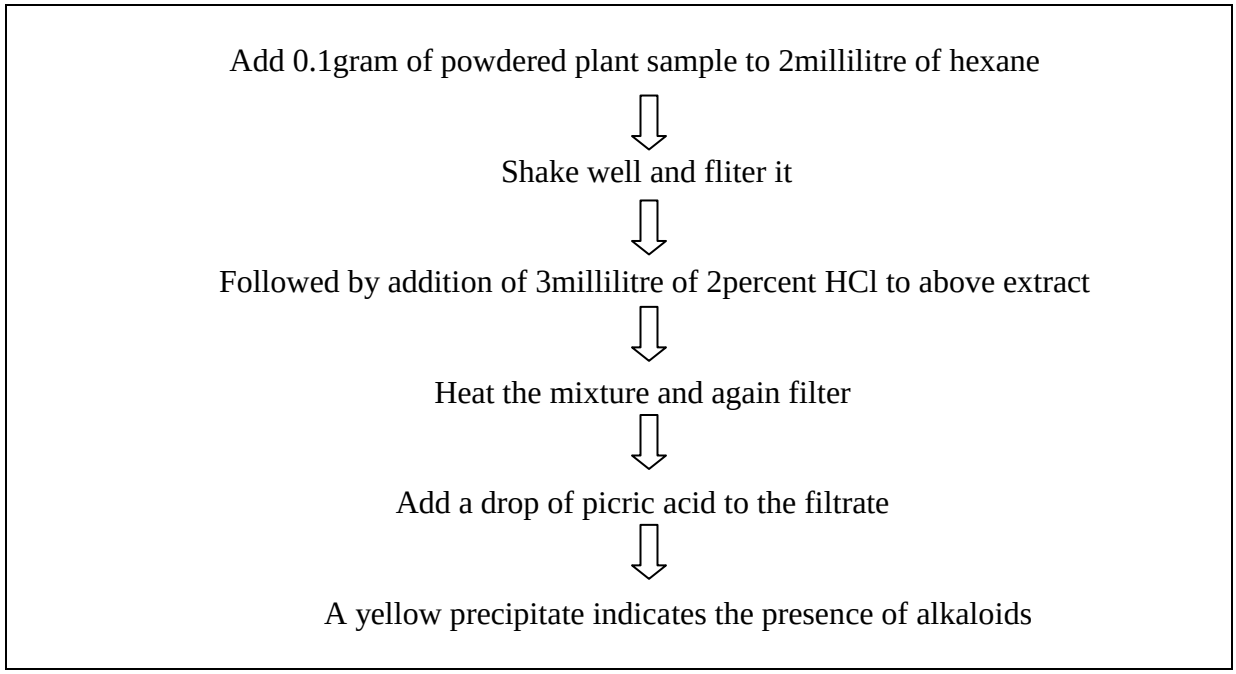



**Figure 4.3 Flowchart representation for Alkaloids test**

### 4.3.3 TERPENOIDS (Salkowski test) (Edeoga, 2005)

**Reagents:**

1millilitre of chloroform

1millilitre of conc. Sulphuric acid

**Procedure:**

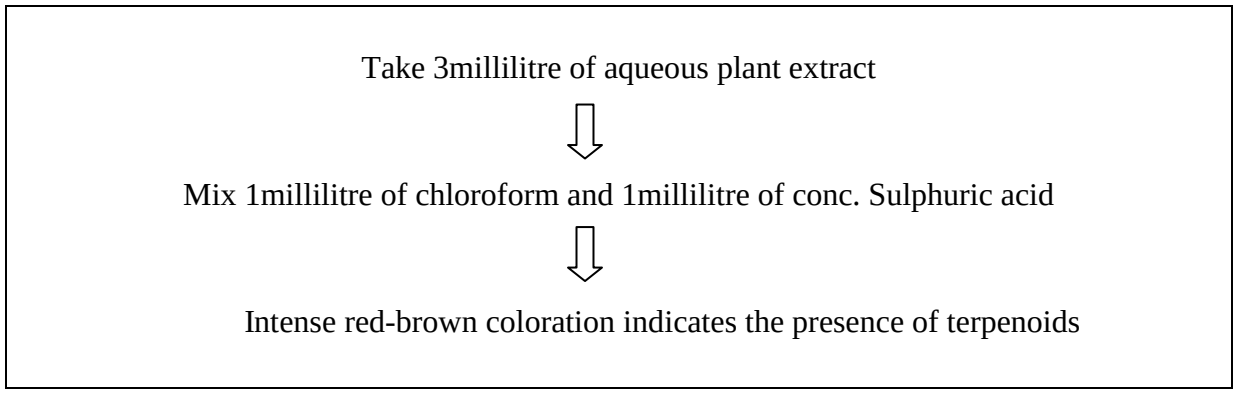



**Figure 4.4 Flowchart representation for Terpenoids test**

### 4.3.4 REDUCING SUGARS (Fehling's test) (Ayoola, 2008)

**Reagents:**

1 milliliter ethanol

1 millilitre of Fehling's solution A and B

**Procedure:**

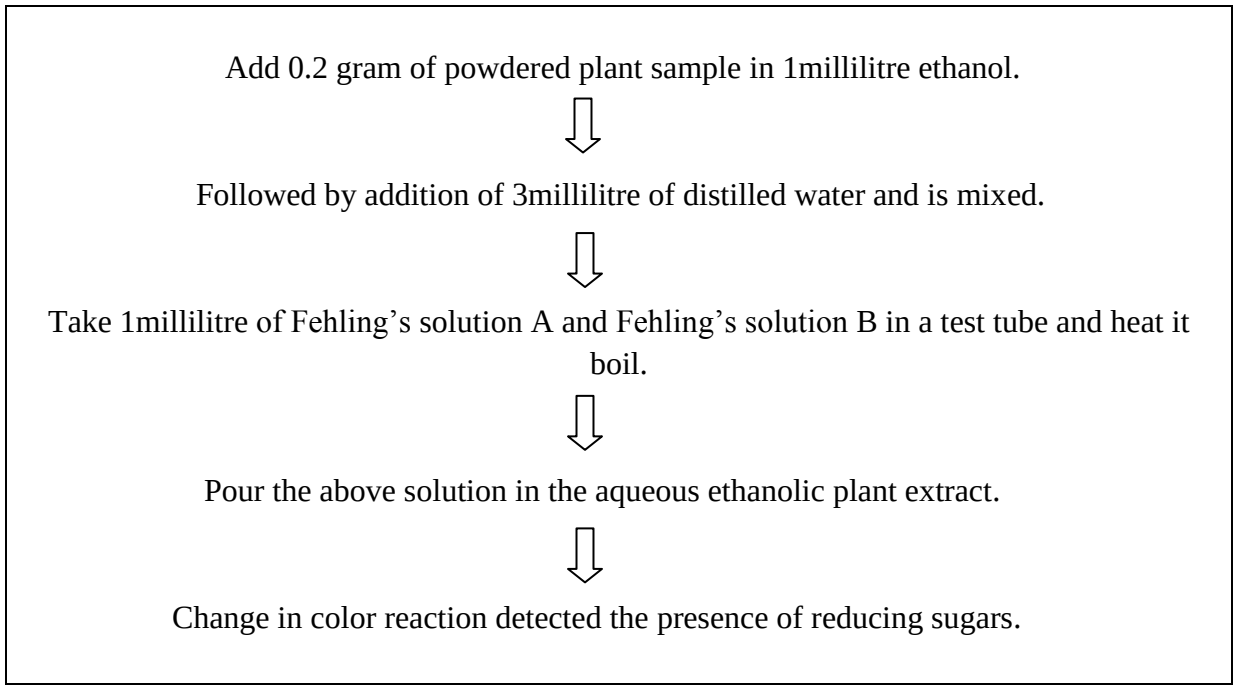



**Figure 4.5 Flowchart representation for Reducing sugar test**

### 4.3.5 SAPONINS (Edeoga, 2005)

**Reagents:**

3 drops of saturated oil (ghee)

**Procedure:**

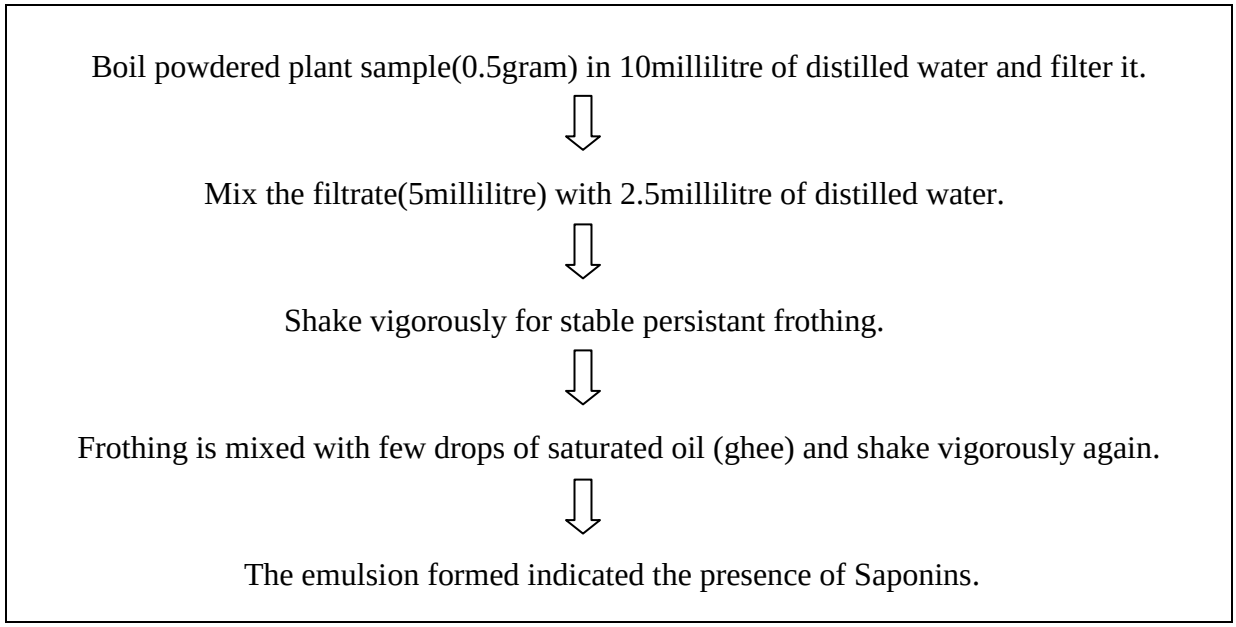



**Figure 4.6 Flowchart representation for Saponins test**

### 4.3.6 TANNINS (Edeoga, 2005)

**Reagents:**

0.1 percent ferric chloride (few drops)

**Procedure:**

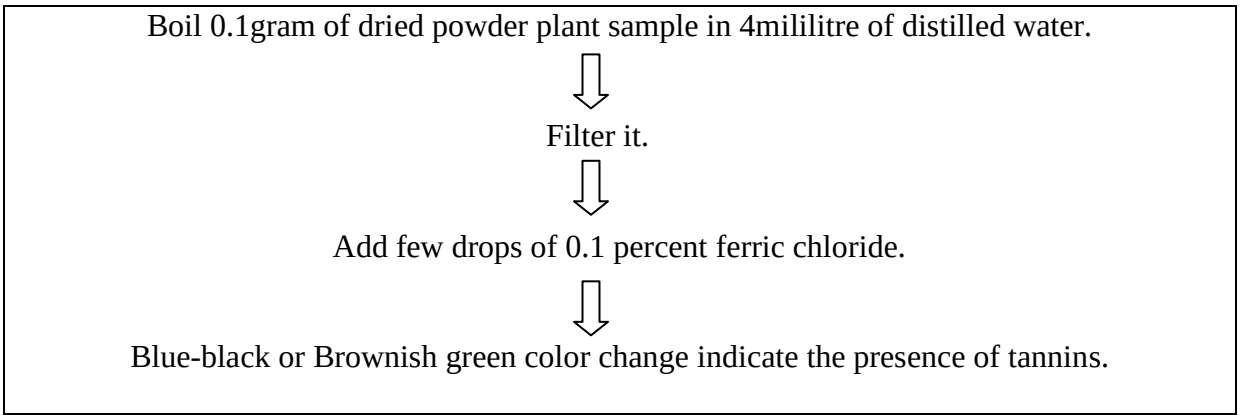



**Figure 4.7 Flowchart representation for Tannins test**

### 4.3.7 PHLOBATANNINS (Edeoga, 2005)

**Reagents:**

1 percent aqueous HCl (2-3 drops)

**Procedure:**

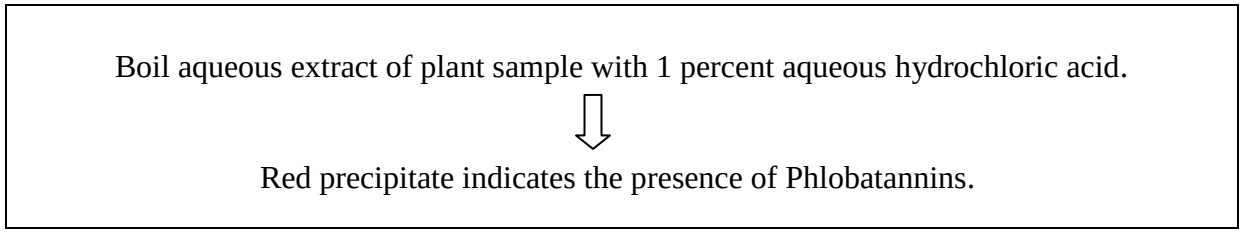



**Figure 4.8 Flowchart representation for Phlobatannins test**

### 4.3.8 ANTHRAQUIONES (Ayoola, 2008)

**Reagents:**

4millilitre conc. Sulphuric acid

3millilitre of chloroform

1millilitre of diluted ammonia

**Procedure:**

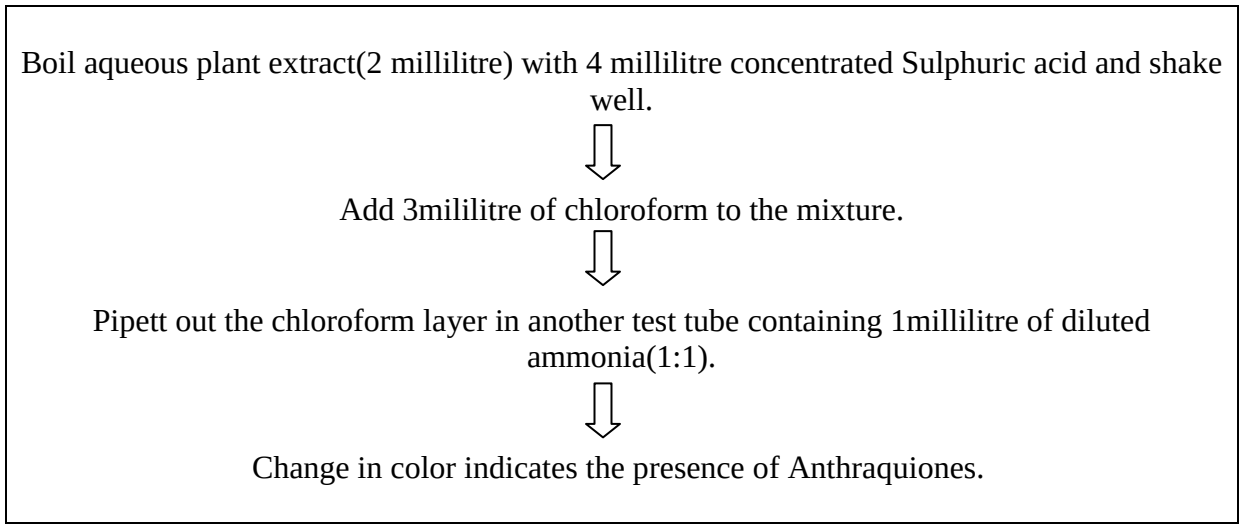



**Figure 4.9 Flowchart representation for Anthraquiones test**

## 4.4 QUANTITATIVE ANALYSIS OF THE SAMPLE

Qualitative tests are performed to determine the contents of active compounds present. Plant material (leaves) was sun dried for 2 weeks. Further it was placed overnight in 1litre of methanol. Use Whatman filter paper to filter the extract. This methanolic plant extract was used for further quantification tests.

**4.4.1 TOTAL PHENOLIC CONTENT:** It was analysed by Folin-Ciocalteu(FC) reagent (Mallick and Singh, 1980).

**Reagents:**

0.5 millilitre of FC reagent

2 millilitre of 20% sodium carbonate

gallic acid

**Procedure:**

Mix plant extract (0.5mililitre) with 0.5 mililitre of FC reagent (1:1 diluted with distilled water).

⇩

Incubate for 5minutes at 22 degree Celsius.

⇩

Followed by addition of 2mililitre of 20 percent sodium carbonate.

⇩

Further incubate the mixture at 22 degree Celsius for 90 minutes.

⇩

Measure the absorbance at 650 nanometre.

⇩

Calculate the total phenolics content (mg/ml) using gallic acid as standard.

**Figure 4.10 Flowchart representation for determining total phenolics content**

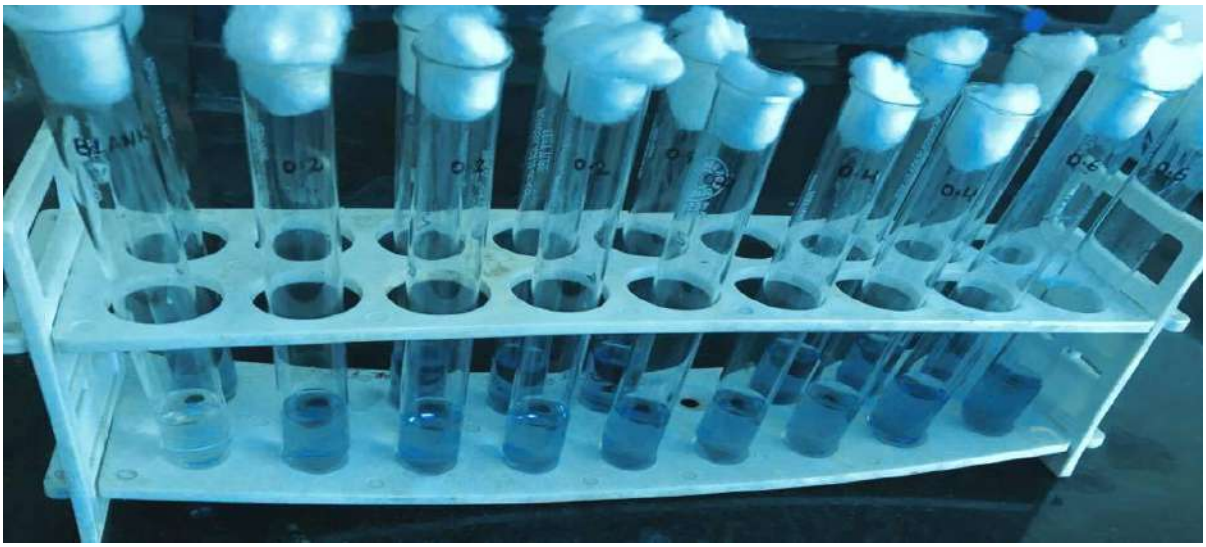


**Figure 4.11 Represents standard solution of gallic acid.**

**4.4.2 TOTAL FLAVONOIDS CONTENT:** It was determined by Aluminium chloride method (Kariyone et al., 1953; Naghski et al., 1951).

**Reagents:**

0.3 millilitre of 5 percent sodium nitrite

8.3 milliliter of 10 percent aluminium chloride

2 millilitre of 1Molar NaOH

Quercetin

**Procedure:**

Mix plant extract (0.5 millilitre), distilled water (0.5 millilitre ) and 5 percent sodium nitrite 0.3 millilitre).

⇩

Incubate for 5min at 25 degree Celsius.

⇩

Followed by addition of 8.3 millilitre of 10 percent aluminium chloride immediately.

⇩

Add 2 millilitre of 1Molar sodium hydroxide to the reaction mixture.

⇩

Measure the absorbance at 510 nanometre.

⇩

Calculate the total flavonoid content by quercetin as standard.

**Figure 4.12 Flowchart representation for determining total flavonoid content**


**Figure 4.13 Represents standard solution of quercetin.**

## 4.5 PLANT TISSUE CULTURE

Murashige and Skoog (MS) is the most commonly used media for plant tissue culture. MS medium have a very high concentration of potassium, ammonia and nitrate that made it common in plant tissue culture.

### 4.5.1 MEDIA PEPARATION

It is the first step in the plant propagation by plant tissue culture (Samantha and Julie, 2012).

**Chemicals and reagents:**

Murashige and Skoog media

3.0 percent sucrose

0.8 percent agar

HCl or NaOH (for pH adjustment)

Salt (NaCl)

**Procedure:**

Firstly, make a total of 600mL of MS agar using 3 percent sucrose, MS salts and distilled water.

⇩

The pH of the medium is maintained to 5.7 plus-minus 0.1 with Hydrochloric acid or sodium hydroxide followed by addition of 0.8 percent agar.

⇩

About 100mL of MS Agar was then prepared for the following six concentrations of sodium chloride: 0mM (negative control), 50mM, 100mM, 125mM, 150mM, and 200mM.

⇩

1-2 percent agar was added to each media.

⇩

The media is then autoclaved at 121 degree Celsius , 15 pounds per square inch for 15 minutes.

⇩

Sterile medium is poured in Petri plate in the laminar flow, and is left to solidify.

**Figure 4.14 Represents the flowchart for Media Preparation.**

### 4.5.2 POURING

The media after autoclaving is poured in the petri plates inside the laminar cabinet under aseptic conditions. The pouring temperature of the media should be 60 degree Celsius.

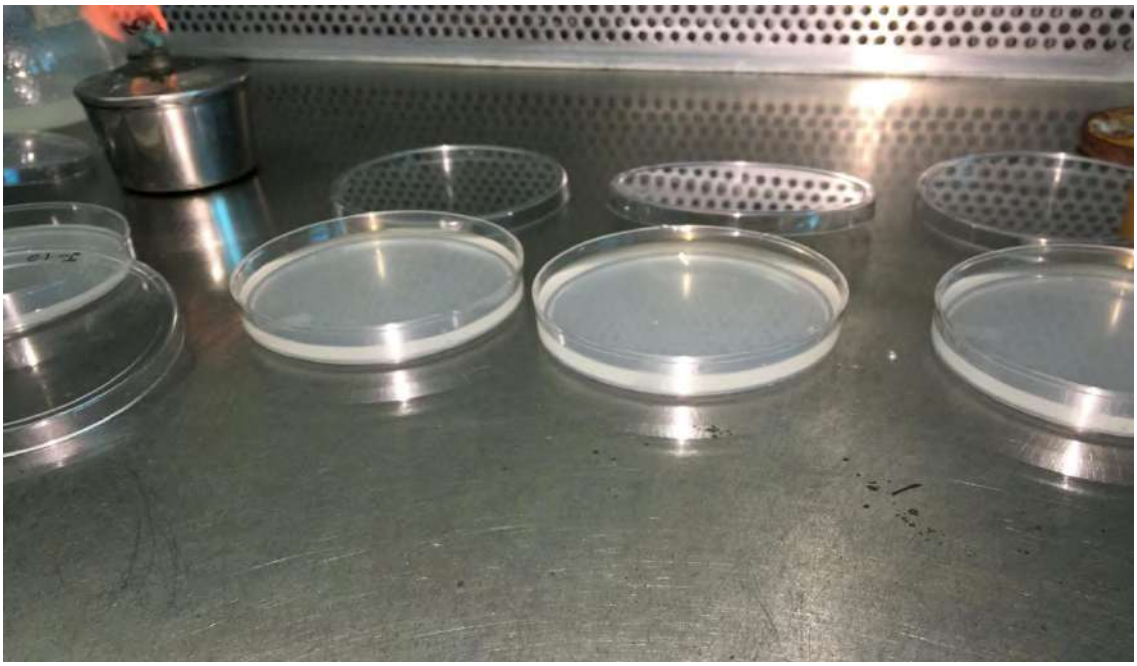
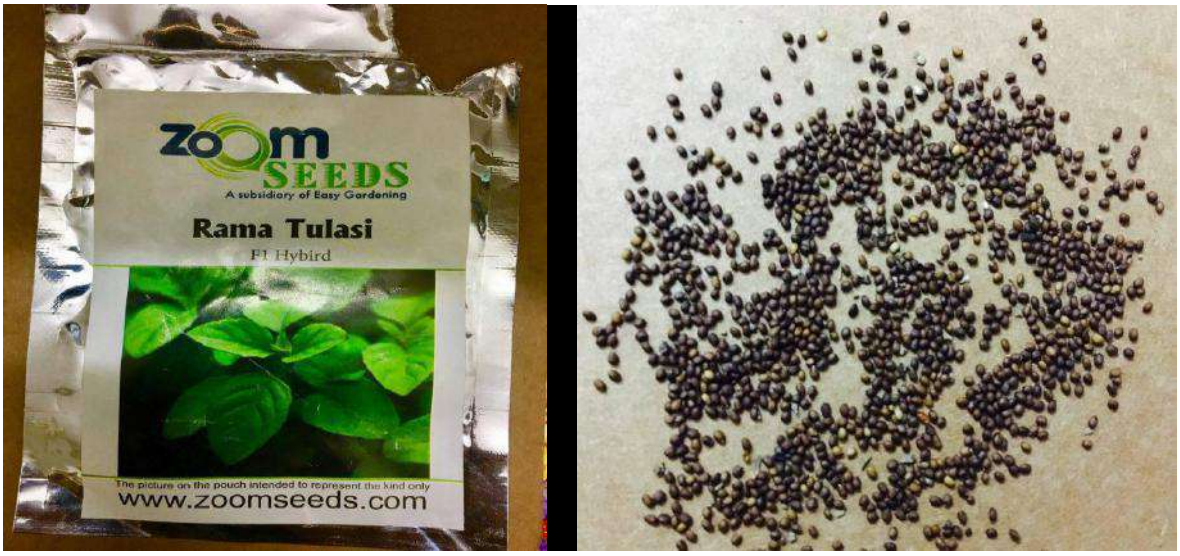


**Figure 4.15 Represents pouring of the media.**

### 4.5.3 SEED COLLECTION

Seeds of *Ocimum tenuiflorum* were collected online from eBay Stores.




**(a)** **(b)**

**Figure 4.16(a) Represents packet of seeds of Rama Tulasi (*Ocimum tenuiflorum*).**

**(b) Represents seeds of *Ocimum tenuiflorum*.**

### 4.5.4 SURFACE STERILIZATION OF SEEDS


**Figure 4.17 Represents surface sterilization of seeds of *Ocimum tenuiflorum.***

**Reagent used:**

2 drops of Tween-20

3-4ml 70 percent Ethanol

25ml sodium hypochlorite

**Procedure:** (Sauer and Burroughs, 1986)

Firstly, the seeds are washed with Tween-20 (2 drops) for 5-10 minutes.

⇩

Rinse 3-4 times with autoclaved water.

⇩

Then, seeds are washed with 70 percent ethanol for 4-5 minutes.

⇩

Again rinse the seeds 3-4 times with autoclaved water.

⇩

Lastly, seeds are washed with sodium hypochloride for 15-20 minutes.

⇩

Rinse with sterile distilled water several times until all traces of sodium hypochlorite are eliminated under aseptic conditions in laminar flow chamber.

⇩

Then, the seeds were transferred onto the Whatman filter paper I and dried prior to inoculation.

**Figure 4.18 Represents flowchart for the surface sterilization of seeds.**

### 4.5.5 INOCULATION

This is done by placing sterilized seeds on contol MS agar plates and plates containing various concentrations of NaCl (0mM, 25mM, 50mM, 100mM, 125mM and 200mM), two plates for each concentration (Samantha and Julie, 2012) with the help of sterilized forceps under the aseptic conditions.

**(a)** **(b)**

**Figure 4.19(a) Represents inoculation of seeds. (b) Represents inoculated petri plates.**

### 4.5.6 INCUBATION

The inoculated plates are wrapped in black charts and kept under dark for 3-4 days. As the shoot begins to develop, the plates are transferred to another room where light and temperature are strictly controlled. It is called culture room. The temperature of the room is maintained at 25 plus-minus 1 degree Celsius under white fluorescent tubes with the intensity of 3000-3200 lux (Aarifa *et al*., 2013). The cultures are incubated for 15-20 days and daily observations are made. Morphogenetic of shoot regeneration in cultures can be observed.

3-5 cm of well proliferated shoots are used for further quantitative metabolite analysis.

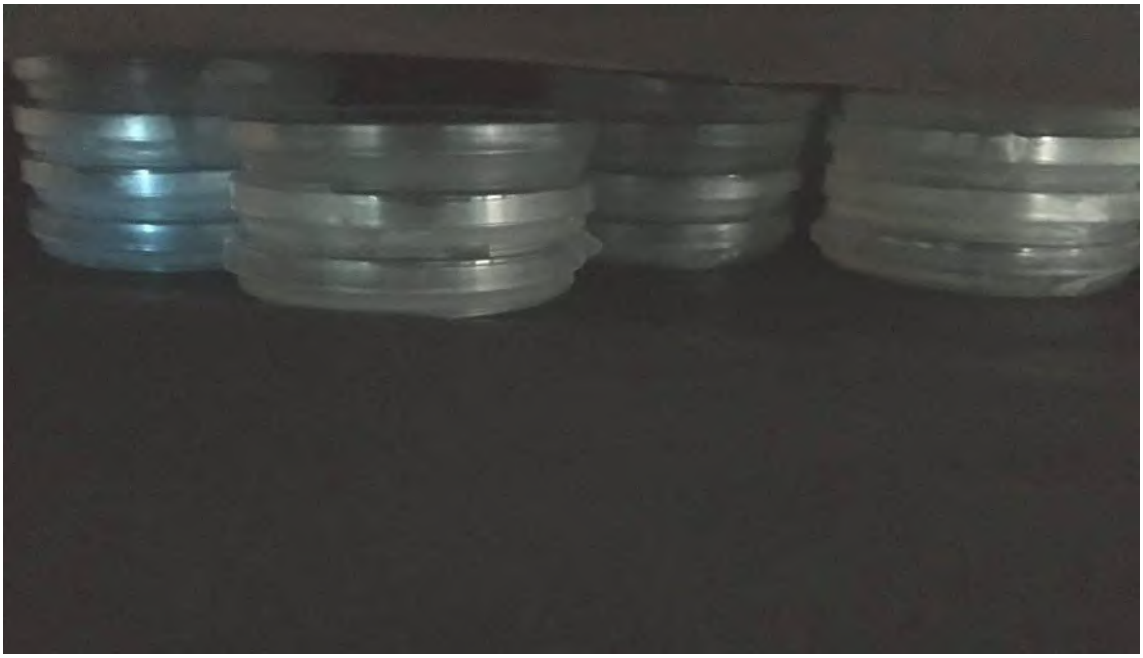


**Figure 4.20 Represents plates incubated at dark.**

### 4.5.8 QUANTITATIVE METABOLITE ANALYSIS:

### TOTAL PHENOLIC CONTENT:

This is analysed by FC reagent (Mallick and Singh, 1980).

### TOTAL FLAVONOID CONTENT:

This is analysed by aluminium chloride method (Kariyone *et al*., 1953; Naghski *et al*., 1951).

### 4.5.9 COMPARISON OF IN VIVO AND IN VITRO ON THE BASIS OF QUANTITATIVE METABOLITE ANALYSIS

Both in vivo and in vitro results are analysed and compared.

# CHAPTER 5

# RESULTS AND DISCUSSION

Plants produce different bioactive compounds as anti-oxidants. *Ocimum tenuiflorum* is known for its vast applications in pharmaceuticals, flavour industry (Cusido *et.al.*,2013). Hence qualitative analysis of the leaf extract of *O. tenuiflorum* was performed by various phytochemical tests followed by quantitation of total phenolic and flavonoid contents spectrophotometrically.

## 5.1 QUALITATIVE TESTS

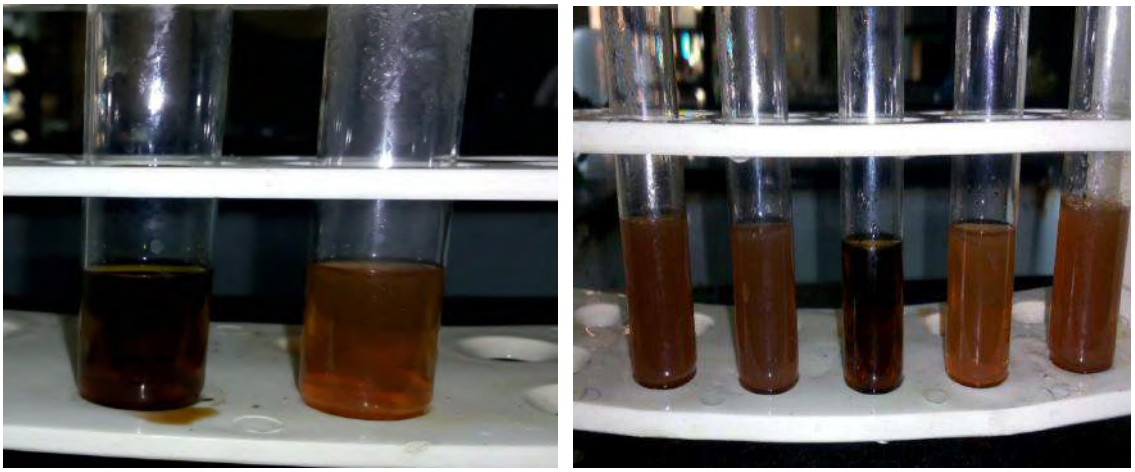
Qualitative analysis of *O. tenuiflorum* leaf extracts showed the presence of flavonoids, alkaloids, reducing sugars, saponins, tannins and anthraquions and absence of terpenoids and phlobatannins. **Table 5.1** shown below represents the results of qualitative tests.

| **PHYTOCHEMICALS TESTS** | ***OCIMUM TENUIFLORUM* (LEAF)** |
|---|---|
| Flavonoids | Positive |
| Alkaloids | Positive |
| Terpenoids | Negative |
| Reducing sugars | Positive |
| Saponins | Positive |
| Tannins | Positive |
| Phlobatannins | Negative |
| Anthraquiones | Positive |

**Table 5.1 Represents the results of qualitative tests.**

### 5.1.1 FLAVONOIDS

Each extract showed yellow coloration that indicated the presence of flavonoids (Sofowara and Nigeria, 1993; Harborne, 1973). Hence, the results were positive. **Figure 5.1(a) and (b)** below represents the color change in the extract.



**(a)** **(b)**

**Figure 5.1(a) and (b) Represents yellow coloration in each extract.**

The above result is proved by Sofowara and Nigeria in 1993 and Harborne in 1973 (Sofowara and Nigeria, 1993; Harborne, 1973). The results are also supported by the report of Sangeeta and Vrunda in 2016 (Sangeeta and Vrunda, 2016).

### 5.1.2 ALKALOIDS

Yellow layer was formed in each extract which showed that alkaloids are present (Sofowara and Nigeria, 1993; Harborne, 1973). Hence, the results were positive. **Figure 5.2** shown below represents the formation of yellow layer.

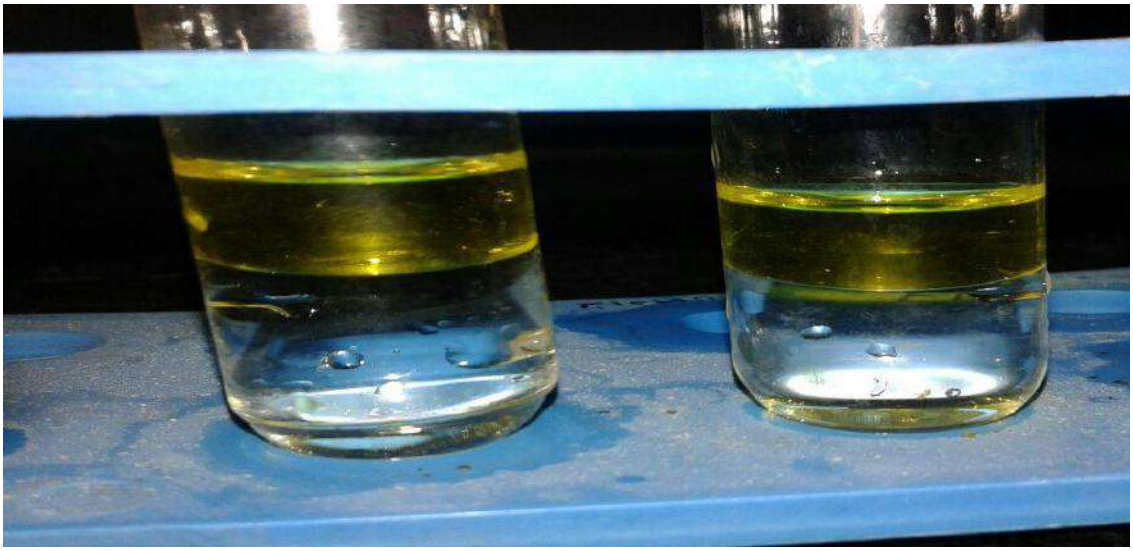


**Figure 5.2 Represents formation of yellow layer in the extract.**

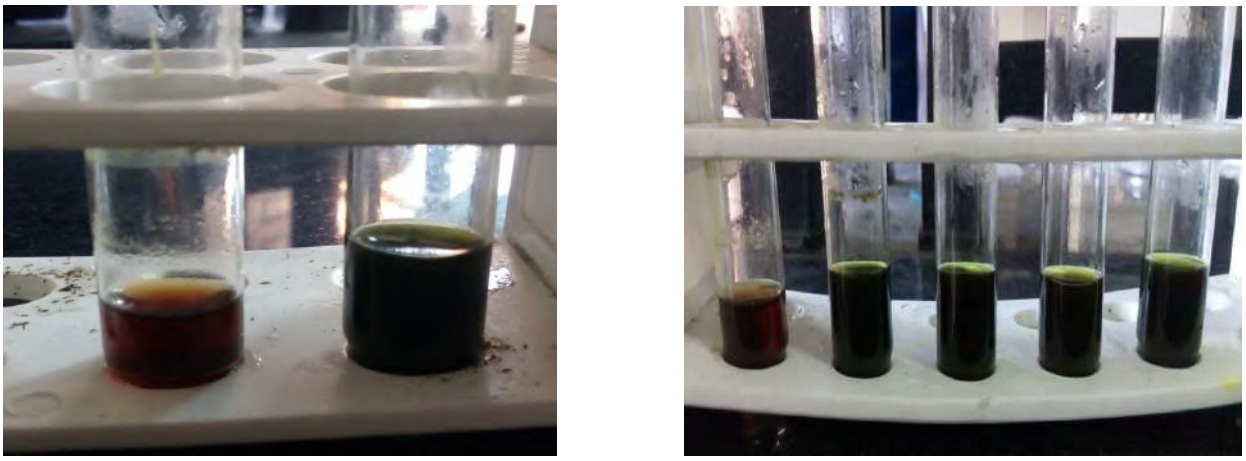
The above result is proved by Sofowara and Nigeria in 1993 and Harborne in 1973 (Sofowara and Nigeria, 1993; Harborne, 1973). The results are also supported by the report of Sangeeta and Vrunda in 2016 (Sangeeta and Vrunda, 2016).

### 5.1.3 TERPENOIDS (Salkowski test)

The leaf extracts showed no intense red-brown coloration that is considered to be the indicative of the presence of terpenoids (Edeoga, 2005). Hence, the results were negative. The result is supported by the report of Sangeeta and Vrunda in 2016 (Sangeeta and Vrunda, 2016).

### 5.1.4 REDUCING SUGARS (Fehling's test)

Change in color reaction detected the presence of reducing sugars (Ayoola, 2008). Hence, the results were positive. **Figure 5.3 (a) and (b)** shown below represents the change in color reaction.



**(a)** **(b)**

**Figure 5.3 (a) and (b) Represents the change in color reaction.**

The above result is proved by Ayoola in 2008 (Ayoola, 2008). The results are also supported by the report of Sangeeta and Vrunda in 2016 (Sangeeta and Vrunda, 2016).

### 5.1.5 SAPONINS

Emulsions were formed in each extract which indicated the presence of saponins (Edeoga, 2005). Hence, the results were positive. **Figure 5.4** shown below represents the formation of emulsion in each extract.

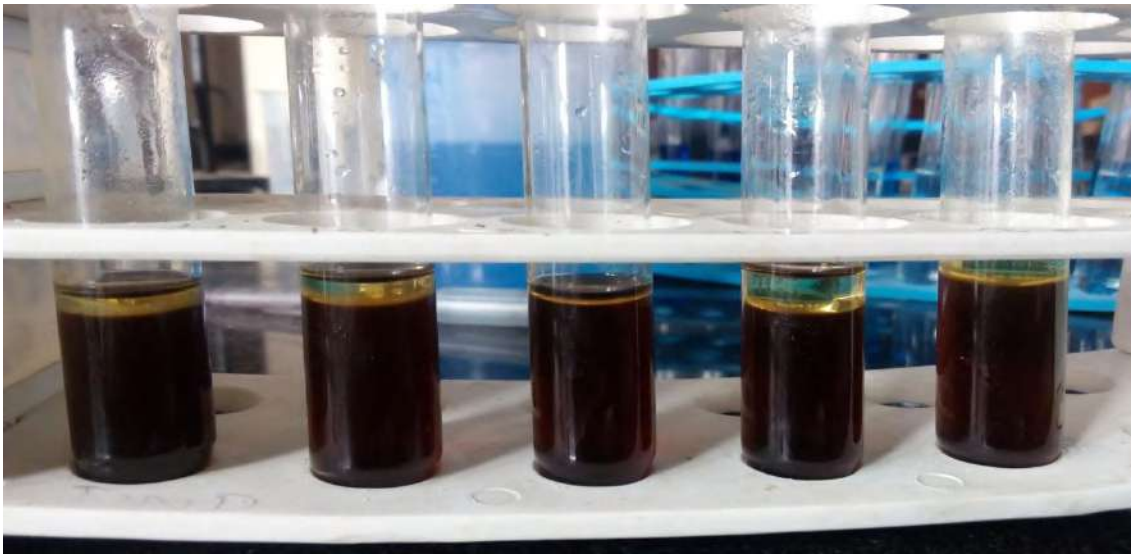


**Figure 5.4 Represents the formation of emulsion in each extract.**

The above result is proved by Edeoga in 2005 (Edeoga, 2005). The results are also supported by the report of Sangeeta and Vrunda in 2016 (Sangeeta and Vrunda, 2016).

### 5.1.6 TANNINS

Blue-black or brownish-green color change indicated that tannins are present (Edeoga, 2005). Hence, the results were positive. **Figure 5.5** shown below represents the brownish green coloration in each extract.

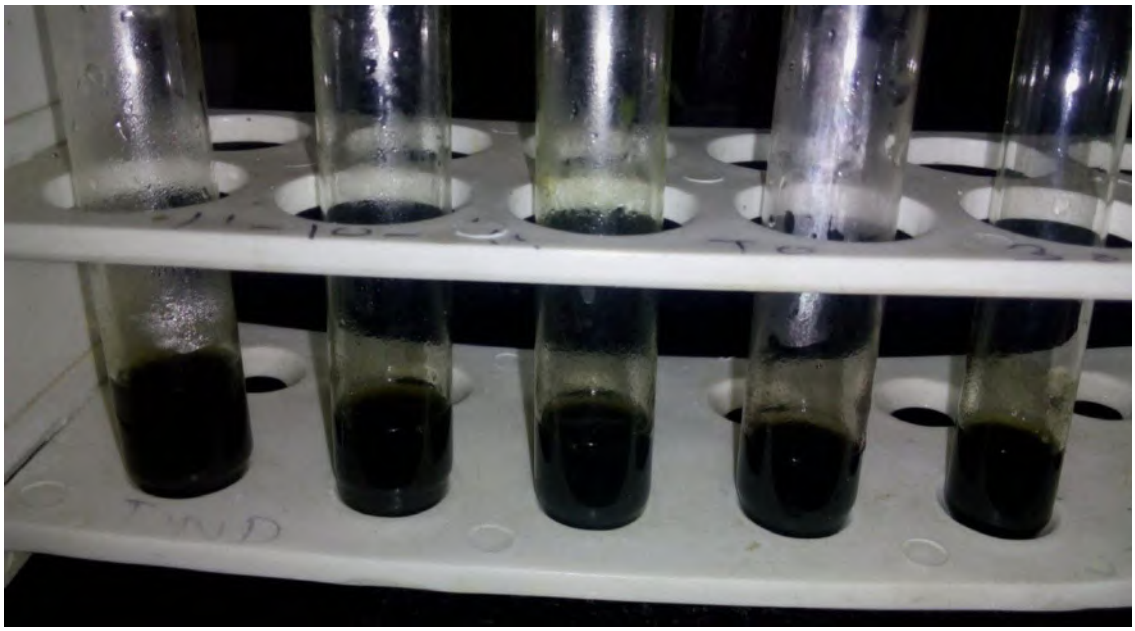


**Figure 5.5 Represents the brownish green coloration in each extract.**

The above result is proved by Edeoga in 2005 (Edeoga, 2005). The results are also supported by the report of Sangeeta and Vrunda in 2016 (Sangeeta and Vrunda, 2016).

### 5.1.7 PHLOBATANNINS

There was no deposition of red precipitate which indicated the absence of phlobatannins in each extract (Edeoga, 2005). Hence, the results were negative. The above result is proved by Edeoga in 2005 (Edeoga, 2005). The results are also supported by the report of Sangeeta and Vrunda in 2016 (Sangeeta and Vrunda, 2016).

### 5.1.8 ANTHRAQUIONES

A chloroform layer is formed above the mixture which is pipette out in another test tube containing ammonia (1:1). Anthraquiones were detected with a change in color (Ayoola, 2008). Hence, the results were positive. **Figure 5.6** shown below represents the formation of chloroform layer.

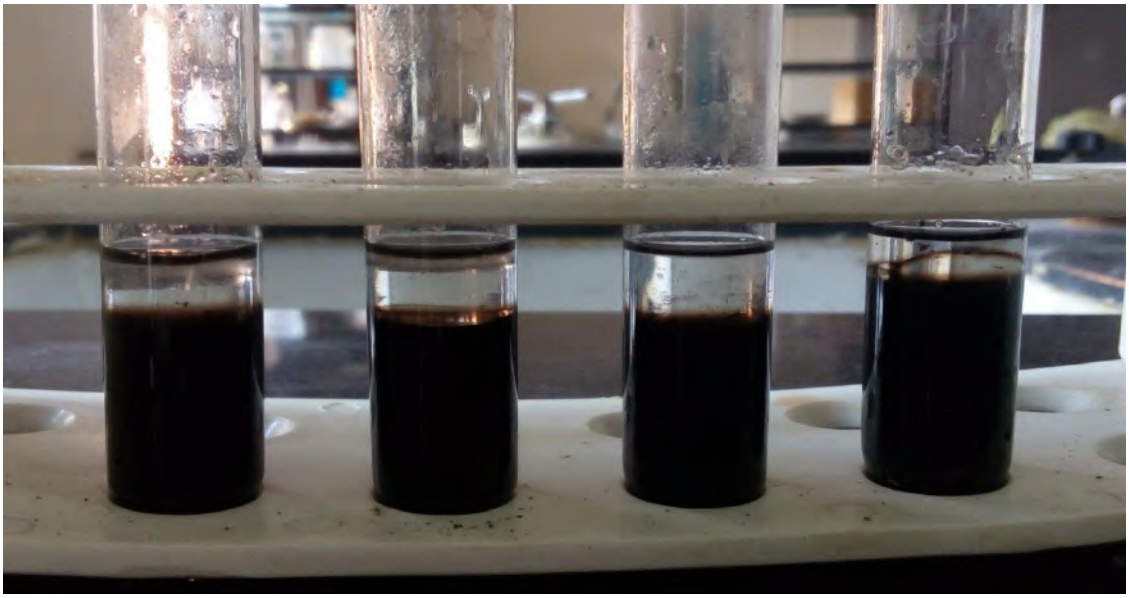


**Figure 5.6 Represents the chloroform layer formed in each extract.**

The above result is proved by Ayoola in 2008 (Ayoola, 2008). The results are also supported by the report of Sangeeta and Vrunda in 2016 (Sangeeta and Vrunda, 2016).

## 5.2 QUANTITATIVE TESTS ( INVIVO)

Formula used : $\frac{\text{O.D of Test}}{\text{O.D of standard}} \times \text{Concentration of standard} \times \frac{\text{Volume made}}{\text{Volume taken}} \times \frac{1}{\text{weight}}$

### 5.2.1 TOTAL PHENOLIC CONTENT

It was observed that the total phenolic content in *O. tenuiflorum* is 0.58 mg/ml (**Table 5.2**). **Figure 5.7** represents the graphical representation of standard Gallic acid.

The above result is proved by Ayoola in 2008 (Ayoola, 2008). The results are also supported by the report of Sangeeta and Vrunda in 2016 (Sangeeta and Vrunda, 2016).

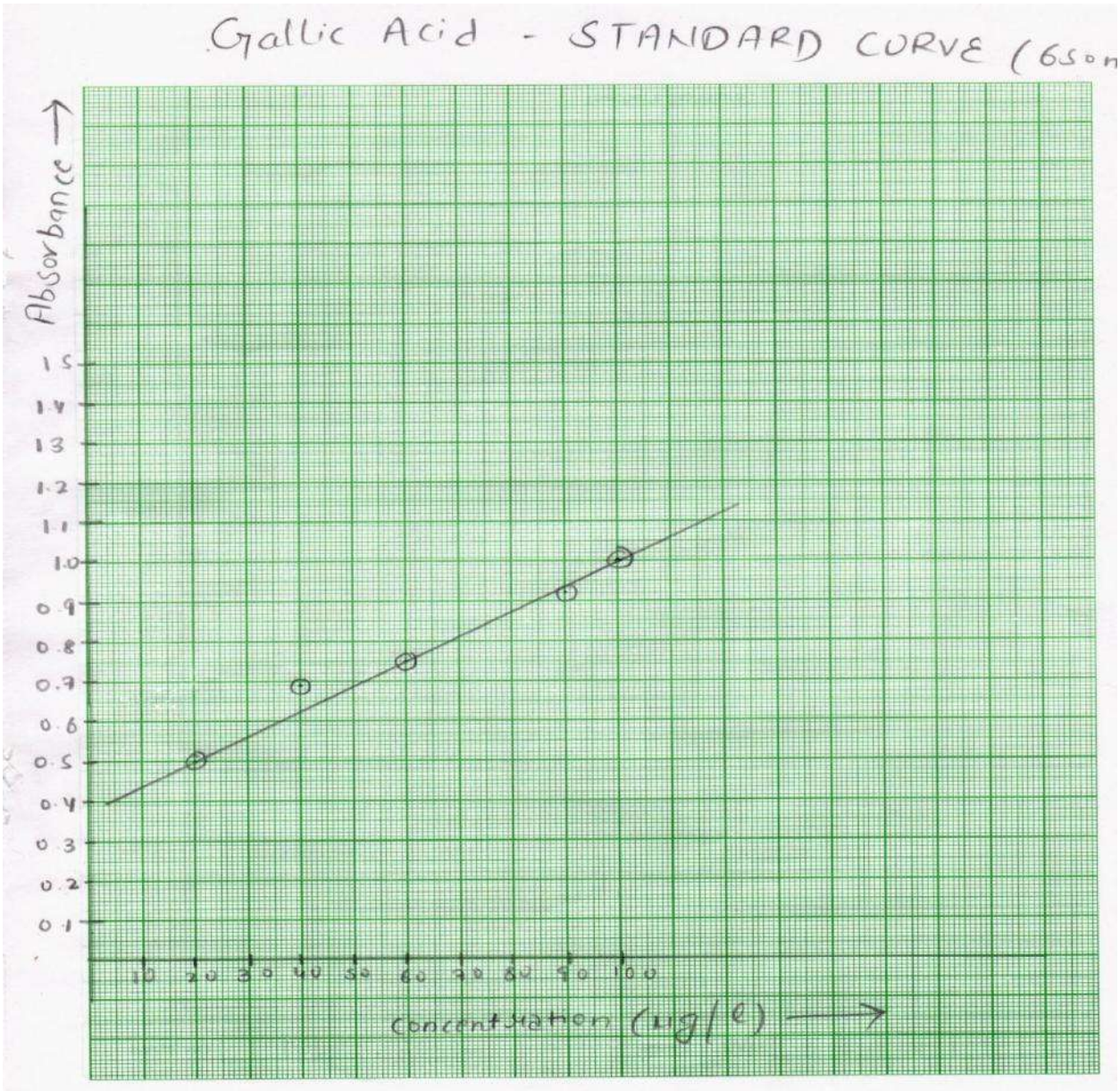



**Figure 5.7 Graphical representation of standard Gallic acid.**

### 5.2.2 TOTAL FLAVONOID CONTENT

It was observed that the total flavonoid content in *O. tenuiflorum* is 4.38 mg/ml (T**able 5.2**). **Figure 5.8** Represents the graphical representation of standard Quercetin.

The above result is proved by Ayoola in 2008 (Ayoola, 2008). The results are also supported by the report of Sangeeta and Vrunda in 2016 (Sangeeta and Vrunda, 2016).

| Plant species | Total phenolic content (mg/ml) | Total flavonoid content (mg/ml) |
|---|---|---|
| *Ocimum tenuiflorum* | 0.58 | 4.38 |

**Table 5.2 Represents results of Quantitative tests for total phenolics and total flavonoids.**

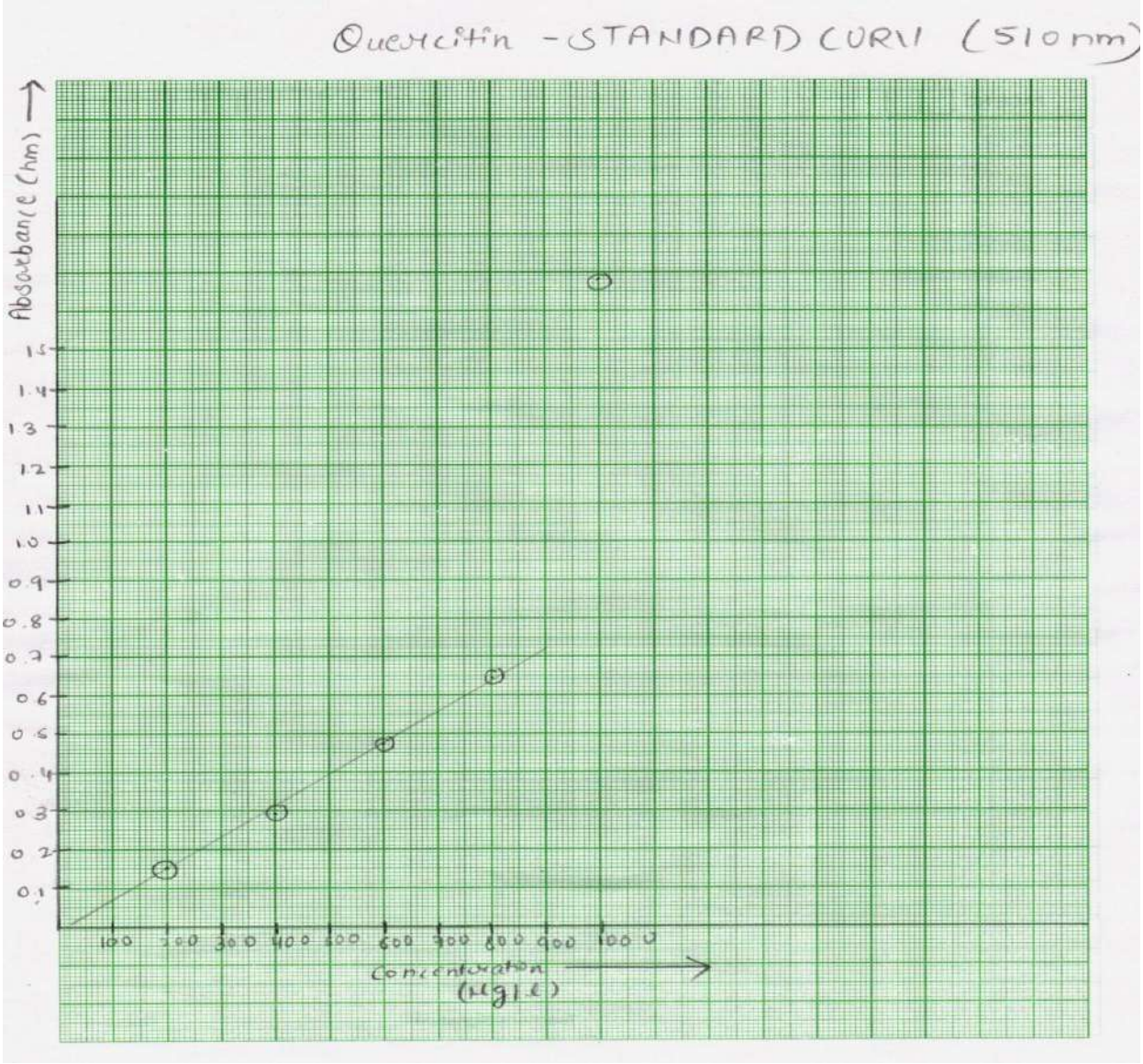



**Figure 5.8 Graphical representation of standard Quercetin.**

## 5.3 PLANT TISSUE CULTURE

### 5.3.1 TIME COURSE GROWTH

**Figure 5.9** illustrates the time course growth of seeds of *Ocimum tenuiflorum* after 4 days of keeping in dark conditions.

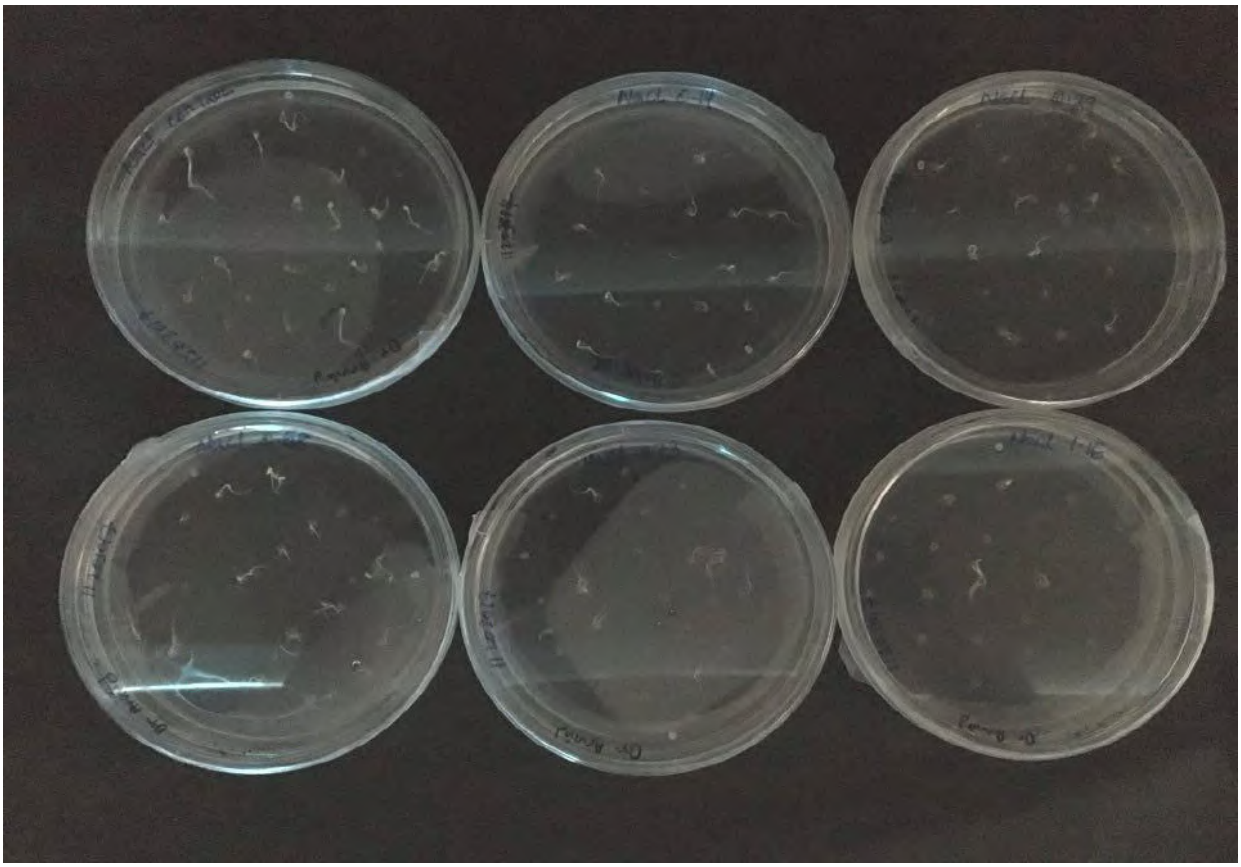


**Figure 5.9 Represents growth after 3-4 days in the Dark.**

On the fourth day, the petri plates were transferred to another room where light and temperature (25 plus-minus 1 degree Celsius) are strictly controlled. **Figure 5.10** illustrates the time course growth of seeds after 7-8 days in the light conditions.

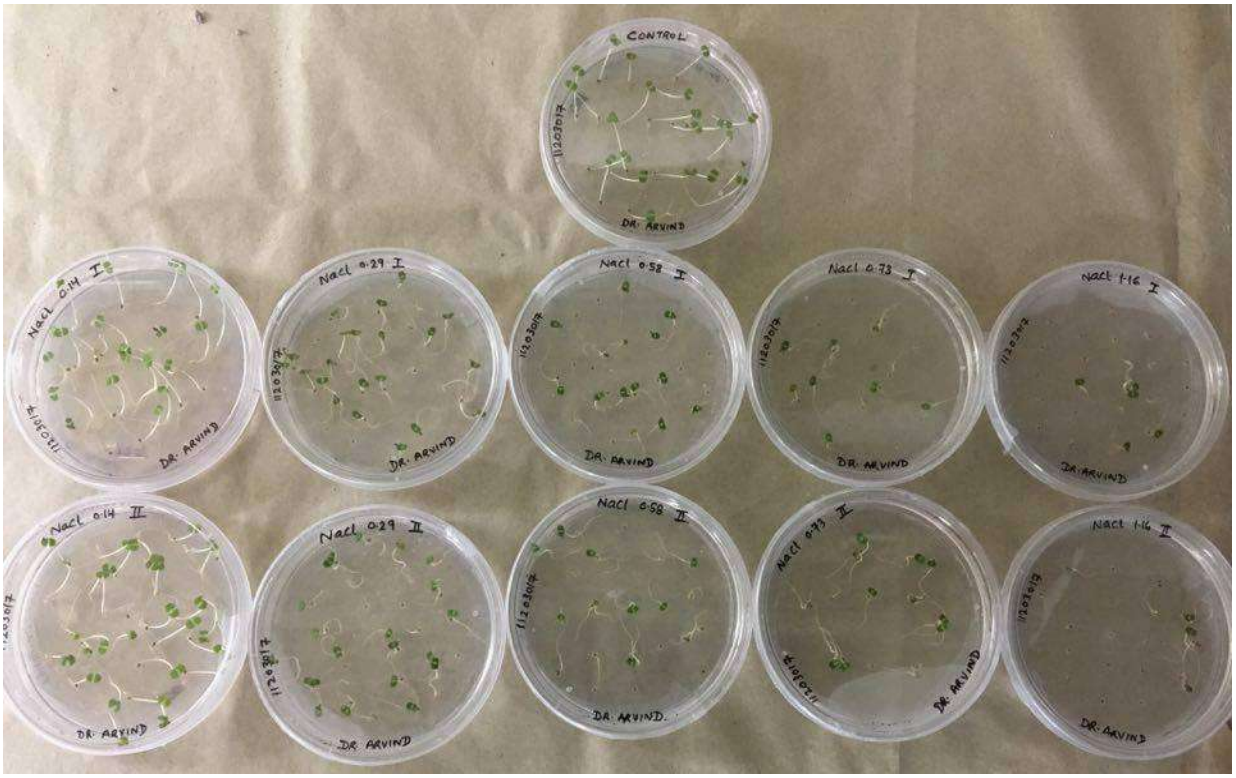



**Figure 5.10 Represents growth after 7-8 days in the light conditions.**

### 5.3.2 PTC OPTIMIZATION

It was observed that the increasing concentrations of NaCl suppressed the growth of shoots and roots. The following results show effects of varying concentrations of NaCl with one replicate each: PTC Optimization.

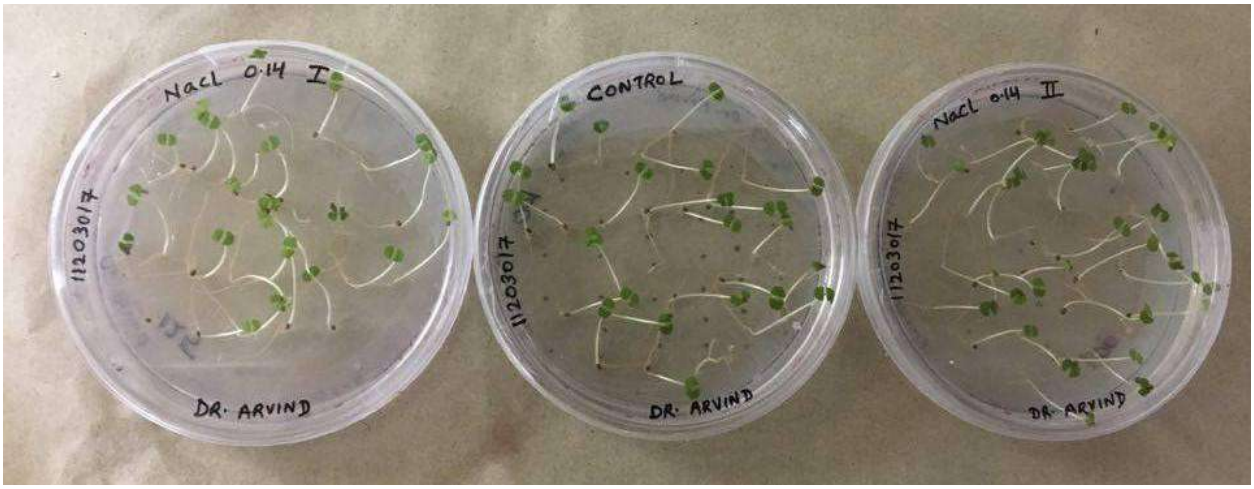



**Figure 5.11 Represents comparison of 25mM (0.14g) NaCl treatment with the control.**


**Figure 5.12 Represents comparison of 50mM (0.29g) NaCl treatment with the control.**


**Figure 5.13 Represents comparison of 100mM (0.58g) NaCl treatment with the control.**


**Figure 5.14 Represents comparison of 125mM (0.73g) NaCl treatment with the control.**


**Figure 5.15 Represents comparison of 200mM (1.16g) NaCl treatment with the control.**

### 5.3.3 MEASUREMENT OF GERMINATION RATE

The percent germination part of this investigation was exceptionally supportive in demonstrating that higher focuses of NaCl hinder plant development. **Table 5.3** represents the effect of varying NaCl concentrations on germination rate from day 4 to day 7. The rate (%) of seed germination was calculated as (G/t) 100, where G is an average number of two replicates of sprouted seeds, and t is an average estimation of two replicates of the total seeds in each test (Lingyun *et al*., 2016) . The table demonstrates that there was not much delay in the germination at lower centralization of NaCl (25 mM) whereas higher groupings of NaCl (from 50mM onwards) were found to delay the process. It was observed that the highest concentration of NaCl (200 mM) almost totally hindered the procedure of germination. As indicated by Huang and Redman (1995), salt induced restraint of seed germination could be ascribed to osmotic anxiety or particular particle toxicity. **Figure 5.16** Represents the average percent germination of *O.tenuiflorum* at different concentrations of NaCl.

| S. No. | NaCl Conc. (mM) | Day 4 (%) | Day 5 (%) | Day 6 (%) | Day 7 (%) |
|---|---|---|---|---|---|
| 1 | 0 (Control) | 95.8 ± 0.70 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 2 | 25 | 91.6 ± 1.41 | 93.05 ± 0.70 | 100 ± 0.00 | 100 ± 0.00 |
| 3 | 50 | 81.9 ± 0.70 | 84.7 ± 0.70 | 87.5 ± 0.70 | 87.5 ± 0.70 |
| 4 | 100 | 63.8 ± 1.41 | 68.0 ± 0.70 | 68.0 ± 0.70 | 70.8 ± 0.70 |
| 5 | 125 | 34.7 ± 0.70 | 37.5 ± 0.70 | 37.5 ± 0.70 | 37.5 ± 0.70 |
| 6 | 200 | 6.9 ± 0.70 | 6.9 ± 0.70 | 9.7 ± 0.70 | 9.7 ± 0.70 |

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

**Table 5.3 Effect of different concentrations of NaCl on seed germination percentage (%) in *O. tenuiflorum*.**

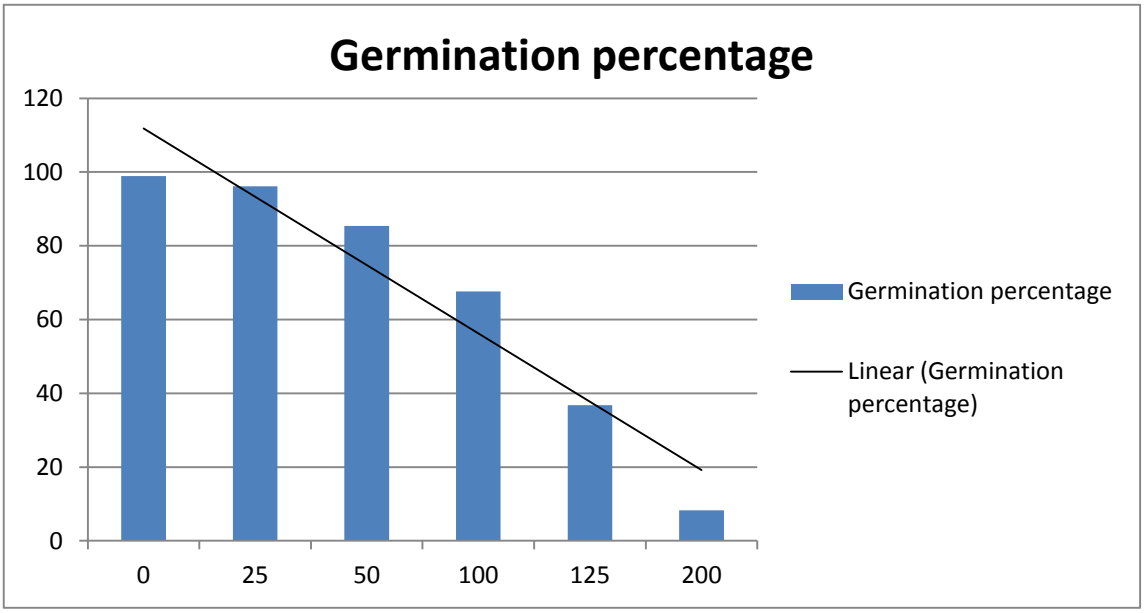



**Figure 5.16 Represents the average percent germination of *O.tenuiflorum* at different concentrations of NaCl.**

### 5.3.4 EFFECT OF INCREASING CONCENTRATIONS OF NaCl ON ROOT LENGTH, SHOOT LENGTH, ROOT/ SHOOT RATIO, TOTAL LENGTH AND FRESH WEIGHT OF *OCIMUM TENUIFLORUM* SEEDLINGS

| Sr.No. | NaCl Conc. (mM) | Root Length (cm) | Shoot Length (cm) | Root / Shoot Ratio | Total length (cm) | Fresh weight (g) |
|---|---|---|---|---|---|---|
| 01 | 0 (Control) | 2.3 ± 0.14 | 2.5 ± 0.14 | 0.92 | 4.8 ± 0.28 | 2.02 ± 0.014 |
| 02 | 25 | 1.4 ± 0.07 | 2.3 ± 0.14 | 0.60 | 3.7 ± 0.21 | 2.00 ± 0.007 |
| 03 | 50 | 1.3 ± 0.07 | 1.8 ± 0.07 | 0.72 | 3.2 ± 0.14 | 2.00 ± 0.007 |
| 04 | 100 | 1.1 ± 0.14 | 1.2 ± 0.07 | 0.91 | 2.3 ± 0.21 | 1.95 ± 0.070 |
| 05 | 125 | 0.7 ± 0.14 | 1.1 ± 0.07 | 0.63 | 1.8 ± 0.21 | 1.01 ± 0.007 |
| 06 | 200 | 0.1 ± 0.07 | 0.3 ± 0.07 | 0.33 | 0.4 ± 0.14 | 0.06 ± 0.021 |

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

**Table 5.4 Represents effect of varying concentrations of NaCl on the seedlings.**

### 5.3.4.1 ROOT LENGTH, SHOOT LENGTH AND ROOT/SHOOT RATIO

In the present study, around 50 percent diminishment when contrasted with control in shoot length was seen at 100 mM NaCl focus as shown in **figure 5.17**, which continuously diminished further with expanding NaCl in the medium. Bijeh keshavarzi *et al*., (2011) proposed that, saltiness prompts decreased water take-up which interfers with cell division and differentiation, in this way influencing the root length and shoot length.

The root/shoot proportion was observed to decrease (**Table 5.4**). The root lengths and in addition shoot lengths were antagonistically influenced with saltiness, notwithstanding, the shoots were observed to be more influenced when contrasted with roots. Turan *et al*., (2010) detailed that in maize, shoot development was a great deal more influenced than root development under saline conditions. As per Jamil *et al*.,(2006), shoots are more touchy and get hampered with saline conditions.

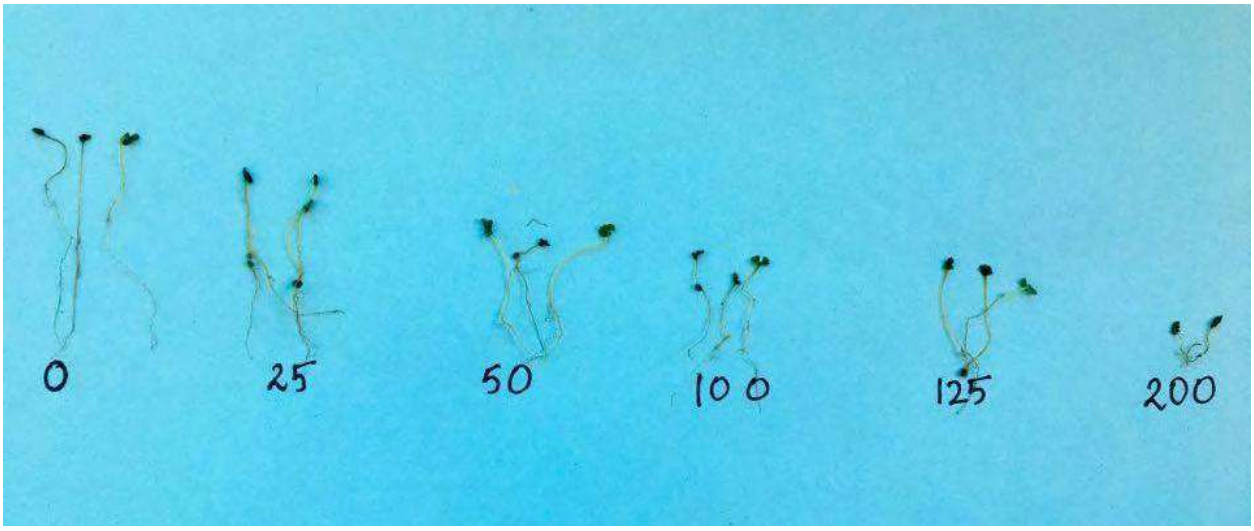



**Figure 5.17 Represents reduction in root length and shoot length of seedlings at different concentrations of NaCl.**

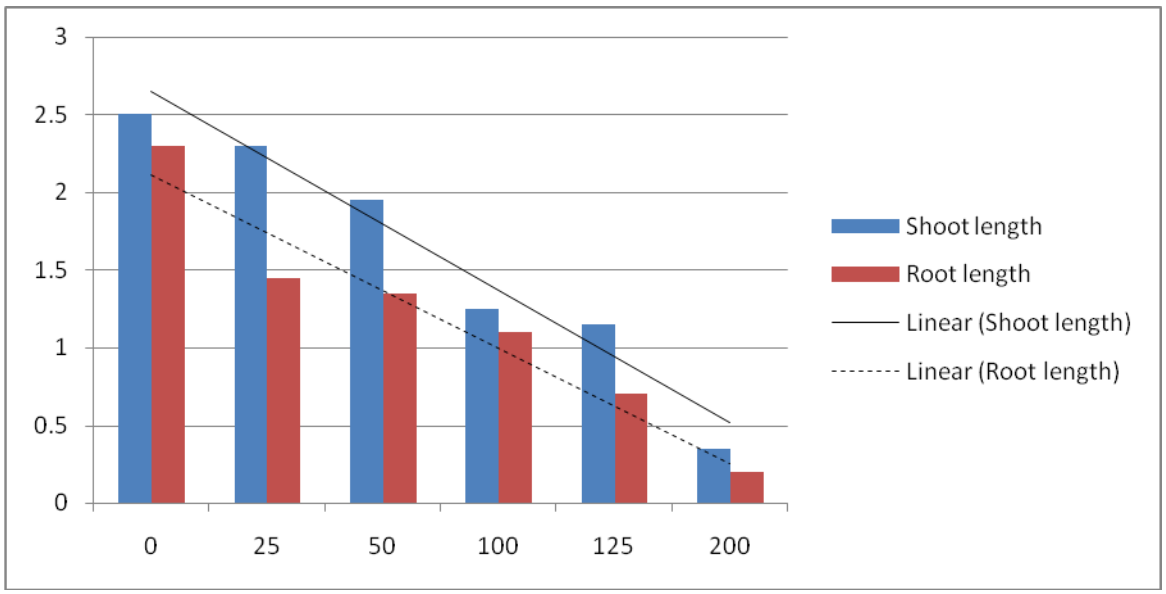



**Figure 5.18 Represents influence of varying concentrations of NaCl on shoot length and root length of the seedlings.**

### 5.3.4.2 TOTAL LENGTH OF SEEDLINGS

In the present review, there is nearly 50 percent decrease in the length of seedling at 100 mM NaCl focus as contrasted with control (**Figure 5.17**). As the root length and shoot length have diminished essentially with expanding saltiness, length of the seedling was additionally seen to diminish. Heidari *et al*. (2011), on the premise of their examinations on *Helianthus annuus* proposed that decrease in plant development is because of diminishing turgor pressure in the cells under saline condition.

### 5.3.4.3 FRESH WEIGHT OF THE SEEDLINGS

**Table 5.4** depicts a decrease in the fresh and dry weight of eight day old seedlings of *Ocimum tenuiflorum*. As per Mahmood and Athar (2003), as salt concentration increments in the medium, plants ingest lesser water bringing on physiological desiccation. This sort of confinement of water ingestion might be in charge of reduction in fresh weight. Abass and Latif (2005) additionally recorded decrease in fresh weight and dry weight of jute seedlings under NaCl stretch. Cha-Um and Kirdmanee (2009) watched a lessening in fresh weight and also dry weight in maize seedlings under NaCl saltiness. As per them, saltiness prompts water deficiency in plants, subsequently bringing about a reduction in fresh weight and dry weight, which might be genuine even in the present study.

## 5.4 QUANTITATIVE TESTS ( INVITRO)

Different concentrations of NaCl (0mM, 25mM, 50mM, 100mM, 125mM and 200mM) were tried to stimulate the accumulation of phenolics and flavonoids in *Ocimum tenuiflorum* (**Figure 5.19**). It was observed that increasing concentration of NaCl increased the accumulation of both phenolics and flavonoids upto 100mM NaCl in comparison to control. It was noted that addition of 200mM NaCl was harmful for the growth. **Table 5.5** shows the effect of NaCl on the accumulation of phenolic and flavonoid contents in *O.tenuiflorum*.

| NaCl Conc. (mM) | Phenolic content | Flavonoid content |
|---|---|---|
| 0 (Control) | 0.82 ± 0.002 | 4.78 ± 0.002 |
| 25 | 0.83 ± 0.001 | 4.81 ± 0.002 |
| 50 | 0.87 ± 0.0007 | 5.19 ± 0.002 |
| 100 | 0.85 ± 0.0007 | 5.05 ± 0.002 |
| 125 | - | - |
| 200 | - | - |

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

**Table 5.5 Effect of different concentrations of NaCl on Phenol and flavonoid content.**

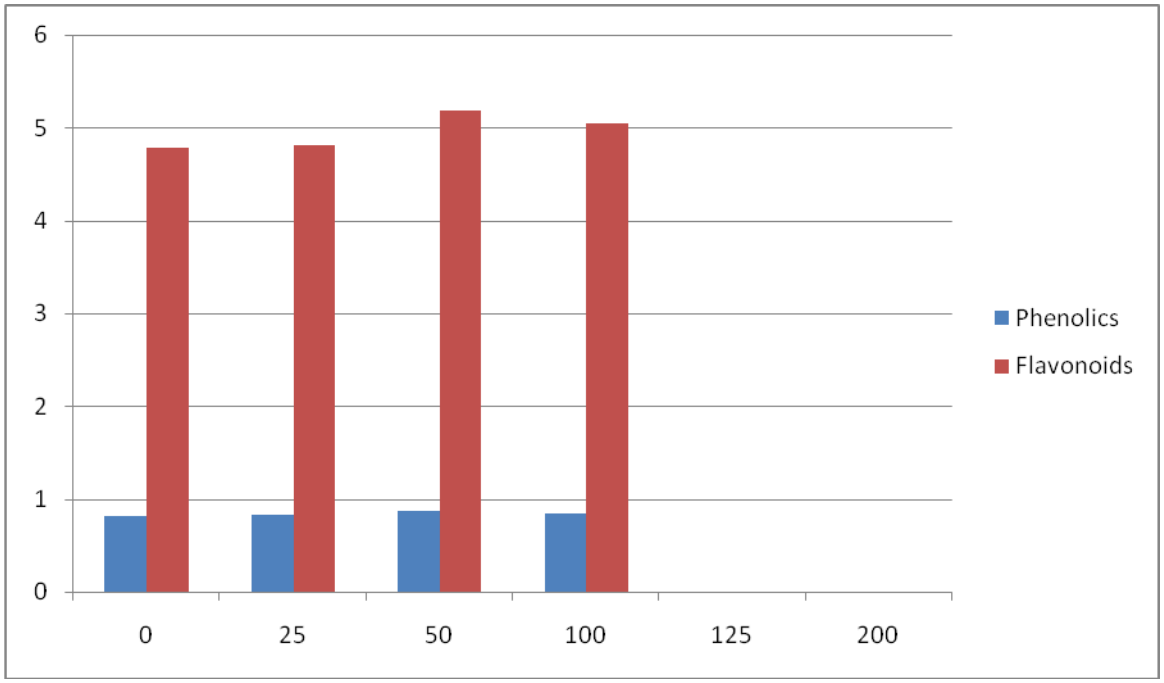



**Figure 5.19 Represents effect of varying concentrations of NaCl on accumulation of phenol and flavonoid in *O. tenuiflorum*.**

## 5.5 IN VIVO AND IN VITRO COMPARISON OF TOTAL PHENOLIC AND TOTAL FLAVONOID CONTENT

Quantitative analysis were performed both in vivo and in vitro of *O.tenuiflorum*. **Table 5.6** shows the results of in vivo and in vitro comparison of total phenolic content and total flavonoid content. It was observed that in vitro results were higher than in vivo results of both phenolic and flavonoid content (**Figure 5.22**).

| Phytochemicals | Invivo | Invitro (at 50mM NaCl Conc.) |
|---|---|---|
| Total Phenolic content (mg/ml) | 0.58 | 0.87 ± 0.0007 |
| Total Flavonoid content (mg/ml) | 4.38 | 5.19 ± 0.002 |

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

**Table 5.6 Represents comparison between invivo and invitro of total phenolic and total flavonoid content in *O. tenuiflorum*.**

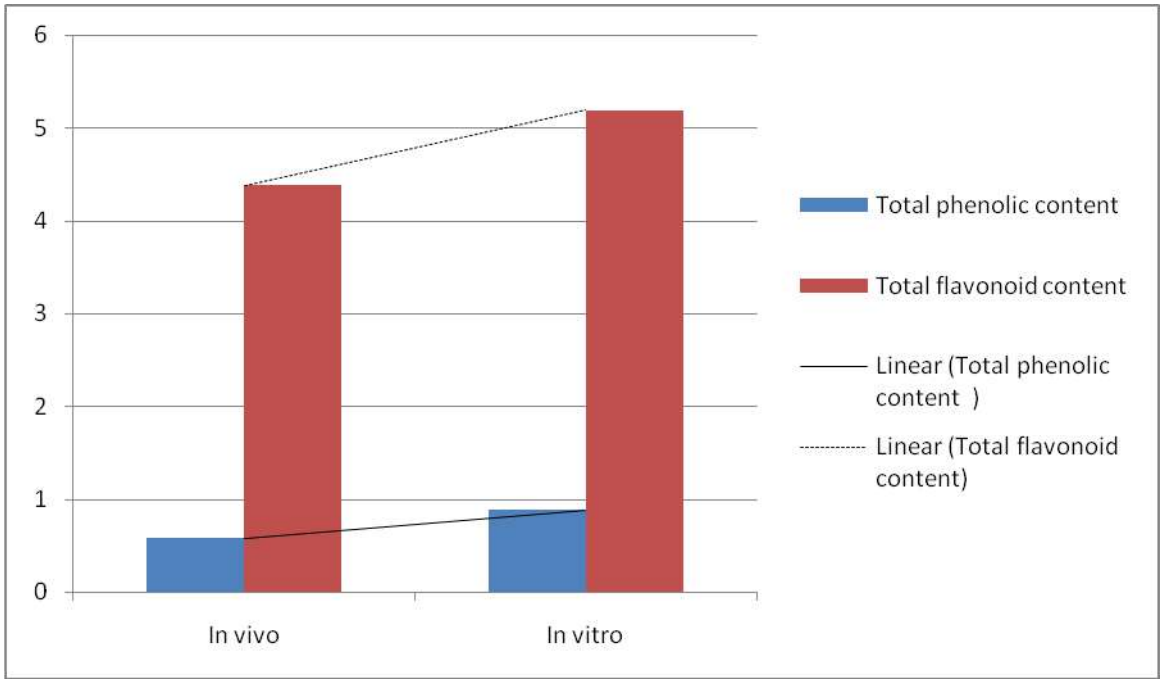



**Figure 5.20 Represents comparison between invivo and invitro of total phenolics and total flavonoids.**

## 5.5 STATISTICAL ANALYSIS

All experiments were carried out with minimum of two replicates per treatment. The results are expressed as mean values ± standard deviation.

## DISCUSSION

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.

Various medicinal plants showed high antioxidant activity (Lachance *et al*., 2001; Zakaria *et al*., 2008; Zivcovic *et al*., 2012). The common antioxidants known in plants are phenolics and flavonoids (Sankhalkar, 2014; Siddhuraju and Becker, 2003). In vivo quantitative tests reported phenolic content to be 0.58 mg/ml and flavonoid content to be 4.38 mg/ml. Various reports show that genus Ocimum contain phenolic compounds in large amounts and thus is largely used in traditional medical systems (Wang *et al*., 2004). At least one hydroxyl ion is substituted with the aromatic ring of both phenolics and flavonoids and both can form chelate complexes with the metal ions thereby getting easily oxidized and are the means for donating electrons to scavenge free radicals (Siddhuraju and Becker, 2003; Sreedam *et al*., 2010). Studies show that higher phenolic content is correlated with increased antioxidant activity (Borneo *et al*., 2008; Katalinic *et al*., 2004). Ocimum shows variation in the antioxidant properties in relation to leaf position (Wongsen *et al*., 2013). Similar observation is shown by number of reports (Kostyuk *et al*., 2001).

The chemical constituents in plants known as phytochemicals have different actions on the human body (Vimala *et al*., 2013). Major bioactive phytochemicals are alkaloid, flavonoid, phenolic, terpenoid, and essential oil (Anwar *et al*., 2006). It is observed that the distribution of these phytochemicals depend on the geographic location of the plant and the solvent system used in the extraction process (Deshpande and Kadam, 2013).

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.

In view of my outcomes, I acknowledge my speculation for various distinctive reasons. My theory expressed that the plants would display various stress responses including shorter root length and an increased production in secondary plant products when exposed to different concentrations of sodium chloride. My outcomes showed a shorter root length and shoot length as well as reduced fresh weight and dry weight as the centralizations of NaCl expanded. It was also observed that the accumulation of phenol and flavonoid also increased with increasing NaCl concentrations. The phenolic and flavonoid contents in control were 0.82 mg/ml and 4.79 mg/ml respectively. The highest phenol (0.88 mg/ml) and flavonoid (5.19 mg/ml) accumulation was observed in media treated with 50mM NaCl. In vitro results were compared with in vivo results, where in vitro results came out to be higher.

It can be hypothesized that antioxidant nature of *O. tenuiflorum* may be due to the increased contents of phenolic and flavonoid in the leaf extracts. However, there is a need for further quantitation and identification of the chemical structure of these phytochemicals by HPLC, GC, MS and NMR. The natural antioxidants are less harmful than synthetic antioxidants so the identification of active constituents in this medicinal plant is thus highly significant in food and pharmaceutical industry. However, for future research applications the physicochemical evaluation of the drug is a very important parameter to study the quality of the plant material.

There are various diverse ways that I could go on to proceed with this analysis later on. I could test even more concentrations of NaCl or concentrate on a smaller range of focuses. I could likewise have a go at testing how varying concentrations of NaCl influence plants that are developed in soil versus agar, and check whether it has a distinction. Testing distinctive NaCl focuses on various sorts of plants could likewise give intriguing outcomes. I could add more repeats to lessen the amount of error in my investigation. With everything taken into account, there are diverse things that I could go further to proceed with this experiment.

# CHAPTER 6

# SUMMARY AND CONCLUSION

Secondary metabolism in the plants synthesis a number of secondary compounds that are highly valuable products. Some of the secondary molecules are produced by the induction of morphological differentiation. It appears as the cells undergo morphological differentiation and maturation during plant growth. A large number of pharmaceutical industries employ secondary metabolites or bioactive constituents or phyto-pharmaceuticals either directly or indirectly occurring in intact plants. However, the supply of these secondary metabolites often have many limitations. Secondly, many important plant sources are getting depleted due to their over-utilization, leading to serious ecological problems. Various strategies have been tired like plant cell immobilization, biotransformation, media manipulation, precursor feeding etc. but all experiments didn't work to produce the desired products in required commercial scale.

Cultured plant cells are recognized as continuous, reliable and potential source of natural products, but because of low yields of desired compounds few cell cultures synthesize secondary metabolites. This is because the basic secondary metabolic regulation in plant cell cultures are not well understood.

To increase the productivity of required secondary compound through *in vitro* culture systems, elicitation is stated as the most preffered technique. Based on their nature, Elicitors are classified into abiotic and biotic. In elicitation the type of elicitor, its dosage, and exposure time are the main factors responsible for successful stimulation of secondary compound productivity in *in vitro* cultures of medicinal plants.

In the past, abiotic elicitors were not much recognised, but at present abiotic elicitors are used effectively to increase the productivity of different commercial important secondary compounds using *in vitro* cultures. On the premise of present review it can be concluded that *Ocimum tenuiflorum* is delicate to NaCl saltiness. Increasing concentrations of NaCl in the development medium antagonistically influenced the rate germination, postponed the procedure of germination. Saltiness likewise antagonistically influenced development as there was decrease in root length, shoot length and fresh weight of the seedlings and increased the accumulation of defense metabolites. Still the exact mechanism of elicitation is not understood till date. Therefore there is a great scope for producing secondary molecules in large scale *in vitro* with the use of elicitors as an agent.

Different phytochemical tests were performed for the qualitative analysis of the bioactive compounds present in *O. tenuiflorum*. Qualitative analysis showed the presence of flavonoids, alkaloids, reducing sugars, saponins, tannins and anthraquions and absence of terpenoids and phlobatannins.

From this study, we conclude that the leaf extract of *O. tenuiflorum* exhibit antioxidant and phytochemical potential. The plant extract contains large amounts of flavonoids than phenolics. Also, the in vitro results of quantitative metabolite analysis are higher than in vivo results. Thus, in vitro culture proves to be a significant tool for enhancing secondary plant products. The study showed that the plant is a source of significant natural antioxidants and may be useful in protection against oxidative stresses. This proves to be highly significant in food and pharmaceutical industry, subject to economic considerations because the natural antioxidants are less harmful than synthetic antioxidants.

# CHAPTER 7
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