INTRODUCTION

1.1 Introduction

Plants support all other life forms and maintain the oxygen content of the air, ecosystems and control the climate. Plants are primary source of vitamins and provide medicine, clothes, shelter and raw materials from which innumerable products are made. These benefits are widely recognized. Therefore, they are essential parts of the world's biological diversity, and human beings are very dependent on plant.

During the past decade, research and investigation on plant potential abilities have been valuable topics in the world. Tissue culture is an experimental technique in which mass of cells are produced from the explants. Tissue cultures raised the knowledge in some areas including differentiation, cell division, and nutrition and cell preservation. Nowadays, cells are cultivated *in vitro* in bulk or as clone from single cells to grow whole plants from isolated meristem, produce callus and develop complete plantlets by organogenesis or by embryogenesis.

Biotechnology offers an opportunity to use the cell, tissue, organ or entire organism by growing them *in vitro* and to genetically manipulate them to get desired compounds.

Since the world population is increasing rapidly, there is an extreme pressure on the available cultivable land to produce food. For other uses such as production of pharmaceuticals and chemicals from plants, the available land should be used effectively. During the past decade, a considerable progress has been made to stimulate formation and accumulation of secondary metabolites using plant cell cultures (Ravishankar & Rao, 2000).

The callus formed can be utilized directly to regenerate plantlets, or be a source of primary and secondary metabolites. The adopted methods for enhancing the secondary metabolites include obtaining efficient cell lines for growth, monitoring of high-growth cell line that produces metabolites of interest, immobilization of cells to enhance yields of extra-cellular metabolites and to facilitate biotransformation, use of elicitors to enhance productivity in a short period of time, permeation of metabolites to facilitate downstream processing, adsorption of the metabolites to partition the products from the medium and to overcome feedback inhibition and scale-up of cell cultures in suitable bioreactors.

Medicinal plants are the earliest known to have various benefits and usage. Based on clay written tablet excavated in Iraq, their historical record was about six thousand years ago which proves that human beings have been depending on plants mainly for food and medicine, apart from other uses. Crude extracts, and whole plants have been used as medicine without knowledge of their active components (Judith, 2000; Endress, 1994).

Plant secondary metabolites are sources of phytochemicals that can be used directly or as intermediates for the production of pharmaceuticals, as additives in cosmetic, food or drink supplements. Consumers preferred to use plants as producers of secondary metabolites (Stafford, 2003).

The use of traditional medicinal plants is popular in China, India, Japan, Pakistan, Sri Lanka, and Thailand. These countries and the African continent are rich sources of medicinal plants (Lemma, 1991).

The range of species and their scope used for healing is vast and many undiscovered existing plants are yet to be described. Currently, it is estimated that more than 50,000 plant species are used worldwide for medicinal purposes (Schippmann *et al.,* 2002).

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This equates to approximately 20% of the world's vascular flora and constitutes the biggest spectrum of biodiversity used by people for a specific purpose (Hamilton *et al.,* 2006).

Medicinal plants are obviously an important global resource in terms of health care, and an important economic resource, traded widely on scales ranging from local to the international places. The trade in medicinal plants is estimated to be 60 billion U.S. dollars per annum (Bank, 2004) with increasing rate of 7% in year (Koul, 2004).

Malaysia is one of the 12 mega-diversity centers in the world with 1200 plant species reported to have medicinal properties. This country is rich in plant genetic diversity, and most of them are used for medicinal purposes (Jamal *et al.,* 2010; Shyun & Rasadah, 2004). Setefarzi (2001) predicted that the market of plant-based medicine and herbs in Malaysia would increase to 1.37 billion U.S. dollars in 2010. The worth of this market was about 527 million U.S. dollars in 2000 (Shyun & Rasadah, 2004).

The history of research on medicinal plants in Malaysia is almost half century but most of these researches focused on natural plants in the last 20 years. Government researches, Institutions of higher learning, other government agencies and private companies manufacturing herbal products are involved in various aspects of medicinal plant research. Focus on the bio prospecting study of medical plant was the first goal of the earlier researches and afterwards, screening of phytochemical begun at the University of Malaya and followed by others universities working on taxonomic, ethno botanical, and bioassay-guided studies (Shyun *et al*., 2004).

Gardenia jasminoides or commonly Gardenia, Cape jasmine or Cape jessamine is a fragrant flowering evergreen tropical plant. This plant originated in the trop-

ical and subtropical regions of Africa, Southern Asia, Australia, and Oceania with its shiny green leaves and fragrant white summer flowers. It has been cultivated in China for at least ten centuries (Keswick *et al.*, 2003) and was introduced to English gardens in the middle of 18th century. Some varieties have been bred for horticulture, with low growing, and large and long flowering forms.

The genus *Gardenia* belongs to *Rubiaceae* family and there are about 200 species of this genus. The name *Gardenia* was given to commemorate Dr. Alexander Garden 1780-1791, (Green, 1965). This species is an evergreen shrub with dark green, glossy and oval. The blooms are waxy and the color ranges from pale yellow to creamy white. They are native to the tropical and subtropical regions of Africa, Southern Asia, Australia, and Oceania (Neal, 1965).

Suffix of "*oides*" means "*-like* "and *G. jasminoides* is "jasmine-like" flower. This genus was registered in 1725s by Edwards's Bot (Roxburgh, 1975), which is a native of South China and there are more than 15 cultivars of this plant in China (Chen *et al.*, 2010).

This plant is an evergreen shrub, growing up to 1-2 meter tall, with sweetly fragrant flower can be used as a cut flower and landscape shrub, also is one of the most popular plants in the USA and many of the European countries (Green, 1965) . The other names of this plant include:

English: Cape jasmine, Malaysia: Bunga Cina, Indonesia: Ceplok Piring, Philippines: Rosal*,* Thailand: Phut Cheen*,* and Vietnamese: Donh Donh.

This plant was chosen for this research due to its medical uses as well as 'magical' uses for treating jaundice, hemorrhage, hepatitis, toothaches, wounds,

sprains, and skin conditions (Choi *et al*., 2007; Lelono *et al.,* 2009; George *et al.,* 1993).

Gardenia is considered very effective as a haemostatic agent and effective in treating injuries to the muscles, joints, and tendons. A yellow silk dye has been made for centuries from the chemical compound 'crocetin', which is extracted from the gardenia berry. Gardenias are widely used as exotic ornamental flowers in corsages, as houseplants, and outdoor plants.

1.2   Objectives of Study

The present study was conducted to investigate the potential of WPM media on plant regeneration and callus induction using leaf explants of *Gardenia jasminoides* Ellis and to determine the anti-bacterial and anti-oxidant activities from *in vivo* leaf extract and in vitro callus extract *G.jasminoides* Ellis. MS (Murashige & Skoog, 1962) and LS (Linsmaier & Skoog, 1965) media have been attempted for micro propagation of *G.jasminoides*. However, there is still no report available about *in vitro* culture of this species on WPM media (Lloyd & Mc Cown, 1980). In this study callus induction of *G.jasminoides* on different media supplemented with various types and concentrations of auxin and cytokinin for investigation of secondary metabolite activities were evaluated.

Literature review

2.1   Botanical Characteristics of *Gardenia jasminoides*

G. jasminoides is native to the south of Japan and China. The leaves are opposite, thick, dark green with lanceolate shape to ovate and can reach 10 cm in length (Fig 2.1). The sweet fragrant, terminal flower, 8-10 cm across, consists of a calyx (with five green fascinated teeth) and a corolla (with six whorls and five to nine white waxy petals). Frequently stamens and pistil(s) are transformed, resulting in sterile flowers. Flower induction and development are influenced by different factors. Stem cutting after flowering is the conventional method for propagation (Hutchinson, 1980).

Figure 2. 1: Leaf and flower of *Gardenia jasminoides* Ellis

2.1.1   **Benefits of *Gardenia jasminoides* Ellis**

Aside from being used a cut flower, in landscape designs oil & scent (flower) for Traditional Chinese Medicine, Gardenia is also used as: fried and charred to stanch bleeding and the husk (fruit without seeds) or flower clears heat from the lungs. The simple dried fruit i.e. (Fig 2.2), commonly is used in heat/fire signs such as Irritability, restlessness, insomnia, delirious speech and a stifling sensation in the chest.

Figure 2.2: Dry and fresh fruit of *Gardenia jasminoides* Ellis

According to Traditional Chinese Medicine (TCM), some other studies have been investigated on this plant:

Gardenia seed clears internal heat and eliminates heart vexation, removing the pathogenic fire, relieving restlessness and inducing diuresis (Dharmananda, 2003; Xinrong, 2003). The effect of ethanol extract of Cape jasmine could be useful in preventing vascular disease (Hwang *et al.,* 2010).

The importance pigments of Gardenia fruit as natural colorants in food science was studied by Mortensen in 2006. *G. jasminoides* extract could be used as chemo-preventive agent in Alzheimer's disease (Choi *et al*., 2007). *G. jasminoides* has antifungal activity against agricultural pathogens with no environmental side effects (Lelono *et al.,* 2009). The fleshy fruit of this plant is a diuretic, stimulant, an emetic and used in lung, jaundice and kidney disorders (George *et al.,* 1993).

Meanwhile, the major components of the fruits of Gardenia are iridoid glycosides (geniposide and related constituents). This colourless iridoids can be converted into pigments of a variety of colors of which blue and red pigments are the most important (Fig 2.3), while polar crocetin derivative is a major factor in the color of medicine. These crocetin derivatives are known for their coloring properties owing to their particular water-soluble behavior, which is the reason for its great application as food, in contrast to most plant families of carotenoids (Van-Calsteren *et al.,* 1997; Giovanni *et al.,* 2003; Mortensen, 2006; Wenhao *et al.,* 2010).
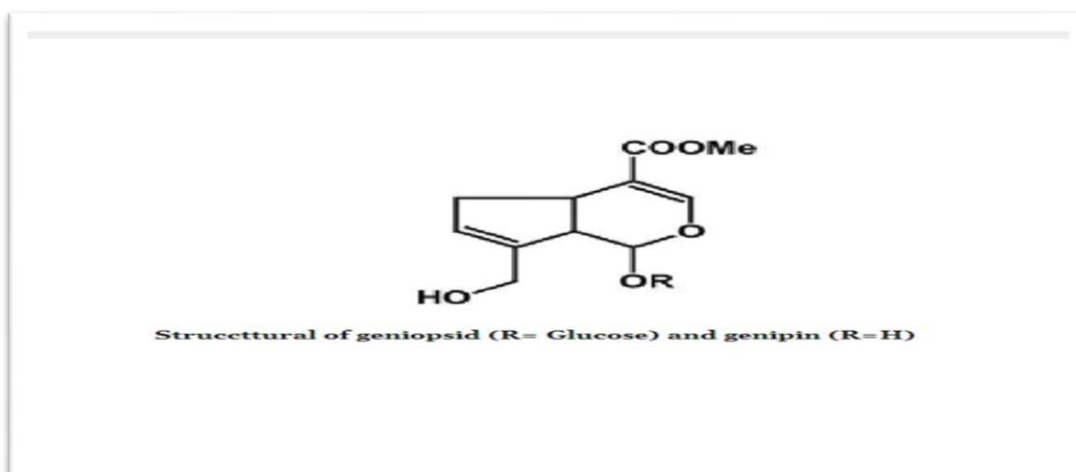

Figure 2.3: Structure of genioposide (R-Glucose) and genipin (R-H)

The carotenoids are considered as the main contributors to the antioxidant capacity of the plant and responsible for a variety of pharmacological effects, such as preventing cardiovascular diseases (Shu-Ying *et al.,* 2005; Xiang *et al*., 2006).

Crocetin was able to improve sleeping problem (Kuratsune *et al*., 2010), and could be used as remedies for the treatment of liver disease (Kotoky & Das, 2008). It can also, inhibit tumor cell proliferation (Magesh *et al*., 2006), Nero and protect cells (Ochiai *et al*., 2004; Ahmad *et al*., 2005), and hepatocytes (Tseng *et al*., 1995).

2.2   Biotechnology

There are various definitions of biotechnology, but Ereky in 1919 as the first person involved in biotechnology stated:" Any process where a biological organism is used to make a product for human source." However, this definition could not include modern biotechnology, moreover does not recognize the incremental use of genetically modified organism.

Modern biotechnology is a mixture of many different sciences such as Biology, Chemistry, Medicine, Computer science, and Mathematics (Manning & Hugh, 2000; Shmaefsky, 2006). It begun in 1980, and at the meantime, the Supreme Court in U.S.A ruled a privilege of genetically modified microorganism to Chakrabarty, whom developed a bacterium capable to collapse of crude oil with proposed to use in treating oil (Supreme court, 1980). Biotechnology is divided into various fields such as bioenergy, bioethical, bioinformatics, bio nanotechnology and agricultural technology. Agriculture is one of the sciences that is clearly suitable to the definition of "using a biotechnological system to make product".

Some of agricultural biotechnology field consists of plant tissue culture, forest bio-technology, marine biotechnology and food technology. Traditional farmers began the earlier biotechnology by domesticating crops, animals and selecting the plant material for propagation and animals for breeding about 10 000 years BC. The result of that exploitation was different from their early forerunners. However, the main goal of using modern and traditional biotechnology is producing superior animals or crops.

Nowadays, modern plant biotechnology is increasing the quality of crop yields. Genes transfer and use of DNA molecular markers and *in vitro* micropropagation are some areas of biotechnologies that are used in reproducing and improving the breed of trees and crops.

2.3   Tissue Culture Technique

According to estimations of the World Health Organization (WHO), more than 80% of the world's population in developing countries relies primarily on herbal medicine for basic health care needs. However, difficulty in cultivation of some of the plants due to specific ecological requirements or low germination rates justified. Plant tissue culture as an alternative method for the preservation of our medicinal and aromatic plants.

Micropropagation of some of the medicinal and aromatic plants has been performed via shoot-tips and auxiliary buds. Stem cutting is the conventional method of propagation of *G. jasminoides*, but this way is slow,therefore, micropropagation could be an alternative method for rapid regeneration. Clonal multiplication has been successfully carried out from auxiliary buds (Pontikis, 1983; George, *et al*., 1993) and shoot tips (Economou & Spanoudaki, 1986b).

2.3.1 Review of Tissue Culture

Gautheret (1985) believed that experiment of Moneceau′s in 1756 could be considered as a preface for the discovery of plant tissue culture. He proved wounds of the *Elm* plants heals naturally by callus formation (Radzan, 2002). In addition, Schwan and Schleiden in 1839, the founders of cell theory stated that living cell is capable of developing into a multicellular organism if put in the proper media and condition.

Tissue culture is possible because of Morgan in 1901 stated that totipotency is the ability of the cell to develop and regenerate into a whole organism" (Dodds & Lorin, 1995; Pierik, 1997).

Sterile small pieces of a whole plant can be used in tissue culture. These pieces are known as explant, may consist of seeds, leaves , roots as pieces of organs, pollen or endosperm. The type of many affect the efficiency of culture initiation. Generally, younger explant (at an early stage of development) with more rapidly growing tissue is most effective. Micro propagation often called tissue culture is another type of asexual propagation where a very small piece of tissue (shoot apex, leaf or even cell) is excised and placed aseptically on a sterile container containing a special culture medium.

The media contain the proper ratio of nutrient, sugar, vitamins with or without jelly agent, and plant growth regulators such as Auxin, Cytokinin, which causes the plant part to grow at very rapid rates to produce new plantlets. All the procedures are done in an aseptic operating room in a laboratory with special ventilated cabinet.

In vitro micro propagation or tissue culture could be divided into two main types of cell growth and differentiation systems: Unorganized tissue that is later led to organized tissue formation (callus) and maintenance of organized tissue (bud tissue

culture) which has a limited and very specific uses such as germplasm maintenance and rapid increase of rare genotypes in colonial propagated materials (woody plant and flower).

Organogenesis or embryogenesis could change unorganized tissue to an organized one. Plenty of information on the best method for taking care of plant material of various species and *in vitro* micro propagation have been collected for many years.

2.3.2   *In Vitro* **Microropagation of Medicinal and Aromatic Plants**

Clonal mass multiplication is necessary for those groups of medicinal plants, which yield costly active principles present in small quantities, but are required in enormous amounts, like, *Catharanthus roseus* (L.) or (Madagascar periwinkle, Sadabahar). Two tons of the leaves which yield only one gram of alkaloid is required to treat a leukemia patient for six weeks. For *Taxus brevifolia* Nutt (Pacific yew), bark of one full mature tree which is two hundred years of age is needed to treat one patient with ovarian cancer (Chaturvedi *et al.,* 2007). Rapid propagation is also essential for endangered plant. *Dioscorea deltoidea* (Medicinal yam), an indigenous species having the highest diosgenin among of *Dioscorea* species, has very long regeneration cycle of 10 years (Chaturvedi *et al.,* 2007; Martin & Gaskins, 1968).

Plants can be regenerated *in vitro* either by somatic embryogenesis or by shoot morphogenesis. Plenty of important Chinese traditional medicinal plants have been successfully regenerated *in vitro*. Each plant has a special group of bioactive compounds.

Taxus tree is one of the anticancer factors known due to its unique mode of action on microtubular cell system. Taxol or plaxitaxol, which is a complex diterpene

alkaloid, is found in the bark of *Taxus* tree. Another example of medicinal usage of plants is latex from *Papaver somniferum* or *Opium poppy*, which is a commercial source of the Codeine, Analgesics and Morphine (Tam *et al.*, 1980; Yoshikawa, 1985; Siah & Doran, 1991).

Ginsenosides which are primary bioactive components of ginseng are a group of triterpenoid saponins (Proctor, 1996; Sticher, 1998) and Berberine in the roots of *Coptis japonica* is an isoquinoline alkaloid (Nakagawa *et al.*, 1982). *D. deltoidea* contained diosgenin which is a forerunner for the chemical synthesis of steroidal drugs and possess tremendous importance to the pharmaceutical industry (Zenk, 1978; Yeh *et al.*, 1994).

There are numerous reports and experiments about micropropagation of different species of ornamental and medicinal plants. For example, camptothecin is a powerful antitumor alkaloid, isolated *in vitro* from *Camptotheca acuminata* (Liu & Li, 2001). *In vitro* flowering of *Withania somnifera* as an antitumor medicinal plant (Saritha & Naidu, 2007) and embryogenic tissues of Ginseng (*Panax ginseng*) has been reported by Asaka *et al* (1993).

There are many reports about production of virus-free-plant via meristem culture method (Tyagi *et al.*, 2010; Alam *et al.*, 2009; Arora & Bhojwani, 1989; Hunter, 1988; Hunter, 1979). Seed and hypocotyl culture of *Ruta graveolens* L. are sources of pharmacologically active molecules (Lièvrea *et al.,* 2005).

Mass propagation of *Paederia foetida* L. as an important medicinal Asian plant reported by Kumar in 1995 (Amin *et al.*, 2003), *in vitro* culture of *Crocus sativus* or Saffron studied by Ajinomoto (Karl *et al.*, 2009a), cell culture of *Gingko biloba* was carried out by Wilson, 1995, rapid micropropagation of *Clitoria ternatea* L. or 'Aparajita' which is Indian medicinal herb, was studied by Pandeya (2010) and

tissue culture of *Jasminum officinale* L. as aromatic plant carried out by Bhattacharya in 2010.

Various experiments on *in vitro* propagation of medicinal and ornamental plant by root culture have been reported (Kubota *et al*., 1995; Pradel *et al*., 1997; Beruto, 2010; Bhojwani & Razdan, 1996b) .

Leaf is one of the most suitable part in isolation of cell. Leaf culture in *Arachis hypogaea* have been reported by Ball (1965) and Joshi (1968). Similar attempt has been done by Edwards and Black (1971) on spinach and crabgrass (Bhojwani & Razdan, 1996a), Geier (1986) used long leaves for *in vitro* propagation of *Anthorium scherzerianum* (Karl *et al*., 2009b), *Hippeastrum, Amaryllis* also was cultured via leaf (Kyte & Kleyn, 1996). Young leaves of *G. jasminoides* Ellis have been studied for callus induction (Al-Juboory *et al*., 1998; Mizukami *et al*., 1987).

shoot tip culture was successfully used in many of medicinal and aromatic plants; like *Aconitum coreanum* reported by Xu *et al* (2004), multiple buds of *Hypericum patulum Thunb* from *in vitro* shoot tip culture was carried out by Baruah *et al* (2001) and Ananthi *et al* (2011) used shoot tip as explants in micropropagation of *Rorippa indica L.*

Micropropagation of *G. jasminoides* Ellis by shoot tips (Economou & Spanoudaki, 1986a; Serret *et al*., 1996; Sayd *et al*., 2010) and microshooting (Hatzilazarou *et al*., 2006) were reported.

Citrullus colocynthis L. was micropropogated via shoot tip and direct shoot regeneration from auxiliary bud (Meena *et al*., 2010). However, shoots tip explants of *Solanum nigrum* was reported by Seridhar & Naidu (2011), micropropagation of *Aloe barbadensis Mill*. through *in vitro* culture of shoot tip explants was reported by

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Baksha *et al* (2005) and shoot-tip culture of *Limonium wrightii* (Hance), an endangered medicinal plant, was achieved (Huang *et al*., 2000).

Shoot regeneration *in vitro* from root pieces was reported from some medicinal and aromatic plants such as Zingiberaceae, *Vernonia amygdalina, Inula helenium* L. (Khalafalla *et al*., 2009; Tripathi & Tripathi, 2003; Stojakowska *et al*., 2004). It is a method of propagation that is potentially applicable to a wide range of species.

Shoot regeneration from root pieces does not offer a continuous method of micropropagation unless there is a ready supply of aseptic root material from isolated root cultures (George *et al*., 2008a). Chuenboonngarm (2001) used the young shoots of *G. jasminoides* Ellis as explant. In addition, ovary culture for callus initiation via immature ovary portion of flower of *G. jasminoides* (George *et al*., 1993), and *in vitro* culture of single nodes and shoot tip from *G. jasminoides* by Duhoky and Rasheed (2010) have been reported.

2.3.3 **Review of PGR's Effect on Plant**

The effects of auxin and cytokinin on shoot multiplication of various medicinal plants were reported (Rout *et al*., 2000; Ahuja *et al*., 1982; Arora & Bhojwani, 1989; Faria & Illg, 1995; Sahoo *et al*., 1997). Although, cytokinin levels was shown to be the most essential for multiplication of many medicinal plants (Bhojwani & Razdan, 1996a; Mao *et al*., 1995; Sharma *et al*., 1993; Chen *et al*., 1995).

The development of axillary meristems and shoot tips of *Atropa belladonna.* L. was stimulated through BA with 0.001-0.05 mg l^{-1} concentration (Benjamin *et al*., 1987) and kinetin 1.0–5.0 mg l^{-1} increased rapid proliferation rate in *Picrorhiza kurroa* (Lal *et al*., 1988). In addition, Barna and Wakhlu (1988) reported that a medium containing a combination of 0.9-1.3 mg l^{-1} kinetin and 0.01 mg l^{-1} NAA gave a higher production of multiple shoots in *Plantago ovate*. In many genotypes for optimal quantity of shoot proliferation, cytokinin with low concentration of auxin is required (Shasany *et al*., 1998; Sharma *et al*., 1993; Rout & Das, 1997; Roja *et al*., 1990). Morever, a single cytokinin could induced embryogenesis in coffee (Yasuda & Fujii, 1985; Bhojwani & Razdan, 1996a).

Benzylaminopurine (BAP) showed high proliferation of *G. jasminoides* compared to 2ip and kinetin Sayd *et al* (2010) using indole-3-butyric acid (IBA) in micro cuttings of *G. jasminoides*, high percentages of root *in vitro* and *ex vitro* were obtained (Pontikis, 1983; Hatzilazarou *et al*., 2006).

Dumitrescu (2002) carried out a successful combination of 0.1 mg l^{-1} IAA and 1 mg l^{-1} BAP for chlorophyll extract on *G.jasminoides* Ellis, and a higher range of shoot proliferation of this plant via BAP reported by Chuenboonngarm, *et al* (2001).

Furthermore, gibberellin increased numbers and length of shoots in *G. jasminoides* and improved the shoot quality rating (Economou & Spanoudaki, 1986b).

Pontikis (1983) revealed long and high-quality of shoot in cape jasmine via 2iP, but in 2011 Chuenboonngarm reported, 2iP made 100% chimeric plants of this species. Based on an Economou's report, BA induced axillary buds in *G. jasminoides* (Economou & Spanoudaki, 1986a).

Using 1.7 mg l^{-1} TDZ with IAA 1 mg l^{-1} produced adventitious shoot on Gardenia (Al-Juboory *et al*., 1998). However, BAP and NAA showed high proliferation compared to 2ip and kinetin on this crop (Sayd *et al*., 2010). In addition, indolic-3-butyric acid (IBA) in micro cuttings of *G. jasminoides* produced high percentages of roots *in vitro* and *ex vitro* (Pontikis, 1983; Hatzilazarou *et al*., 2006). Combination of 0.2 mg l^{-1} 2, 4- D and Kinetin on suspension culture of *G. jasminoides* Ellis produces salicin from salicyl alcohol (Mizukami *et al.*, 1987; Kubota *et al.*, 1995).

2.3.4 Explant Sterilization

Purnima and Sabita (2010) sterilized the surface shoot and bud of *Crataeva adansonii* and *Jasminum officinale* L. an ornamental and medicinal plant as explant with 0.1% (w/v) mercuric chloride solution for 5 min followed by 5–6 rinses with autoclaved distilled water followed by detergent (0.5 ml 20% Extran) and 0.1% (v/v) tween-80 in 250 ml sterile conical flasks by continuous shaking for 20 min. In tissue culture of *Myrtus,* Barbara et al (2010) used a few drops of liquid dish soap, followed by 70% ethanol for 30 seconds and sterilized with NaOCl solution, (1.2% of active chlorine) for 20 minute and rinsed twice by autoclaved distilled water.

For *G. jasminoides* Ellis Al-Juboory *et al*, (1998) sterilized the explants using 1% (v/v) sodium hypochlorite (NaOCl) solution containing 0.1% tween-20 for 10 min, and three separate rinses with sterile distilled water for five min each. However, Chuenboonngarm *et al*, (2001) sterilized the shoot of *G. jasminoides* using of two time clorox 15% and 10% (v/v), respectively for 10 minute, both supplemented with 0.25% (v/v) Tween-20. Sayd *et al.,* 2010 followed Chuenboonngarm et al (2001) method but he used 0.1% mercuric chloride for second step instead of clorox 10%

Economou & Spanoudaki, (1986a), sterilized vegetative shoot tip explants of *G. jasminoides* by immersing it in 0.1% captan solution (w/v) for 10 min followed by a soaking in 1% sodium hypochlorite (v/v) solution that had been supplemented with 5 drops Tween 20 for 15 minutes.

2.4   Secondary Metabolites

Secondary metabolism in plant was used by one of the great pioneers of plant physiology, Julius Sachs in 1873. In his published textbook he wrote:
"We can designate as by-products of metabolism such compounds that are formed by metabolism, but are no longer used for the formation of new cells. Any importance of these compounds for the inner economy of the plant is as yet unknown" (Karl *et al.,* 2009b).

Kossel in 1891 introduced the term "secondary", which implies, the secondary metabolites do not have an important effect for plant life and are present only incidentally, but primary metabolites are present in every living cell capable of dividing (Edreva *et al.,* 2008). In last decade, secondary metabolites, have been changed to a subject of dramatically increasing interest relevant to their important practical application for medicinal, nutritive and cosmetic purposes (Kumar & Shekhawat, 2009).

In the first of the 1970s, plant tissue culture had achieved a developmental status employing methods of microbial fermentation techniques and antibiotic production to be used for large-scale cultures from plants, in order to avoid the above mentioned problems of imports of raw materials.

Nowadays, traditional medicinal systems utilize plant-based medicines, and are experiencing a revival worldwide. This has resulted in enormous pressures on biodiversity, and the destruction of valuable biotopes particularly in developing countries involved in meeting the demands of global markets. Tissue culture could provide alternatives.

2.4.1   Secondary Metabolites in Plants

Plants in natural environment produce a diversity of compounds from a single highly purified molecule to highly complex molecule. Furthermore, some of the metabolites are produced by certain stereo specific reactions, which are carried out only by plant system. e. g. Digicoxin (Karl *et al.,* 2009b; George 1995; Seong *et al.*, 2010).

In recent years, there has been a sudden rise in consumer demand for the natural plant derived products. This has led to increased use in the development of biotechnological methods and plant products for the production of such compounds (Seong *et al.,* 2010). Ever since Routien and Nickell (1956) suggested to use of plant tissue culture for commercial exploitation, many plants have been screened for potential metabolites (George, 1995).

The low yield of the metabolites from plant tissue culture, as compared to intact plant, has been the main factor in the commercial development of many of the compounds (Tab 2.1). In addition, some results equal or higher than intact plant in secondary metabolites have been reported (Knorr, 1989; Xinrong, 2003).

Among of various *in vitro* produced plant metabolites, only a small number have been found to have the requirements of a market's price and size, which are the two major factors in the commercialization of any compound (Endress, 1994).

The product cost of vanillin production from cell culture has been brought down by manipulating the culture conditions. The use of immobilization technique has the potential to bring down the price of vanilla (Dziezak, 1986; Prosper-Cabral *et al.,* 2007).

2.4.2 **Antioxidant Compounds**

Antioxidant compounds reduce the risk for chronic diseases including cancer and heart disease and play an important role as a health-protecting factor. They consist of a group of molecule capable of inhibiting the oxidation of other molecules that have health enhancing effects in our bodies such as vitamins, minerals and enzymes. Most of these compounds in a typical diet are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties.

Some compounds, such as gallates, have strong antioxidant activity, while others, such as the mono-phenols are weak antioxidants. Free radicals harm our immune system leading to many degenerative diseases. They are atoms that cause damage to our cells. These atoms are formed by our cells being exposed to a variety of substances such as smoke, pollutions, radiations, chemicals, drugs, alcohol, and pesticides.

Antioxidants works by donating an electron to the free radicals to convert them to harmless molecules. This protects cells from oxidative damage that leads to aging and various diseases.

Many types of minerals and vitamins are classified as antioxidants but they are not the same. Some antioxidants, including enzymes and other molecules are made in our cells and some other essential antioxidants such as vitamins C, E, and selenium must come from our diets (Aruna *et al*., 2001; Ramamoorthy & Awang, 2007). Various plants and spices such as *Ocimum sanctum*, *Piper cubeba* L., *Allium sativum* L., *Terminalia bellerica*, *Zingiber officinale Roscoe* and several Indian and Chinese plants have been reported to possess antioxidant activity. Majority of this activity is

due to the flavones, isoflavones, anthocyanin, flavonoids, coumarin lignans, catechins and isocatechins (Aqil *et al.,*, 2006; Khalaf *et al.,* 2008).

Antioxidant-based drug formulations are used for the prevention and treatment of complex diseases like Atherosclerosis, Stroke, Diabetes, Alzheimer's disease and Cancer (Devasagayam *et al.,* 2004; Edreva *et al.,* 2008; Hsin-Sheng, 2004; Choi *et al*., 2007; Aruna *et al.,* 2001; Karl *et al.,* 2009a).

2.4.3 **Antioxidant Activity Screening Methods**

These screening methods are popular due to their high speed and sensitivity:

a. **Total Phenolic Content (TPC)**

Polyphenols in plants possess an ideal structural chemistry for free radical scavenging activity. These diverse group of phenolic compounds include flavanols, flavonols, anthocyanins, phenolic acidsand many others. Antioxidative properties of this group arise from their high reactivity as electron donors or hydrogen from the ability of the polyphenol derived radical to stabilize and delocalize the unpaired electron and from their potential to chelate metal ions. The amount of total phenol content can be determined by Folin-Ciocalteu Reagent (FCR) method (Chanda & Dave, 2009; Riceevans *et al.,* 1997).

b. **Total Flavonoid (TF)**

The amount of total flavonoid content can be determined by aluminum chloride method. Quercetin or catechin can be used as a positive control. The

flavonoid content is expressed in terms of (mg 1^{-1} of extracted compound) standard equivalent (Chanda & Dave, 2009).

c. Free Radical Scavening Assay

1,1-diphenyl-2-picrylhydrazyl free radical scavenging (DPPH) assay, this method is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant compounds. DPPH is based on the reduction of DPPH in methanol solution in the presence of a hydrogen–donating antioxidant due to the formation of the non radical form DPPH-H (Khalaf *et al.*, 2008).

d. Superoxide Anion Radical Scavenging (SO) Assay

The superoxide anion is a weak oxidant. It gives rise to generation of powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress. Numerous biological reactions generate superoxide anions that are highly toxic species. Measurement of the superoxide anion scavenging activity of the extracts was based on the method described by (Liu *et al.,* 1997) with slight modification of (Oktay *et al.,* 2003). Superoxide radicals are generated non-enzymatically in PMS–NADH systems by the oxidation of NADH and assayed by the reduction of Nitro Blue Tetrazolium (NBT).

e. Xanthine Oxidase Method

Xanthine oxidase (XO) is one of the important biological sources of oxygen-derived free radicals that contribute to oxidative damage to living tissues that are involved in many pathological processes such as inflammation, atherosclerosis, cancer and aging (Chiang *et al.,* 1994; Cos *et al.,* 1998).

In vitro bioassays are used to examine test materials for xanthine oxidase inhibition, as inhibitors of xanthine oxidase may be potentially useful for the treatment of gout or other XO-induced diseases .

Two different assays can be used to determine superoxide anion-scavenging activity: the enzymatic method with cytochrome C and the no enzymatic method with nitroblue tetrazolium (NBT).
that enzymatic method, superoxide anions can be generated by xanthine and xanthine oxidase system (Sweeney *et al.,* 2001).

2.4.4 Review of previous experiments

Ramamoorthy and Awang (2007) tested antioxidant activity of *Morinda citrifolia* (as a very old folk medicinal plant) fruit extracts. They tried various solvent such as butylated hydroxyl toluene and tannic acid and were analyzed for their antioxidant activity by peroxide value method and diphenyl picryl hydrazyl (DPPH) radical scavenging method. Khalaf *et al* (2009) and Edreva *et al* (2008) investigated antioxidant activity of methanolic extract of *Camellia sinensis* L. (green and black tea) leaves powdered, rhizomes of *Zingiber officinale Roscoe* (Gingers), seeds of *Trigonella foenum-graecum* L. (Fenugreek), cloves buds *Eugenia caryophyllus* (Spreng), *Piper nigrum* L. (Black Pepper), *Elettaria cardamomum* L. (Cardamom)

and *Piper cubeba* L. (Sweet Pepper) by free radical scavenger activity method (DPPH).

Antioxidant activity of *Ginkgo biloba* and *Panax ginsen* were measured by Mantle *et al* (2000). They used *in vitro* extract and methanol as a solvent. The SOD assay has been used in this experiment, O'Sullivan et al (2011) also used this method for screening antioxidant activity in different medicinal plants. His essay has been evaluated via methanol extract (*in vitro*) and SOD kit .

A comparison between antioxidant activity of callus extract and *in vivo* grown extracts of *Asparagus officinalis* was reported by Khorasani *et al* (2010). The experiment was carried out via SOD assay and DPPH method, they used ethanol as a solvent.

In vivo and *in vitro* methanol extracts of *Gardenia jasminoide* Ellis was tested for antioxidant activity by DPPH method. All *in vitro* extracts growth on MS medium supplemented with different concentrations of various auxin compared to other hormones and intact plants showed higher levels of antioxidant content (Sayd *et al.,* 2010).

High potential antioxidant activity of methanol fruit extract via DPPH method (Chen *et al*., 2010; Chen *et al*., 2008) and water fruit extract of *G. jasminoide* by superoxide dismutase like (SOD-like) and DPPH assay (Debnath *et al.,* 2011) have been reported.

Table 2.1: Secondary Metabolites by Plant Cell Cultures (George, 1995; Edreva *et al.*, 2008; Khalfalla *et al.*, 2009)

Metabolite	Obtained form	Application
Shikonin	*Lithospermum Erythrorhyzon*	Pharmaceutical, cosmetic
Indole alkaloids	*Rauwolfia serpentina*	Pharmaceutical
Indole alkaloids	*Catharanthus roseus*	Pharmaceutical
Berberine	*Coptis japonica*	Pharmaceutical
Rosmarinic acid	*Coleus blumei*	Flavour
Artemesin	*Artemesia annua*	Artimalarial
Carotenoids	*Many sources*	Food colourant
Diosgenin	*Dioscorea spp*	Contraceptive
Stevioside Rebaudioside	*Stevia rebaudiana*	Sweetener
Vanillin	*Vanila planifolia*	Flavour
Glycyrrhizin	*Glycyrrhyza glabra*	Sweetener
Capsaicin	*Capsicum annum* *Capsicum frutescens*	Pungent food additive
Betaxanthins	*Beta vulgaris*	Food colourant
Digitoxin	*Digitalis Lanata*	Pharmaceutical
Nicotine	*Nicotiana tabaccum*	Insecticides

2.5   Antibacterial Activity

Waksman (1942) stated the term "antibiotic" to describe any substance produced by a microorganism that is antagonistic to the growth of other microorganisms in high dilution. This definition excluded synthetic antibacterial substances and compounds that kill bacteria but are not produced by microorganisms. Many antibacterial compounds are relatively small molecules with a molecular weight of less than 2000 atomic mass units.

Despite the fact that pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased. In general, bacteria have the genetic ability to transmit and obtain resistance to drugs, which are used as therapeutic agents (Cohen, 1992; Nascimento *et al.*, 2000).

Such a fact is cause for concern, because of the numbers of patients in hospitals who have suppressed immunity, and have new bacterial strains, which are multi-resistant. Consequently, new infections can occur in hospitals resulting in high mortality. Using extracts from phytochemicals obtained from plants, with known antibacterial properties, can be of great importance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency (Sabahat & Tariq, 2009). Natural products as medicinal agents has become progressively popular. However, The lack of standardized methods also makes direct comparison of results between studies impossible. The various methods used are disc diffusion, well diffusion, agar dilution and broth dilution. (Pati & Kurade, 2005). The diffusion and dilution methods are routinely used in antibacterial susceptibility testing and have been widely used for many years to accurately measure antibacterial activity (Janssen *et al.,* 1987).

Antibacterial activity based on broth dilution technique show enormous variations in methodology and the choice of surfactants and solvents such as Tween 20, Tween 80, Dimethyl sulphoxide (DMSO) and Ethanol (Flamini *et al.,* 1999; Hammer *et al.,* 1999; Pati & Kurade, 2005).

Tween 80 has various effects on bacteria at concentrations as low as 0.05%, 0.5% and 1%. These effects observed include a bacteriostatic action, inhibition of nucleic acid synthesis and alteration of fatty acid composition, respectively. Furthermore, tween 80 has been showed most accurate results as an emulsify oil for testing the antimicrobial activity of the hydrophobic and viscous essential oils in Broth dilution method (Hood *et al.,* 2003).

2.5.1 Methods and Review on Antibacterial Activity Assay

Some of the common methods for screening antimicrobial activity test include: Agar Absorption Assay, Agar Dilution Assay, Disc Diffusion Assay, Well Diffusion Assay and Broth dilution assay.

Sabahat and Tariq (2009) has used disc diffusion method for screening antibacterial activity in *Origanum vulgare* (oregano) against 111 gram-positive bacterial isolates belonging to 23 different species related to three genera. The inhibitory activity of *Vatairea macrocarpa* on *Klebsiella* spp and *Staphylococcus aureus* were reported (Matos *et al.,* 1988). Another study by Lemo (1992), showed antibacterial and antifungal (*C. albicans*) activity of essential oils from leaves of *Croton triangularis*.

A study of five bacteria species proved that ethanol extracts from 70 % of the plants were toxic to cells and only one of the species of *Combretum duarteanum* showed antibacterial activity (Nascimento *et al.,* 1990).

In 1988 the toxicity of extract from *Arthemus sativa*, which is known to have antibacterial activity, was reported (Carvalho *et al.,* 1988; Nascimento *et al.,* 2000). Antibacterial activity from *Mikania triangularis*, known as "Thin leaf guaco", was tested against five genera of bacteria and three genera of yeast, and its activity against *Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Staphylococcu aureus* and *Staphylococcus epidermidis* has been proven (Cruz *et al.,* 1996; Choudhar *et al*., 2011).

Effects of phytochemical and the antimicrobial activity of anacardic acid on *S.aureus, Brevibacterium ammoniagenes, Streptococcus mutans* and *Propionibacterium acnes* were observed (Izzo *et al.,* 1995).

Antibacterial effect of Thyme (Geraniol) , Lavender and Rosemary via disc diffusion method with Mueller–Hinton agar (MHA) as basal medium against *Haemophilus influenzae*, *Streptococcus pyogenes*, *S.aureus* and *E. coli* has been reported (Shigeharu *et al.,* 2001). *In vitro* dried extracts of *Pimpinella anisum, Cinnamomum cassia, Coriandrum sativum, Juniperus oxycedrus, Glycyrrhiza glabra* (Ates & Erdorul, 2003) and *crude petroleum* ether extract obtained from leaf callus tissue of *Decalepis hamiltoni* against various bacterial species for antibacterial potential by agar diffusion methods and (MHA) were studied (Thangavela *et al.,* 2011).

In this study, antibacterial activity of *in vitro* and *in vivo* extracts of *Gardenia jasminoides* Ellis by disk diffusion method with MHA as basal medium against selected bacteria was evaluated.

2.6   Cell Suspension Cultures and Somatic Embryogenesis

Callus cultures, has been known in two categories: compact or friable. In compact callus, the cells are seen in densely aggregated, however in friable callus the cells are not fitting tightly to each other and the callus becomes soft and easily could be separated.

Friable callus provides the substance to form cell-suspension cultures. Explants from particular cell types or some plant species tend not to form friable callus, making cell-suspension initiation a difficult task.

Sometimes, friability callus can be improved by repeated subculturing or by manipulating the medium components and even by culturing it on medium with a low concentration of agar or semi-solid medium. Friable callus into a liquid medium that is usually the same compound as the solid medium used to the callus culture and then agitated, single cells and/or small cluster of cells is released into the media under proper conditions, the released cells continue to grow and divide and finally producing a cell-suspension culture. For quick build up the cell numbers should be used relatively large inoculums when commencing cell suspensions. However, some toxic products released from stressed or damaged cells, and could build up to harmful and deadly levels and shall be removed the large cell cluster during subculturing.

Maintaining of cell suspensions is same as a culture in conical flasks. Repeated subculturing into fresh media sequentially cultures them, the results in dilution of the suspension and the beginning of another growth cycle. The dilutions degree during subculture is very important and should be determined scientifically for each culture.

The other method to direct tissue extraction for products that cannot be chemically synthesized is by using of cell suspension cultures and callus for the production of a known secondary metabolite (González-Rábade *et al.,* 2011).

Basic of somatic embryogenesis is the development of somatic cells into somatic embryos (Arnold *et al.,* 2002) through characteristic embryological developments without gametic fertilization (Schumann *et al.,* 1995). Somatic embryogenesis due to high production of regenerates, lower frequency of chimeras and incidence of somaclonal variation is a reliable micropropagation method (Ahloowalia, 1991) and also can be induced to occur directly or indirectly by modulating tissue culture conditions *in vitro* (Sharp *et al.,* 1980; Namasivayam, 2007).

Embryos directly develop on the surface of explants in direct somatic embryogenesis but there is an intermediary step of cell suspension culture or callus formation in indirect somatic embryogenesis (Williams & Maheswaran, 1986). Direct or indirect somatic embryogenesis can be achieved in a plant species by manipulating the plant growth regulators and explant types (Siong *et al.,* 2011; Ali, *et al.,* 2007).

2.7   Double Staining Test

Double staining procedure described by Gupta & Durzan (1987) allows densely cytoplasmic cells and highly vacuolated cells (Emons, 1994) be distinguished, which constitute most multicellular aggregates (Filonova *et al*., 2000).

This method, because of two stain, acetocarmine and Evan's blue for staining cells, has been called double staining.

Based on Gupta and Durzan (1987) in this method:

1)   Embryogenic cells have large nuclei and dense cytoplasm. These nuclei stain an intense, bright red with acetocarmine. Strands in the cytoplasm also show an affinity for acetocarmine and stain bright red. Acetocarmine is used to detect Glycoproteins, Chromatin, and DNA in cytochemical studies (Sharma & Sharma, 1980).

2)   Smaller nuclei, which are associated with formation of suspensions derived from embryonal cells, react with Evan's blue to further differentiate the embryogenic mass.

This method easily distinguished embryogenic masses from non-embryogenic cells (Bozhkov *et al.,* 2002; Dos *et al.,* 2002; Steiner *et al.,* 2005; Hiraoka *et al.,* 2004; Bhansali & Singh, 2000; Jain & Gupta, 2005; Gupta & Durzan, 1987).

Materials and Methods

3.1   Incubation Conditions

Temperature, light and humidity are important parameters in culture room. Temperature usually applied in the culture incubation room is approximately 25 ºC while some of the tropical species usually require higher temperatures.

Light is another essential parameter for morphogenetic processes like shoot and root initiation and somatic embryogenesis. Quality, intensity and photoperiod are very critical to the success of certain culture experiments (Murashige, 1977). Exposure to light for 12–16 hours per day under 35–112 mmol m^{-2} s^{-1} provided by cool, white fluorescent lamps is usually recommended.

3.2   Basic Processes in Tissue Culture

The major procedures normally performed in a tissue culture laboratory are:

    A.   Glassware washing

    B.   The media preparation

    C.   Sterilization (equipment and media)

    D.   Explants preparation and aseptic for transferetion

    E.   Culturing and growth explants

3.3 Basic Organization of Laboratory

Each plant tissue culture laboratory should be having three areas:

1) General laboratory (This area provides enough space for common task doing individually or in-group working).

2) Aseptic area for explants transfer

3) Culture rooms (The conditions of these rooms such as light, temperature and humidity must be controlled).

3.4 General Laboratory Area

This place has arranged with most of the equipment:

➢ **Washing area:** should be having a big washbasin (alkaline and acid resistance is preferable) taps water; at least two sinks and table and racks for the proper drying glassware place.

➢ **Refrigerator:** For keeping some chemicals, prepared media, PGRs.

➢ **Autoclave:** This is one of the vital equipment in most of biology laboratory which using for sterilization media, glassware, water and instrument. High pressure and temperature during the specific time (121ºC and 15 Psi between 15- 21 minutes) is a proper sterilization method even for hardy fungus and spores.

➢ **Hotplate, Stirrer:** For preparation media or hormone or making solid or semi-solid and measuring the pH during media stirring.

➢ **pH meter:** Required for measuring pH.

➢ **Water distiller:** To provide high quality water.

> **Balance:** A triple beam could be useful, measuring of material and chemical is essential in plant tissue culture laboratory.

3.5   Culture Area

In this area temperature, light quality, relative humidity and photoperiod should be taken into consideration. Some of the varieties need more than 5,000 Lux, but most of them require an illumination between 500 to 3,000 Lux and others just need darkness as in the case of *in vitro* tube induction.

Culture shelves can be metallic or wooden, and should be painted white, otherwise arrangement, and number of the shelves, where containers and tubes with the cultures are placed, will vary according to the room's dimensions.

3.6   Aseptic Transfer Area

Preferable a separate room and as clean as possible, Still-air boxes or Laminar flow hood with ultraviolet (UV) is installed. Aseptic procedure will be doing inside the chamber.

3.6.1   Chamber Sterilization Steps

- ✓   Spraying 70% (v/v) ethanol
- ✓   Dry with paper towel's
- ✓   Turning on the air flow 45 minute before
- ✓   Turning on the (UV) for 15-20 minutes before startting work

3.6.2   Preparing Ethanol Solution

Ethanol solution was prepared by:

1)   Ethanol 100%

2)   Distilled water

3)   Graduated cylinder 100 ml

70 ml of ethanol was measured by graduated cylinder and the volume was adjusted to 100 ml with distilled water.

3.7   *In vitro* Culture Establishment Stage

For this step, a clean place that guarantees the quality, uniformity, and strength of the material for marketing and research at the final stage is selected. The selected plants can develop and grow through the process of thermotherapy and meristem culture. These types of plants will be used as a source of explants for the production process. In some infection cases, antibiotics threat until the complete elimination of infection symptoms is necessary. Otherwise, if pathogen free cases, entire buds are taken and placed in a temporary culture medium where they will be observed for one or two weeks.

3.8 Production Stage

The propagation range depends on the species:

These ranges commonly present as a reference in most of the micropropagated plants that have been taken. In the same crops, propagation range may vary according to the phytohormones in the culture medium. The average time of each propagation cycle is between three or four weeks for each step , depends on these three causes:

I.   The environmental conditions

II.   Species behaviour

III.   Culture medium

3.8.1 Preparation MS Media

To prepare one litter MS medium, these items were used:

➢ MS (Murashige & Skoog, 1962) powder with Gamborg vitamins

➢ Gelling agent (gellan gum)

➢ Carbon source (sucrose)

➢ Distilled water

MS powder (4.43 g) and 30 g of sucrose were weighed out and were added into 1000 ml beaker filled with distilled water (800 ml) on a magnetic stirrer, and then stirred until fully dissolved.

The volume was adjusted to 1000 ml with distilled water (removed the beaker from the stir plate and the medium was poured into a graduated cylinder, the volume

was brought up to 1000 ml and the medium was poured back into the beaker and was stirred.).

The pH was adjusted to 5.8 using one or two drops of 0.1N NaOH or HCl. In the final step, 5 g of gellan gum was added and media were stirred and the solution was heated until agar was completely dissolved and the media were cleared.

If adding hormones to the media was required, pH adjusting should be done after adding the appropriate concentration of hormones by using prepared stock hormones.

MS medium were dispensed into proper container sealed with aluminium foil and were autoclaved for sterilization (21 minutes under 121ºC and 15 psi).

3.8.2   WPM Medium Preparation Method

To prepare one litter WPM medium (Lloyd & Mc Cown, 1980), these items were used:

- ➢ WPM powder with vitamins

- ➢ Gelling agent (gellan gum)

- ➢ Carbon source (sucrose)

- ➢ Distilled water

WPM powder (2.41 g) and 30 g of sucrose were weighed out and were added into 1000 ml beaker filled with distilled water (800 ml) on a magnetic stirrer, and then stirred until fully dissolved.

The volume was adjusted to 1000 ml with distilled water (removed the beaker from the stir plate and the medium was poured into a graduated cylinder, the volume was

brought up to 1000 ml and the medium was poured back into the beaker and was stirred.). The pH was adjusted to 5.8 using one or two drops of 0.1N, NaOH or HCI.

In the final step, 5 g gellan gum was added and media were stirred and the solution was heated until agar was completely dissolved and the media were cleared. If adding hormones to the media was required, pH adjusting, should be done after adding the appropriate concentration of hormones by using prepared stock hormones.

WPM medium were dispensed into proper containers, sealed with aluminium foil and were autoclaved for sterilization(21 minutes under 121ºC and 15 psi).

3.8.3   Hormone Stock Preparation

Most plant tissue culture laboratories prepared their plant growth regulators as stock solutions. The stock solution is a concentrated solution of a desired chemical. When the chemical is needed, a small amount of stock solution is added to a medium. This avoids having to weigh out frequent and small amounts of plant growth regulators.

To prepare, 1 g l^{-1} hormone stock solutions for tissue culture these chemicals were used:

➢ Plant growth regulators

➢ Desired solvent

➢ Distilled water

Plant growth regulator (100 mg) was added to a 100 ml volumetric flask and 3-5 ml of solvent was added to dissolve the powder. Once completely dissolved, volume topped up with distilled/ deionized water. One ml of the stock solution in one litter of medium will yield a final concentration of 1.0 g l^{-1} of the plant growth.

In this experiment different types of auxin such as NAA, IBA, IAA, 2, 4- D and two types of cytokinin (Kn, TDZ) were used. However, NaOH 1Normal, was used as a solvent for all PGR's, except TDZ (DMSO or Dimethyl Sulfoxide was used as a solvent for TDZ).

Table 3.1: Plant Growth Regulators Concentration Conversions and Chemical Specifications (phytotechlab, 2011)

Plant Growth Regulator	ABA	BAP	2,4-D	IAA	IBA	Kinetin	NAA	TDZ
Mol. Weight	264.3	225.3	221.0	175.2	203.2	215.2	186.2	220.2
Preparation and Storage								
Solvent	NaOH/KoH 1 N	NaOH/KoH 1 N	NaOH/KoH 1 N	NaOH/KoH 1 N	NaOH/KoH 1 N	NaOH/KoH 1 N	NaOH/KoH 1 N	DMSO
Diluents	Water	Water	Water	Water	Water	Water	Water	Water
Powder Storage	-20 to 0 C	RT	RT	- 0 C	0-5 C	- 0 C	RT	RT
Liquid Storage	-20 to 0 C	0-5 C	0-5 C	- 0 C	- 0 C	- 0 C	0-5 C	0-5 C
Sterilization	CA/F	CA/F	CA	CA/F	CA/F	CA/F	CA	CA/F

CA= Co autoclave with other media components
F= Filter Sterilize
CA/F= Co autoclave with media components, however, some loss of activity may occur
RT = Room temperature

3.9   Plant Materials

For initiation of callus cultures, young leaves were collected from 7-year-old field grown plants, from UM botanical garden and nurseries. The young, healthy and without infection or disease leaves were washed in running tap water for 30 minutes, followed by washing in liquid detergent (Teepol, Sigma Aldrich brand).

3.10   Sterilization Techniques

The young leaves were washed using tap water with some drops of teepol for 45 minutes, according to the reviewed literatures (Chuenboonngarm *et al.,* 2001; Sayd *et al*., 2010) next steps were carried out inside the laminar flow.

Explants were immersed in 70 % (w/v) ethanol for 1 minute, were soaked with 70% (w/v) Clorox for 15 minutes, were rinsed one time with sterile distilled water, and were soaked 3 minutes in 0.1 g l^{-1} Mercuric Chloride. In the final step, explants were rinsed for five times with sterile distilled water each for 3 minutes.

The scalpel, forceps, and petri dishes were wrapped in thin aluminium foil and were sterilized by autoclaved at 121 ºC for 20 minutes. Distilled water was sterilized by autoclave. Laminar flow and surface of all equipment were scrubbed with cotton dipped in ethanol 70% (v/v). The manipulation was carried out under strict aseptic conditions inside the laminar airflow bench fitted with a bactericidal UV.

The laminar airflow was sterilized by spraying 70% (v/v) ethanol and UV rays continuously for 20 minutes. Gloves were sterilized with 70% alcohol before inoculation.

3.11   Inoculation

The sterilized leaf explants were cut (50 mm X 50 mm) using a sterile blade and were cultured in sterile containers, containing 30 ml MS (Murashige & Skoog, 1962) and WPM (Lloyd & Mc Cown, 1980) basal media supplemented with various concentrations of plant growth substances consisted of: 2,4-D, NAA, IBA, IAA, TDZ and Kn. The concentrations of PGRs were (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 mg l^{-1}) and hormones added prior to autoclaving. Each flask contained three explants. The cultures were maintained in air-conditioned culture room at 24 ºC, with 16 hours light and 6 hours dark conditions.

The light source was fluorescent tube (40 watt) and the intensity of each light was 1000 Lux at the level of cultures. This experiment was carried out with five replications. MS and WPM without hormones were considered as controls media. After every one month, sub-culturing was performed in the same media and hormone concentrations and after six months callus were weighed, and shoot numbers and root length were measured.

3.12 Secondary Metabolites Assay

Extract Preparation

Six-month-old callus obtained from the different treatments. The callus were weighed and dried at culture room temperature. At the same time, some young and healthy leaves from explants source were obtained and dried in the oven. The dried plant materials were ground using an electric grinder. The extraction was done at room temperature.

For preparation *in vivo* extract, 100 g of dried and ground leaf were soaked in methanol (99%) for 3-5 days separately.

Extracts from callus were also obtained. Depending on the weight of the callus, between 3 to 10 g of dried and ground callus were soaked in methanol (99%) for 3-5 days separately.

The soaked material was stirred every 18 hours using a sterilized glass rod. The final extracts were passed through Whatman filter paper No.1 (Whatman Ltd., England). The filtrates obtained were concentrated under vacuum on a rotary evaporator at 40 °C and stored at 4 °C for further use.

The stock solution of callus extracts (100 g l^{-1}) was prepared by dissolving a known amount of dry extract in 5% tween 80. Extracts were kept inside the refrigerator for further study.

3.12.1 Antibacterial Activity

A. Media Preparation

Disk diffusion method was used in this experiment. Mueller-Hinton or MH was purchased as prepared agar plates.

Mueller-Hinton preparation method:

Mueller-Hinton or MH powder (2.43 g) and of bacto agar (17 g) were weighed out. One-liter beaker was filled with 500 ml of distilled water after a magnetic stir bar was added and stir at medium speed. The MH powder and bacto agar were added to the beaker and then removed the beaker from the stir plate and the medium was poured into a graduated cylinder. The volume was brought up to 1000 ml. The medium was poured back into the beaker and stirred for three minutes.

The solution was autoclaved at 121°C for 15 minutes, dispensed to a depth of 4 mm (approximately 25 ml) in 100 mm petri dishes under laminar flow and allowed to solidify at room temperature. The petri dishes were sealed by using of para film and were stored at 4 to 8 °C for further experiment. Mueller-Hinton agar is stable for approximately 70 days from the date of preparation.

B. Equipment Sterilization

Paper disks (10 mm) were made by punching of five-layer Whatman filter paper No.1 (Whatman Ltd., England). A 50 ml distilled water, 50 ml of extract solvent (5% tween 80), in either 50 mM phosphate-buffered saline (PBS; pH 7.2, containing 0.8% NaCl), and two wrapped forceps with aluminum foil were autoclaved for sterilization(21 minutes under 121ºC and 15 psi).

C. Preparation of bacteria

Four common bacteria (*Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus, and Staphylococcus aureus)* were collected from Microbiology Division of Institute of Biological Sciences, University of Malaya and then were grown in nutrient broth medium to yield a final concentration of 107 colonies-forming units (CFU) ml^{-1}, and were kept in a refrigerator.

D. Nutrient Broth or NB medium Preparation

An 80 mg of the Nutrient Broth or NB medium was weighed and dissolved in 100 ml of purified water inside a conical flask. The solution was mixed magnetic stirrer bar and after wrapping by aluminum foil was autoclaved at 121ºC for 15 minutes.

The media (10 ml) were dispensed under laminar flow into sterile test tubes and each species of bacteria was incubated by sterile cotton swab on two test tubes.

E. Antibacterial Assay

The test bacteria (0.1 ml) were streaked on Mueller Hinton medium plates using sterile cotton swabs. Sterilized filter paper discs were soaked in tween 80 extracts (100 g l^{-1}) and were placed in the center of test bacteria plates. The plates were incubated 24 hours and were kept for another 24 hours at room temperatures. The diameters of the inhibition zones were measured after 48 hours of inoculation. Tetracycline disc (30 μg) and PBS were used as the positive and negative controls, respectively.

3.12.2 Antioxidant assay

Superoxide dismutase (SODs) has been evaluated by SOD kit (Cayman Chemical Company, USA) and according to kit's manual, these steps were followed:

A. Reagent Preparation

1) Assay Buffer (10X)

A 3 ml of Assay Buffer concentrate with 27 ml of HPLC-grade water for assaying 96 wells were diluted. This final Assay Buffer (50 mM Tris-HCl, pH 8.0) containing 0.1 mM Di ethyl enetriamine pentaacetic acid (DTPA) and 0.1 mM Hypoxanthine was used to dilute the radical detector. The solutions were stored at 4 ℃, this diluted assay buffer was stable for at least two months.

2)   Sample Buffer (10X)

A 2 ml of Sample Buffer concentrate with 18 ml of HPLC-grade water for assaying 96 wells were diluted. This final sample buffer (50 mM Tris-HCl, pH 8.0) was used for preparing the SOD standards, diluting the xanthine oxidase, and SOD samples prior to assaying. The solutions were stored at 4º C. This diluted sample buffer was stable for at least six months.

3)   Radical Detector

The factory prepared vials contained 250 µl of a Tetrazolium salt solution. Prior to use, 50 µl of supplied solution was transferred to another vials, was diluted with 19.95 ml of diluted assay buffer, and was covered with a thin aluminum foil. The diluted radical detector was stable for two hours. This volume of radical detector was enough for 96 wells.

4)   SOD Standard

The prepared vials contained 100 µl of bovine Erythrocyte SOD (Cu/Zn). The enzyme was ready to use as supplied. The thawed enzyme was stored in ice.

5)   Xanthine Oxidase

These prepared vials contained 150 µl of xanthine oxidase. Prior to use, 50 µl of the supplied enzyme was transferred to another vial, and was diluted with 1.95 ml of Sample Buffer. The Thawed and diluted xanthine oxidase were stored in ice. The diluted enzyme was stable for one hour.

B. Assay Protocol

Plate Set Up:

The wells on the plate were used as the Fig 3.1

A-G = Standards

S1-S41 = Sample Wells

Figure 3.1: A typical layout of SOD standards and samples

C. Standard Preparation

A 20 μl of the SOD Standard were diluted with 1.98 ml of sample buffer (dilute) to obtain the SOD stock solution. Seven clean glass test tubes were taken and were marked them A-G. The amount of SOD stock and sample buffer (dilute) was added to each tube as described Table 3.2.

Table 3.2: Superoxide Dismutase standards

Tube	SOD Stock (µl)	Sample Buffer (µl)	Final SOD Activity (U/ml)
A	0	1000	0
B	20	980	0.025
C	40	960	0.05
D	80	920	0.1
E	120	880	0.15
F	160	840	0.2
G	200	800	0.25

D.   Performing the Assay

a)   SOD Standard Wells: 200 µl of the diluted radical detector and 10 µl of standard ,(Tubes A-G) per well were added to the designated wells on the plate.

b)   Sample Wells: 200 µl of the diluted radical detector and 10 µl of *in vitro* and *in vivo* extracts (100 g l^{-1}) were added to the wells.

c)   A 20 µl of diluted xanthine oxidase to all the wells as quickly as possible was added.

d)   Carefully were shook the 96-well plates for a few second to mix and covered with the plate cover.

e)   The plate was incubated the plate on a shaker for 20 minutes at room temperature.

f)   The absorbance was read at 440-460 nm using a plate reader.

E. Calculations

   i.   The average absorbance of each standard, *in vivo*, and *in vitro* extracts were calculated.

  ii.   Standard A's absorbance was divided by itself and the other standards and samples extract absorbance to yield the liberalized rate.

 iii.   Plot the liberalized SOD standard rate (LR) (from step 2 above) as a function of final SOD activity (U ml^{-1}) from superoxide dismutase standard table.

 iv.   The SOD activity of the samples using the equation obtained from the linear regression of the standard curve substituting the liberalized rate (LR) for each sample was calculated.

One unit was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. SOD activity was standardized using the cytochrome 'C' and xanthine oxidase coupled assay.

According to factory manual, SOD was calculated by this formula:

SOD (U/ml) = [(sample LR - y-intercept / slope) x 0.23 ml / 0.01 ml] x sample dilution

RESULTS
AND

DISCUSSION

4.1   Initiation of Callus and Maintenance

Initiation of callus was observed from the young leaf explants after two weeks of culture on the MS and WPM medium supplemented with different plant growth regulators such as NAA and IAA. For maintenance of good growth, callus was sub cultured every month, onto MS and WPM medium supplemented with the same hormone and concentration (e.g. from MS supplemented with 1mg l^{-1} to fresh MS with 1 mg l^{-1} of NAA).

Callus with different colour were noted (Fig 4.1), when the media was supplemented with TDZ 1, 1.5 mg l^{-1} and IBA 1 mg l^{-1}. The same was reported by Eeckhaut, et al in 2010.

Figure 4.1: Greenish and yellowish callus from 1 mg l^{-1} TDZ and 1.5 mg l^{-1} IBA after 5 weeks

In order to check whether the callus formation were embryogenic or not, double staining test was done. The results showed that embryogenic cells were formed on callus grown with various auxin (Fig 4.2a) and non-embryogenic on callus formed on MS supplemented with TDZ and Kn (Fig 4.2b).
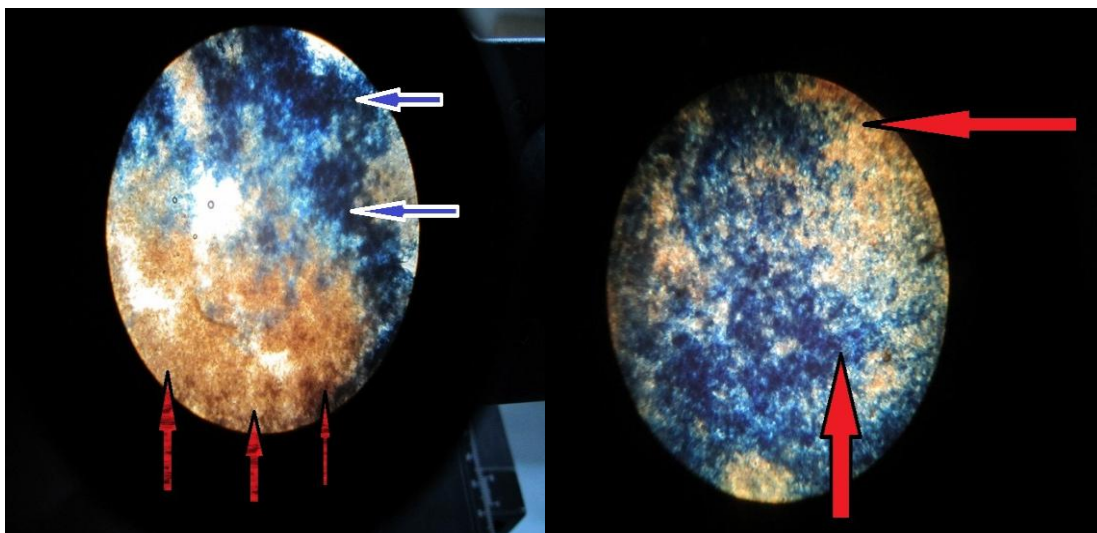
a) embryogeniccells   b) Non-embryogenic cells

Figure 4.2: Early stage embryos after double staining, embryonal heads stained red (acetocarmine) and suspensors stained blue (Evan's blue).

4.1.1 Callus Formation in MS Medium

Callus formation from this species was observed after two weeks of cultures. The types of callus obtained were type one from leaf explant.

The α-Naphthalene acetic acid (NAA) is synthetic auxin and is commonly used in tissue culture media (Bhojwani & Razdan, 1996a). Based on the obtained data, the explants responded to various concentrations of NAA after 14 days of culture.

As shown in Fig 4.3, leaf explants cultured on MS medium supplemented with NAA (1, 1.5, 2, 2.5, and 3 mg l^{-1}) showed higher percentage of callus formation compared to other concentrations. Callus formation was 98 % using 1 and 1.5 mg l^{-1} and 100 % using 2, 2.5, and 3 mg l^{-1} concentration of NAA. Callus induction dropped when concentration of NAA increased from 3.5 to 5 mg l^{-1}.

2, 4-D or 2, 4-Dichlorophenoxyacetic acid is very effective for the induction and callus growth and for somatic embryogenesis *in vitro* conditions (Bhojwani & Razdan, 1996c; Philip, 1984; King, 1984). Callus formation of leaf explants on MS medium supplemented with 2 and 2.5 mg l^{-1} 2, 4-D was 100% and using 1.5 and 3 mg l^{-1} showed 99 and 98 % , respectively. Other concentrations of this hormone gave less than 80 % callus formation.

Indole-3-acetic acid (IAA) is one of the most commonly detected natural auxin, used to induce callus, meristem and shoot culture (Moshkov *et al*., 2008). In many of plant species, the effect of IAA was similar to indole-3-butyric acid (IBA) but IAA is the least stable in the medium (Bhojwani & Razdan, 1996c).

Different concentrations of IAA demonstrated various percentages of callus formation (from 17% until 99%), but the highest percentage (99% and 84%) were

observed on 3.5 and 3 mg l^{-1} of IAA. In contrast, callus from leaf explants were formed 100% on 2.5 and 3 mg l^{-1} concentration of IBA and formation was observed more than 80 % between 3.5 until 5 mg l^{-1} concentration of IBA.
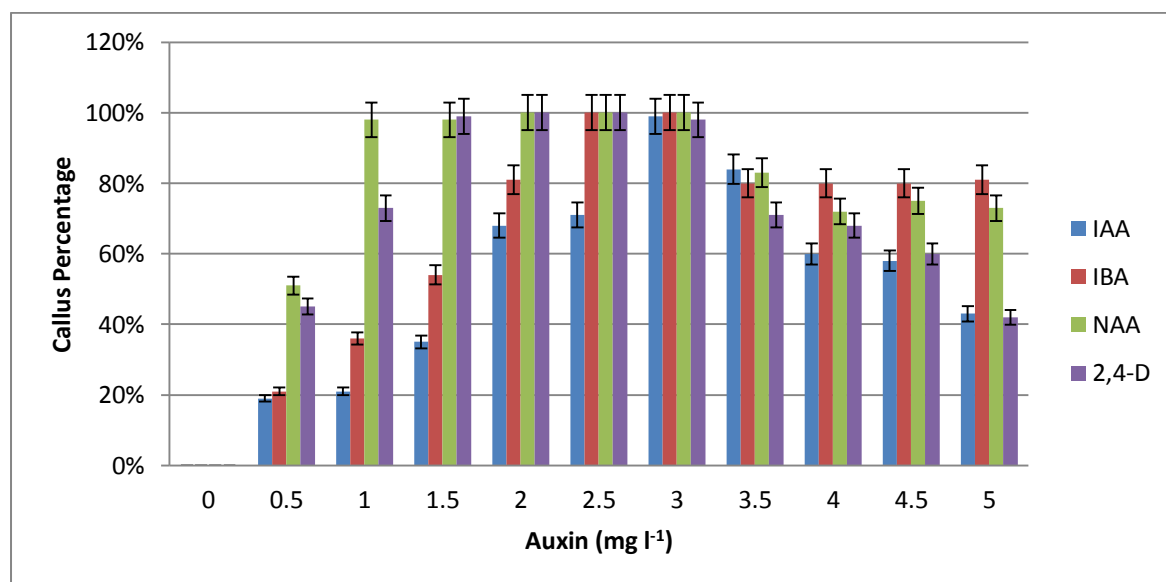
Figure 4.3: Percentage of callus formation of *G. jasminoides* in MS medium supplemented with different type of auxin at various concentrations

Since 1982, N-phenyl-N′-1, 2, 3-thiadiazol-5-ylurea or Thidiazuron which is known as TDZ, has been used as a cytokinin in several studies on shoot multiplication and especially more effective than the other cytokinin with recalcitrant woody species (Lu, 1993). Moreover, this cytokinin in some species is used to obtain higher rate of somatic embryogenesis than other hormones, also cytokinin alone has been found to substitute for both auxin and cytokinin in many species. In some plants, a higher rate of somatic embryogenesis is obtained with TDZ than with other PGRs. (Von, 2007).

Based on Figure 4.4, callus formation started (73%) from 0.5 mg l^{-1} and increased to 84% when MS medium supplemented with 1 mg l^{-1} of TDZ, this percentage fluctuated slightly (between 78% until 70%) on 0.5 to 3 mg l^{-1} and plunged to

45% on 5 mg l^{-1}. Kinetin (Kn) or 6-furfurylaminopurine is often used in culture media for cell suspensions, callus induction, growth and induction of morphogenesis while higher concentrations can be used to induce rapid multiplication of meristems and shoots (Harisha, 2007). However, MS medium supplemented with 1, 1.5, and 2 mg l^{-1} of Kn showed 69, 65 and 60% callus formation, respectively. Percentage of callus formation rapidly dropped to 32% on 3 mg l^{-1} until 11% on 5 mg l^{-1} of kinetin.

Data analyzed showed no significant differences in callus formation between various used auxin on WPM medium, in contrast, MS medium showed significant differences between IAA and other auxin. In addition, Kinetin and TDZ added to MS medium showed statistical differences compare to WPM. There was a little difference between these two hormones on WPM medium but TDZ showed more variation on callus formation compared to Kn.

Shoot formation from leaf explants of *G. jasminoides* Ellis was reported by Duhoky & Rasheed, (2010) and Sayd *et al.*, (2010), but the present results contradict their results. When leaf explant was cultured on MS and WPM media supplemented with a combination used auxin with TDZ and Kn, no shoot formation was observed.
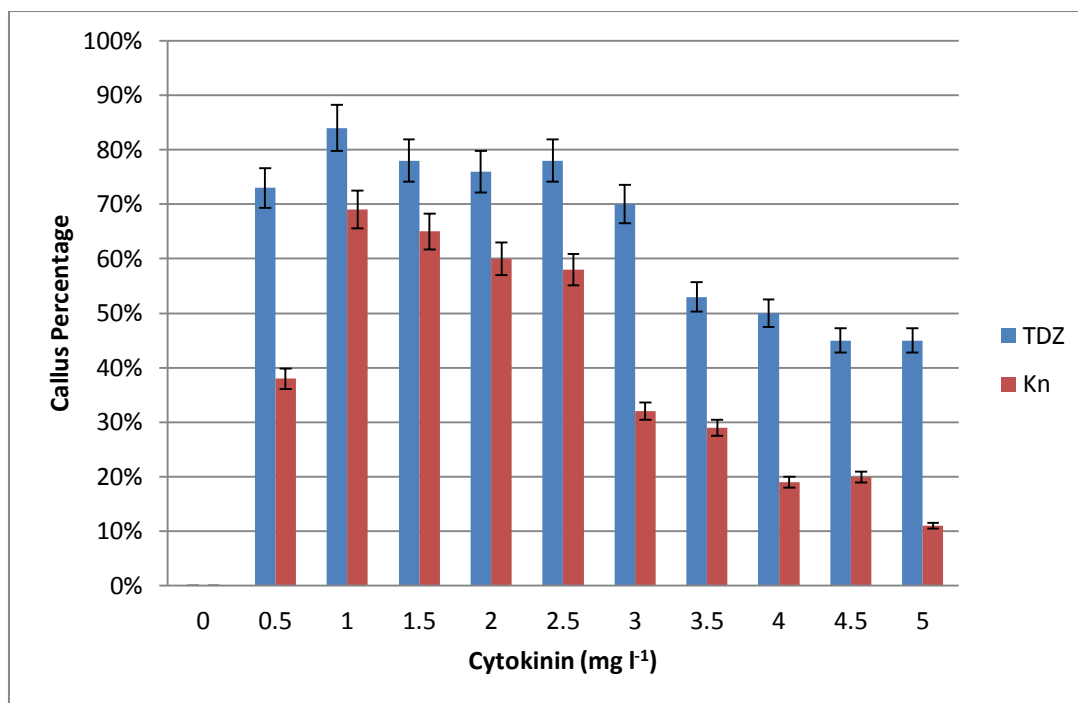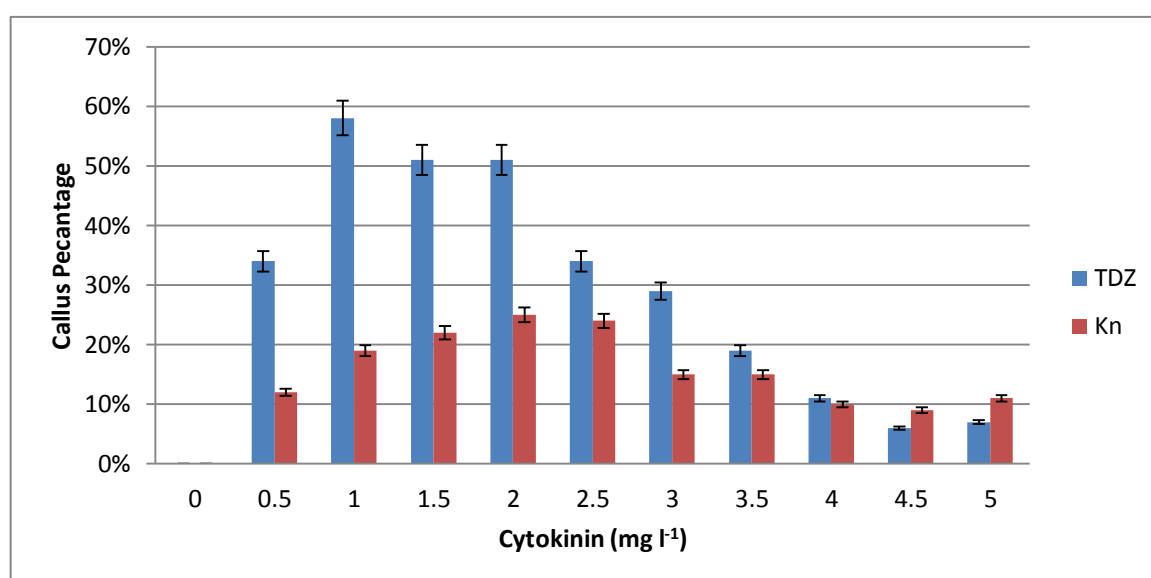
Figure 4.4: Percentage of callus formation on MS medium supplemented with different types of cytokinin at various concentrations

Figure 4.5: Callus induction and root formation from leaf explant of *G. jasminoides* Ellis on MS medium supplemented with 1.5 mg l^{-1} NAA after 2.5 months

4.1.2   Callus Formation in WPM Media

Leaf explants cultured on WPM medium supplemented with different concentrations of NAA except in media without hormone and 0.5 mg l^{-1} of NAA produced more than 80% callus. Based on the figure 4.6, callus formation was observed (73%) on 0.5 mg l^{-1} and 100% on 2, 2.5, and 3 mg l^{-1} concentrations of NAA. No significant difference was observed in the callus percentage between 3.5 to 5 mg l^{-1} of NAA. Callus (29%) on WPM medium supplemented with different concentrations of 2, 4-D was formed on 0.5 mg l^{-1} of hormone and increased to 88% on the 2 mg l^{-1}. The callus formation was maintained at 90% on 2.5 and 3 mg l^{-1} of 2, 4-D and dropped to 76% on higher concentration of hormone (5 mg l^{-1}).

Different concentrations of IAA showed various percentages of callus formation, but 100 % was formed in 2.5, and 3 mg l^{-1} of IAA. WPM medium showed more than 80% of callus formation on 3.5 and 4 mg l^{-1} of IAA; however, on 0.5 mg l^{-1} concentration of this hormone callus was not formed.

Indole-3-butyric acid (IBA) is another synthetic and common auxin for plant tissue culture and for obtaining root initiation in conventional cuttings (Bhojwani & Razdan, 1996 b; Machakova *et al.,* 2008).
The percentage of callus formation on IBA was very close to callus formation on NAA with the same concentrations. A hundred percent callus formation was noted in 2, 2.5, and 3 mg l^{-1} of the IBA and callus percentage was more than 95% between 3.5 until 5 mg l^{-1} of the IBA from leaf explants.
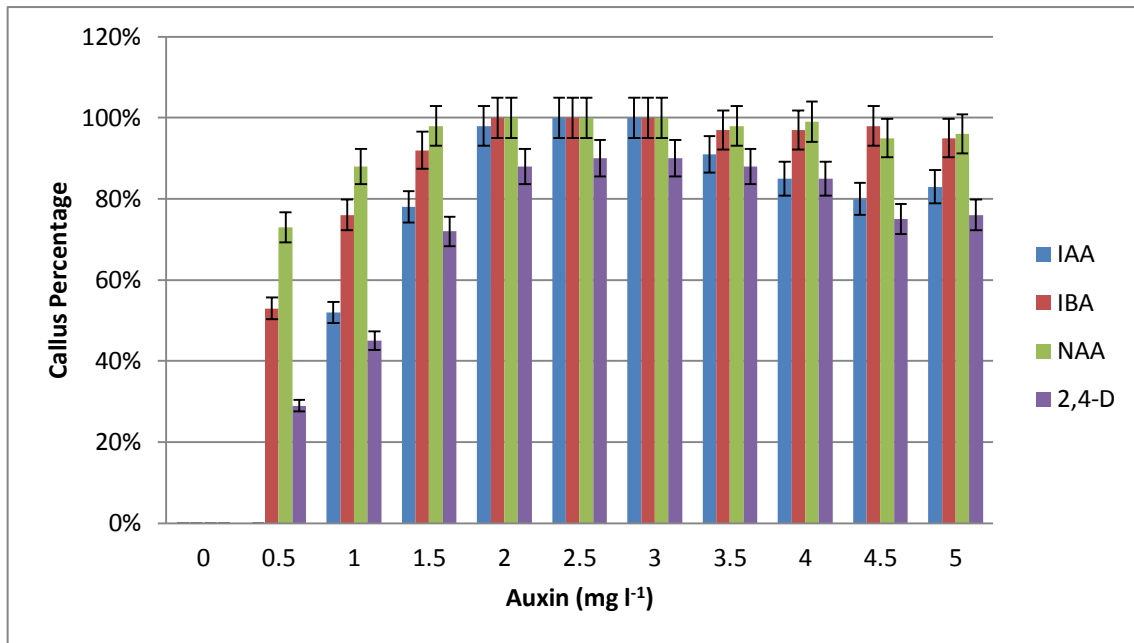
Figure 4.6: Percentage of callus formation in WPM medium supplemented with different type of auxin at various concentrations

Figure 4.7: Callus induction and root formation from leaf explant of *G. jasminoides* Ellis on MS medium supplemented with 1.5 mg l⁻¹ NAA after 2.5 months

Figure 4.8 shows WPM medium supplemented with 1 mg l^{-1} of TDZ gave higher percentage of callus (58%). However, equal percentages (51%) were observed at concentrations of 1.5 and 2 mg l^{-1} of TDZ. In addition, callus percentage suddenly declined from 34% to 7% on concentration of 2.5 to 5 mg l^{-1} of TDZ. Percentage of callus on WPM supplemented with kinetin started (12%) from 0.5 mg l^{-1} and reached to the highest amount (24 and 25%) on 2 and 2.5 mg l^{-1} and fell to lowest percentage (9%) on 4.5 mg l^{-1} .

Figure 4.8: Percentage of callus formation on WPM medium supplemented with different types of cytokinin at various concentrations

4.2.1 Comparison of WPM and MS Media Supplemented with Various Concentrations of NAA for Callus Formation

As data shown in fig 4.9, the horizontal axis represents the concentration of NAA with fixed increment of 0.5 mg l^{-1} and vertical axis represent the percentage of callus formation. Based on observations and collected data, callus formation from leaf explants on MS medium started (51%) on 0.5 mg l^{-1} and rose slightly to 98% on 1 mg l^{-1}, a peak (100%) of callus was observed in 2, 2.5, and 3 mg l^{-1} of NAA.
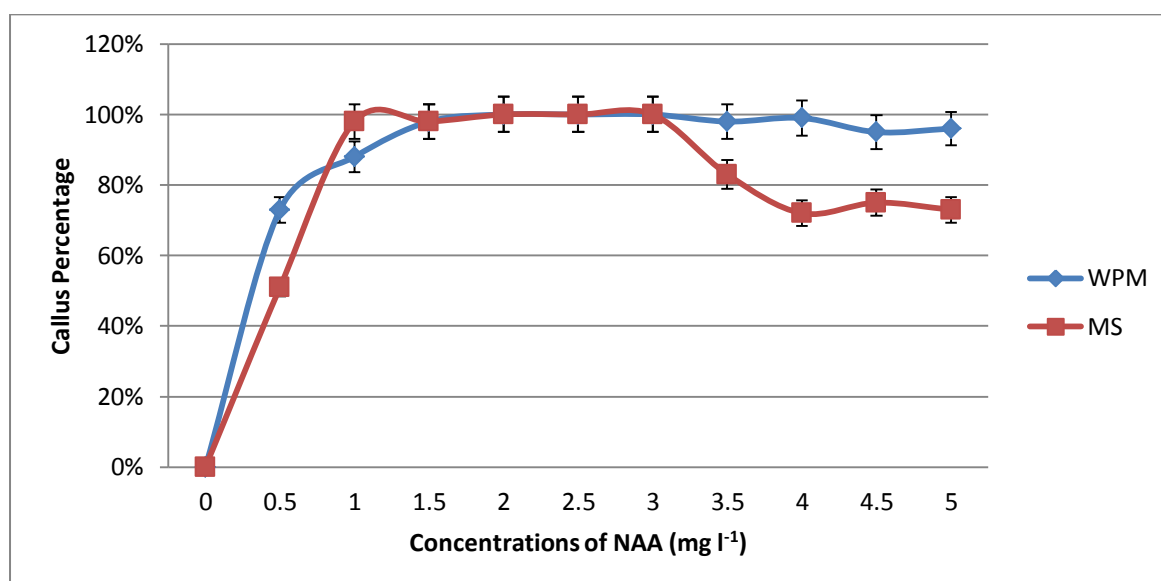
Figure 4.9: A comparison between WPM and MS media supplemented with NAA for callus formation

Formation of callus gradually declined to 83% on 3.5 mg l^{-1} until 73% on 5 mg l^{-1}. Based on fig 4.9, callus was formed (73%) on WPM medium supplemented with 0.5 mg l^{-1} NAA from leaf explants and increased to 88% and 98% in concentrations of 1 and 1.5 mg l^{-1} NAA. Maximum percentage of callus formation (100%) was observed in WPM medium with 2, 2.5, and 3 mg l^{-1} of NAA. However, the callus formation maintained between 98 and 99% on 3.5 and 4 mg l^{-1}. Behbahani *et al*

(2011) compared these media and reported that WPM medium was better than B5 and MS medium in *Lecythidaceae* family.

A comparison between callus formation of leaf explants of *G. jasminoides* Ellis on MS and WPM media showed the optimum callus (100%) was formed on 2, 2.5, and 3 mg l^{-1} NAA when added to MS and WPM media.

Raising concentration of hormone (3 to 5 mg l^{-1}) caused the callus formation to decrease (100% to 70%) on MS medium, but percentage of callus maintained (100% to 95%) between 3 to 5 mg l^{-1} on WPM medium.

The results of MS medium supplemented with NAA are in agreement with previous reports about *in vitro* culture of *G. jasminoides* (Sayd *et al.*, 2010; Duhoky & Rasheed, 2010) , but there are no reports available about *in vitro* culture of *G. jasminoides* Ellis on WPM medium.

4.2.2 Comparison of WPM and MS Media Supplemented with Various Concentrations of IBA for Callus Formation

There was a steep rise on percentage of callus formation when WPM and MS media supplemented with IBA at various concentrations.

Based on fig 4.10, leaf explants of this species formed 53% callus on WPM medium with 0.5 mg l^{-1} of IBA and increased (76% to 92%) on 1 and 1.5 mg l^{-1} of IBA.
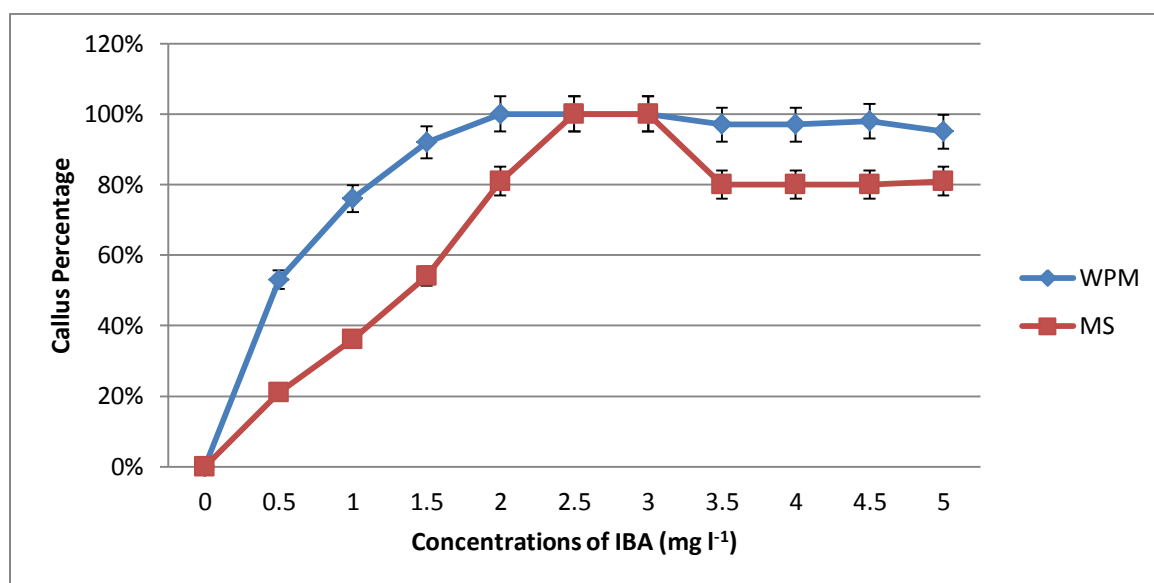
Figure 4.10: A comparison between WPM and MS media supplemented with IBA for callus formation

One hundred percent (100%) of callus were formed on WPM with concentrations of 2, 2.5, and 3 mg l^{-1} of the IBA (Fig 4.10), however, callus maintained between 95 until 98% on higher concentrations (4, 4.5 and 5 mg l^{-1}).

Callus formed (21%) on MS medium with 0.5 mg l^{-1} of IBA and increased (36, 54, and 81%) on 1, 1.5, and 2 mg l^{-1} concentrations of this hormone, respectively.

MS medium with 2.5 and 3 mg l^{-1} of IBA showed 100% callus formation and maintained at 80% on higher concentrations of this hormone (4, 4.5, and 5 mg l^{-1}).

These results are similar with those reported by Sayd *et al* (2010) and George (1995). They reported with optimum concentration of IBA (2-3 mg l^{-1}) for callus induction of *Gardenia* on MS medium.

Figure 4.11: Callus formation from leaf explant in WPM supplemented with 2.5 mg l-1 IBA after 4 months

4.2.3 Comparison of WPM and MS Media Supplemented with Various Concentrations of IAA for Callus Formation

According to figure 4.12, collected data and observation of callus formation, MS medium with 3 mg l^{-1} showed 99% callus formation on the peak of this curve and at 3.5 mg l^{-1} showed 84% callus formation. These results proved former report by Duhoky & Rasheed (2010) about MS medium with the optimum concentration of IAA at 3- 4 mg l^{-1}.
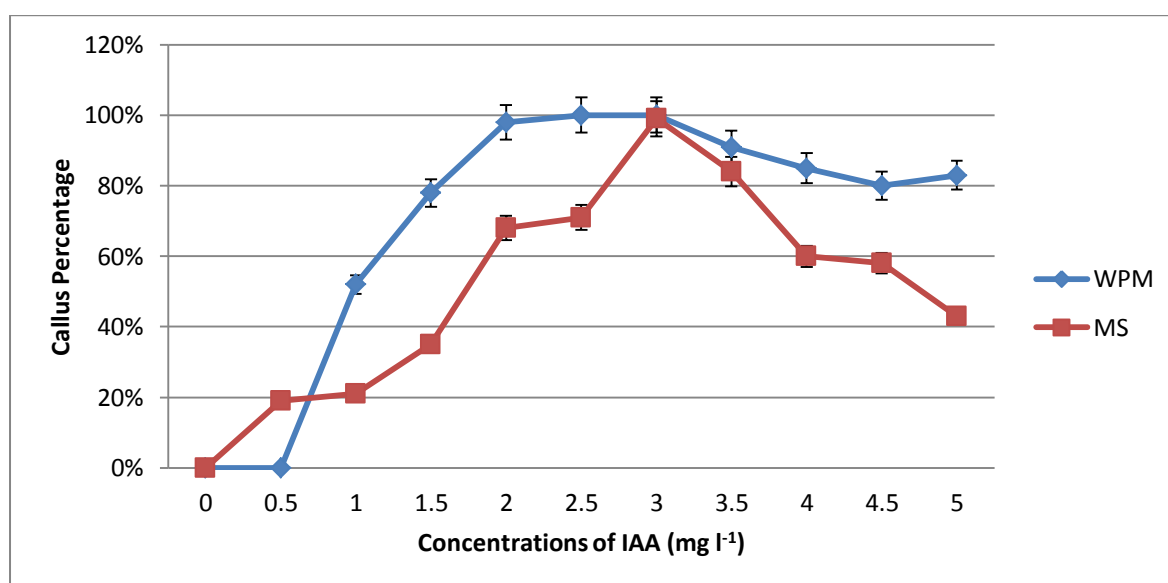
Figure 4.12: A comparison between WPM and MS media supplemented with IAA for callus formation

However, callus was induced (19%) on 0.5 mg l^{-1} IAA and gradually increased to 35% and 71% when MS medium was supplemented with 1.5 and 2.5 mg l^{-1} of this type of auxin, and dropped to 60% and 43% with higher concentrations (4 and 5 mg l^{-1}) of IAA.

WPM medium showed callus formation (51%) from 1 mg l^{-1} concentration of IAA and grew (78 and 98%) on 1.5 and 2 mg l^{-1} of IAA. Callus percentage reached to the highest percentage (100%) on 2.5 mg l^{-1} and was maintained at this level when con-

centration rose to 3 mg l^{-1}. Callus induction dropped to 91% on 3.5 mg l^{-1} until 83%

on 5 mg l^{-1} IAA.

4.2.4 Comparison of WPM and MS Media Supplemented with Various Concentrations of 2, 4-D for Callus Formation

Based on previous reports, 2, 4-D is one of the more effective auxin for callus formation. Figure 4.13 represents the effect of this hormone on callus formation in MS and WPM media. Induction of callus started from a concentration of 0.5 mg l^{-1} 2, 4-D on MS and WPM media with 45% and 29%, respectively.
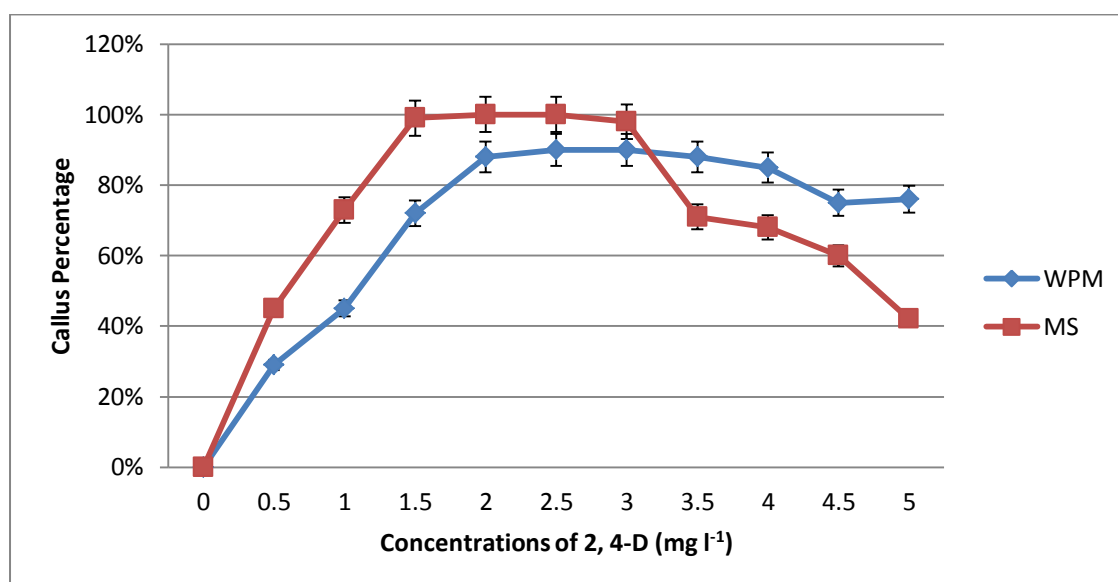
Figure 4.13: A comparison between WPM and MS media supplemented with 2, 4-D for callus formation

Percentage of callus surged to 100% on 1.5, 2, and 2.5 mg l^{-1} and 98% on 3 mg l^{-1} concentration of hormone and fell to 42% on 5 mg l^{-1} on MS medium.

This result is in agreement with that reported by George (1995) and Sayd *et al* (2010). They reported optimum callus formation (100%) of *G. jasminoides* Ellis on MS medium supplemented with concentration of 2 - 3 mg l^{-1} of 2, 4-D from leaf explants. Formation of callus slowly increased in WPM medium (45 and 72%) on 1 and

1.5 mg l^{-1} and reached to higher amount (88 until 90%) between 2 – 3.5 mg l^{-1} and gradually dropped to 76% on 5 mg l^{-1} of 2, 4-D.

4.3 Root Elongation Study

A. MS medium

Based on collected data and observed results, MS and WPM media supplemented with various concentrations of auxin demonstrated different effect on root formation and root elongation from leaf explants of *Gardenia jasminoides* Ellis after six months.
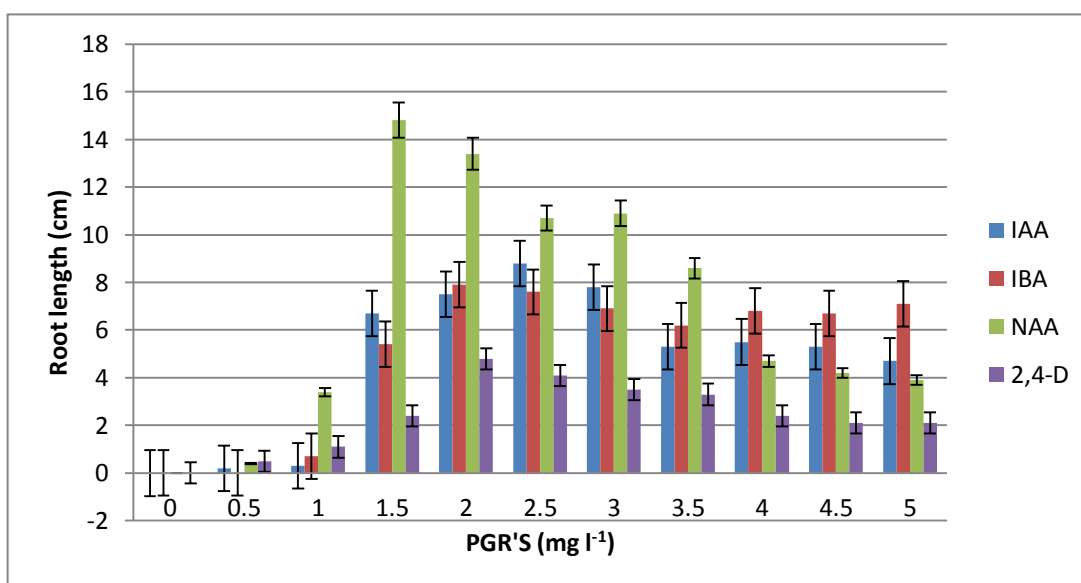
Figure 4.14: Root elongation on MS medium

Rooting started on both media supplemented with various auxin after the fifth weeks. Figure 4.14 shows, among different types and concentrations of used auxin in MS medium, NAA (1.5 and 2 mg l^{-1}) showed higher response for root length (14.8 and 13.4 cm). Roots formation started 0.4 and 3.4 cm from lower concentrations (0.5

and 1.0 mg l^{-1}) of NAA and rose to (10.7 and 10.9 cm) on 2.5 and 3 mg l^{-1} and decreased to (3.9 cm) 5 mg l^{-1} of this hormone (Fig 4.17).

Roots formed (0.2 and 0.3 cm) on MS medium supplemented with 0.5 and 1 mg l^{-1} IAA and rose (8.8 and 7.8 cm) on 2.5 and 3 mg l^{-1} of IAA. The root elongation fell (5.3 and 4.7 cm) at 4.5 and 5 mg l^{-1}. In addition, root formation started on MS medium (0.7 and 5.4 cm) supplemented with 1.0 and 1.5 mg l^{-1} of IBA after four weeks. Root elongation gradually increased (7.9 and 7.6 cm) on 2 and 2.5 mg l^{-1} of IBA. However, the level of elongation showed between 6.2 to 6.9 cm on higher concentrations of IAA (3.0, 3.5, 4.0, 4.5, and 5 mg l^{-1}).

MS medium supplemented with 0.5 mg l^{-1} 2, 4-D showed 0.5 cm root length. This rate increased at the higher levels (4.8 and 4.1 cm) on 2 and 2.5 mg l^{-1} of 2, 4-D.

The level of root growth declined from 3 mg l^{-1} (3.5 cm) to 5 mg l^{-1} (2.2 cm). However, MS medium showed statistically difference when supplemented with NAA comparing to IAA and IBA.
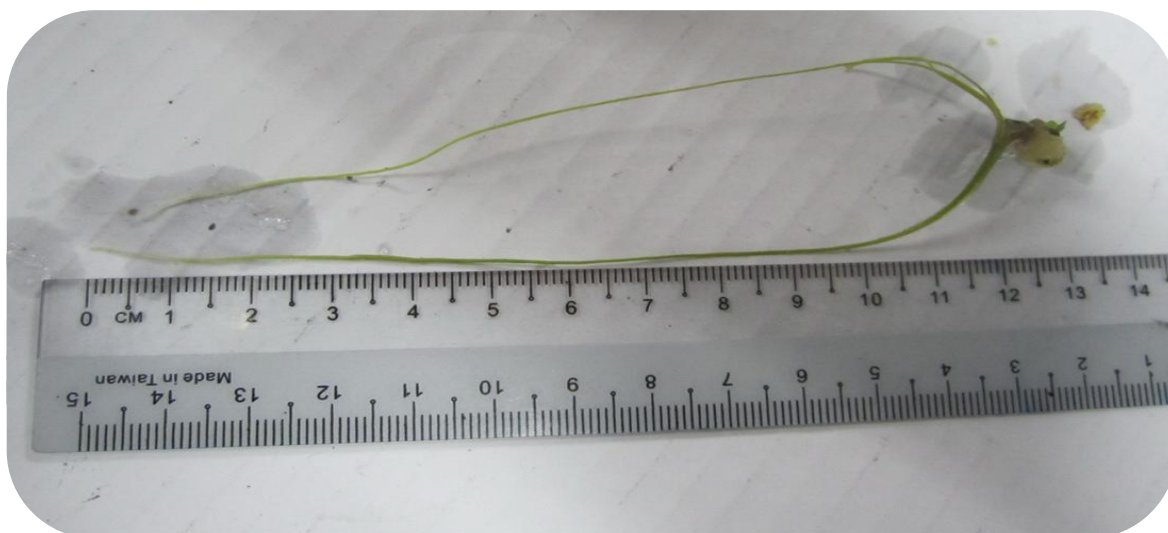
Figure 4.15: Rooting formation in MS medium supplemented with 2 mg l^{-1} NAA after 6 months

B. WPM Medium

WPM medium supplemented with various auxin showed different results of root formation. Figure 4.16, represents longest root length (18.3 and 18.7 cm) on two concentrations of IAA (4.5 and 5 mg l^{-1}), respectively. Rooting formation started from 0.7 cm in a concentration of 0.5 mg l^{-1} and gradually increased to 13.5 cm in 4 mg l^{-1}.
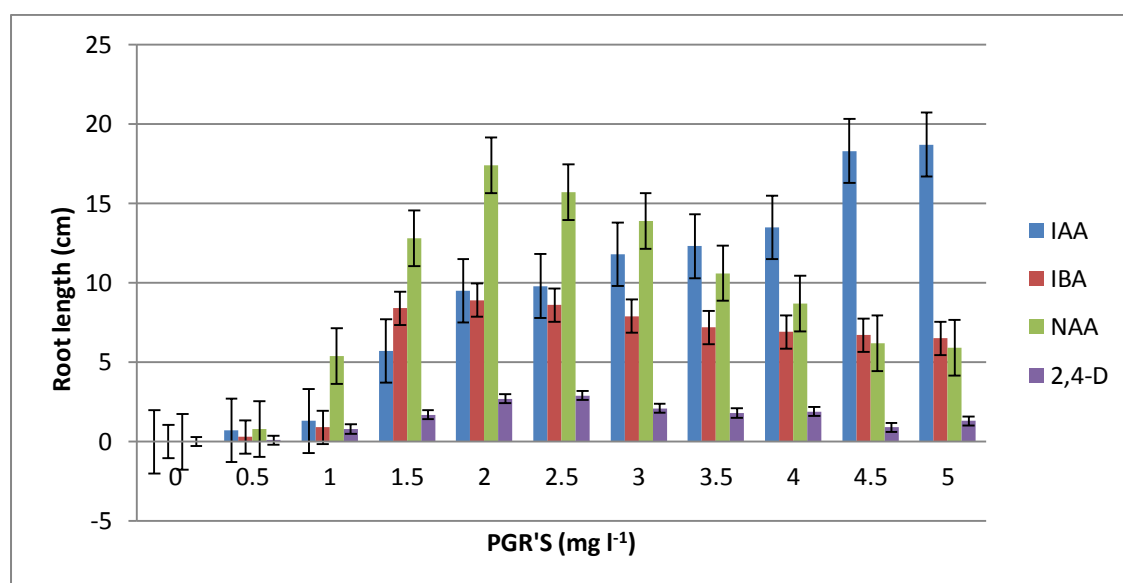

Figure 4.16: Root elongation on WPM medium

In addition, higher root length was observed in WPM media supplemented with 2, 2.5, and 3 mg l^{-1} NAA (17.4, 15.7, and 13.9 cm). Roots formed from 0.5 mg l^{-1} (0.8 cm) and increased to 12.8 cm (1.5 mg l^{-1}). Decreasing of root growth started (10.6 cm) from 3.5 mg l^{-1} until (5.9 cm) 5 mg l^{-1} of NAA (Fig 4.11).

Figure 4.17: Rooting in WPM supplemented with 1 mg l^{-1} IAA one week after the second subculture

Roots formed (0.3 cm) on 0.5 mg l^{-1} of IBA on WPM medium and slightly elongated (0.9 cm) on 1 mg l^{-1} of this hormone. The higher length of roots (8.4, 8.9, and 8.6 cm) was observed in WPM medium supplemented with 1.5, 2, and 2.5 mg l^{-1} of IBA (Fig 4.10), respectively. Level of root length almost stabilized (7.9 to 6.5 cm) between 3.0, 3.5, 4.0, 4.5, and 5.0 mg l^{-1} of the IBA.

Roots were formed (0.1 cm) on WPM medium supplemented with 0.5 mg l^{-1} of 2, 4-D. In addition, concentrations of 2 and 2.5 mg l^{-1} of 2, 4-D showed maximum growth of root length (2.7 and 2.9 cm) on WPM medium.

2, 4- Dichloro phenoxy acetic acid (2, 4-D) is one of the synthetic auxin. There are some successful reports of using this auxin for *in vitro* propagation (Tyagi *et al.,* 2010; Godo *et al.,* 2010) and also on *Gardenia jasminoides* Ellis (Mizukami *et al.,* 1987; George & Ravishankar, 1995). On root elongation study, data analyses showed significant differences in WPM medium supplemented with IAA, NAA, and IBA, respectively.

4.3.1 Comparison of MS and WPM Media Supplemented With NAA on Root Formation

NAA or α-Naphthalene acetic acid was reported as one of the effective auxin for *in vitro* root formation when applied singly (Ładyżyński & Rybczyński, 2009; Sujana & Naidu, 2011) or supplemented with other hormones (Tripathi & Tripathi, 2003; Sanavy & Jami, 2003).

Figure 4.18: Root elongation in MS media supplemented with 2 mg l^{-1} NAA after 4 months from leaf explant of *G. jasminoides* Ellis

Al-Juboory (1998) reported that 1 mg l^{-1} NAA was effective for optimum root elongation for micro cutting explants of gardenia and the same hormone with 2 to 5 mg l^{-1} could give root formation. In addition, MS medium supplemented with 4 mg l^{-1} of NAA showed the highest average number for root length from leaf explants in *G. jasminoides* Ellis (Duhoky & Rasheed, 2010).
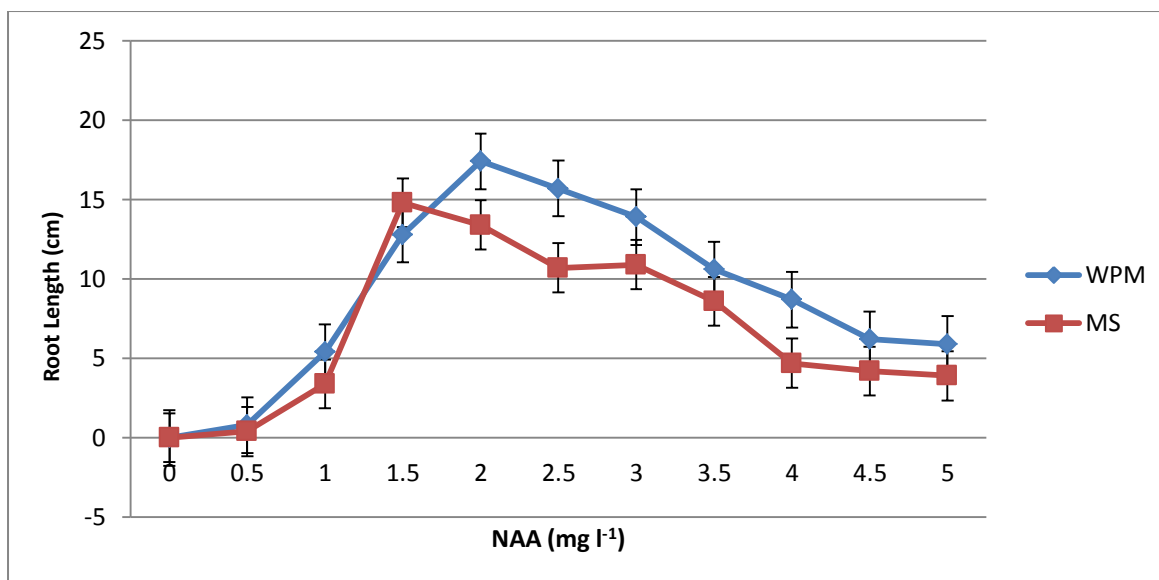
Figure 4.19: A Comparison between MS and WPM media supplemented with various concentrations of NAA for root elongation

In the present study, the data were analyzed by ANOVA and length of roots on MS and WPM medium supplemented with various concentrations of NAA were compared using Duncan's multiple comparison test (DMCT).

Based on the data analyzed, there are significant differences between concentrations of 2, 2.5, 3 and 4 mg l^{-1} of NAA supplemented to MS and WPM media ($p < 0.05$).

The sudden growths (14.8 and 13.4 cm) were observed on MS medium supplemented with 1.5 and 2 mg l^{-1} NAA and maintained (10.7 and 10.9 cm) on 2.5 and 3 mg l^{-1} of hormone (Fig 4.18), root length dropped (8.6 to 3.9 cm) from 3.5 to 5 mg l^{-1} NAA on MS medium. On the other hand, abrupt growth in root length (12 cm) on WPM started from 1.5 mg l^{-1} and grew (17.4, 15.7, and 13.9 cm) on 2, 2.5, and 3 mg l^{-1} of NAA.

Level of root length, gradually decreased (10.6 cm) on 3.5 mg l^{-1} of NAA added to WPM and dropped (8.7, 6.2, and 5.9 cm) on 4, 4.5, and 5 mg l^{-1} of the hormone (Figure 4.19).

4.3.2 Comparison of MS and WPM Media Supplemented With IAA for Root Formation

Indole-3-acetic acid (IAA) is a known as auxin for promoting roots *in vitro* (Al-Amin *et al.,* 2009; Kaladhar *et al.,* 2011; Komal, 2011) and also *in vivo* (George *et al.,* 2008b; Siegel & Galston, 1953). However, Duhoky & Rasheed (2010) reported (8 mg l^{-1}) that IAA has formed the highest number and length of roots in *G. jasminoides* Ellis (3.40 and 3.50 cm roots respectively).

The data were analyzed by ANOVA and length of roots on MS and WPM media supplemented with various concentrations of IAA were compared using Duncan's multiple comparison test (DMCT).

Statistically differences were noted between concentrations 3, 3.5, 4 and 5 mg l^{-1} of IAA supplemented to MS and WPM media respectively (P < 0.05). The concentrations between 3.5 to 5 mg l^{-1} of the IAA are strongly significant.

Furthermore, root formation on WPM medium supplemented with IAA grown rapidly with increased hormone concentration. However, differential growth was observed in MS medium. Gradual growth (0.2 and 3.0 cm) started from low concentrations (0.5 and 1.0 mg l^{-1}) of the IAA and increased at 2.0 and 3.0 mg l^{-1} showed the peak of root length growth (8.8 and 7.8 cm).

Root elongation dropped (5.3 to 4.7 cm) on higher concentration of IAA (from 3.5 to 5 mg l^{-1}) on MS medium (Figure 4. 20).
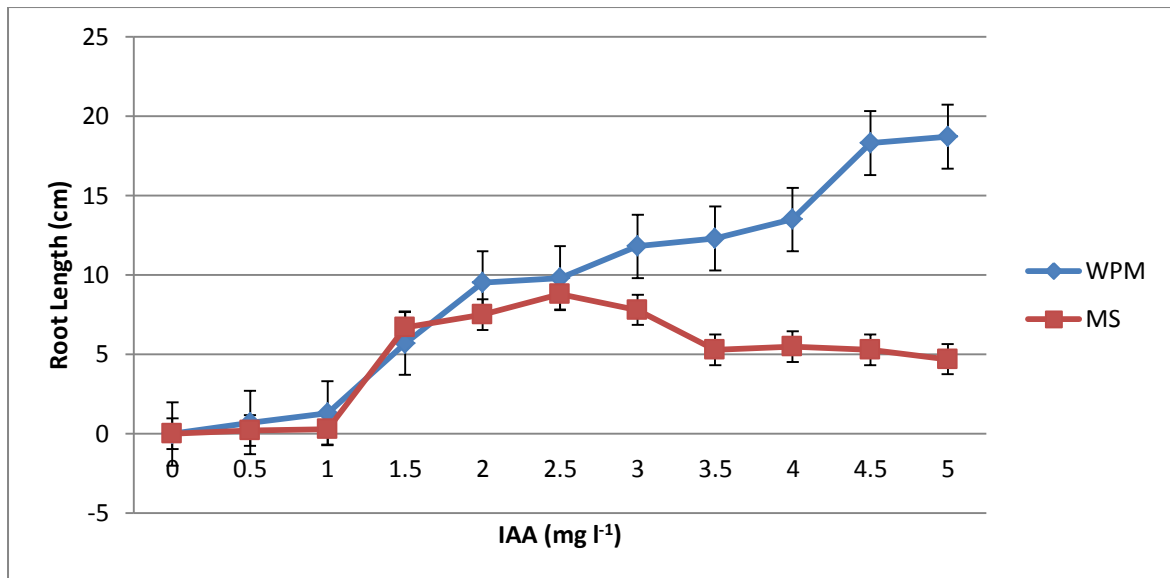
Figure 4.20: A Comparison between MS and WPM media supplemented with various concentrations of IAA for root elongation
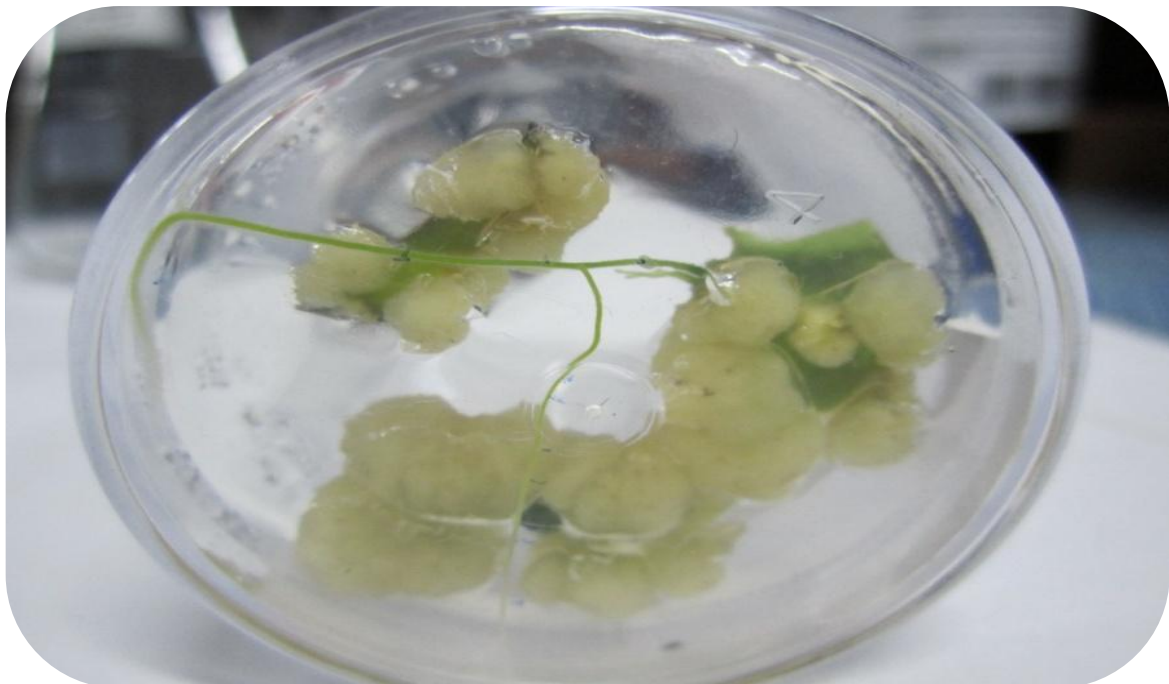

Figure 4.21: Rooting in WPM supplemented with 2 mg l^{-1} IAA 4 weeks after the second subculture of leaf explant of *G. jasminoides* Ellis

4.3.3 Comparison of MS and WPM Media Supplemented with IBA On Root Formation

Applying IBA showed a higher rooting rate in WPM medium over time. The optimum rooting observed between 1.5 until 2.5 mg l^{-1} of the IBA in WPM and dropped on higher concentration, but this value in MS medium demonstrated in 2 mg l^{-1} with the highest rate and minimum and maximum difference between rooting of both media was observed in 1 and 1.5 mg l^{-1} respectively (Figure 4.24).

Indole-3-butyric acid or IBA is used in the same manner as IAA and is accepted around the world as a propagating and rooting hormone for *in vitro* ornamental and fruit grafting and cuttings. There are some reports of optimum rooting from explants between, 1-2 mg l^{-1} of IAA or IBA (Meyer, 1982; Eeckhaut *et al.,* 2010; George *et al.,* 2008a; Bhojwani & Razdan, 1996a). However, the results of IBA are in agreement with former reports (George *et al.,* 1993; Al-Juboory *et al.,* 1998; Hatzilazarou *et al.,* 2006).
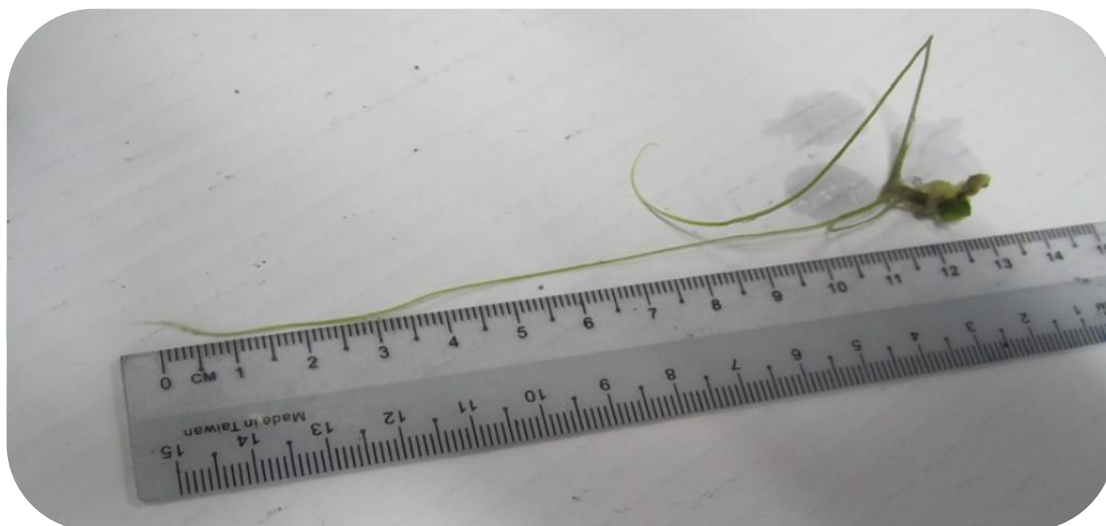
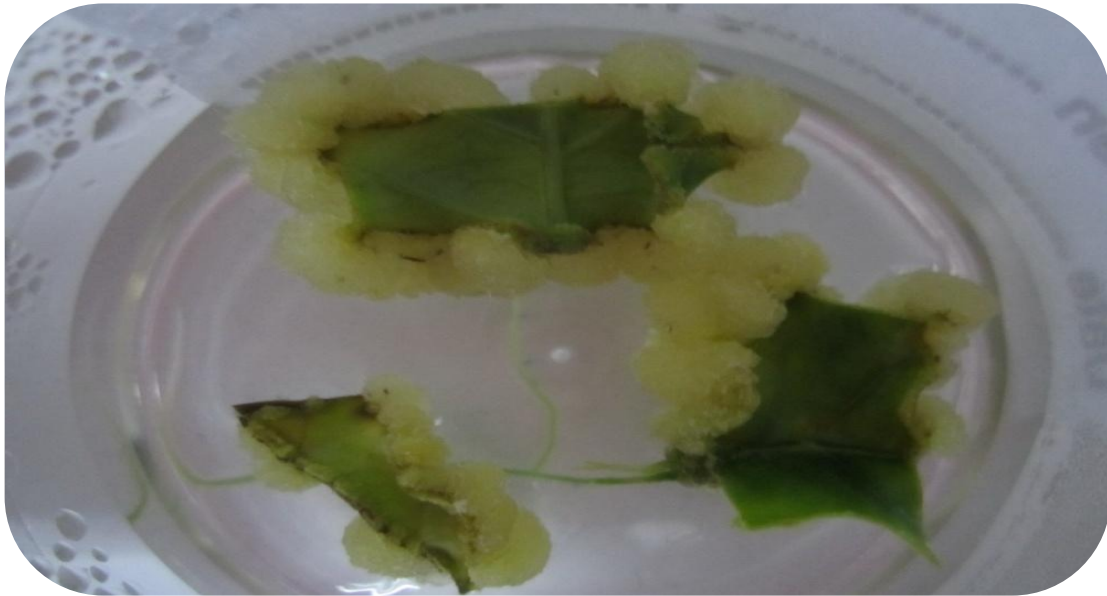**Figure 4.22: Root elongation in WPM medium supplemented with 2 mg l^{-1} IBA after 4 months**

Figure 4.23: Rooting in WPM medium supplemented with 2 mg l^{-1} IBA 3 weeks after the second sub culture
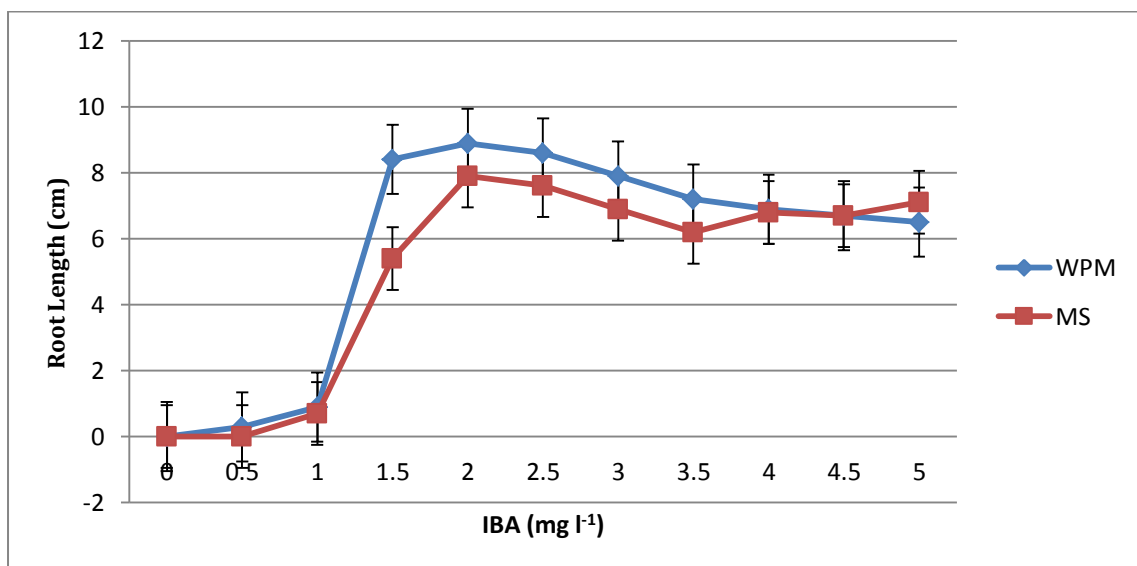

Figure 4.24: A Comparison between MS and WPM media supplemented with various concentrations of IBA for root elongation

4.4   Fresh and Dry Weight of Callus

In many cases, the evaluation of experimental results is by determination of fresh and dry weight of callus (Karl *et al*., 2009a). Figures 4.25 and 4.27, revealed the minimum and maximum weight of callus in both media. Fresh grown callus on MS medium supplemented with NAA (3 mg l^{-1}) and Kn (5 mg l^{-1}) were 34.23 g and 3.39 g; respectively. In addition, 30.04 g and 3.78 g were measured as fresh weight callus, cultured on WPM medium supplemented with (2.5 mg l^{-1}) NAA and (4 mg l^{-1}) Kn.
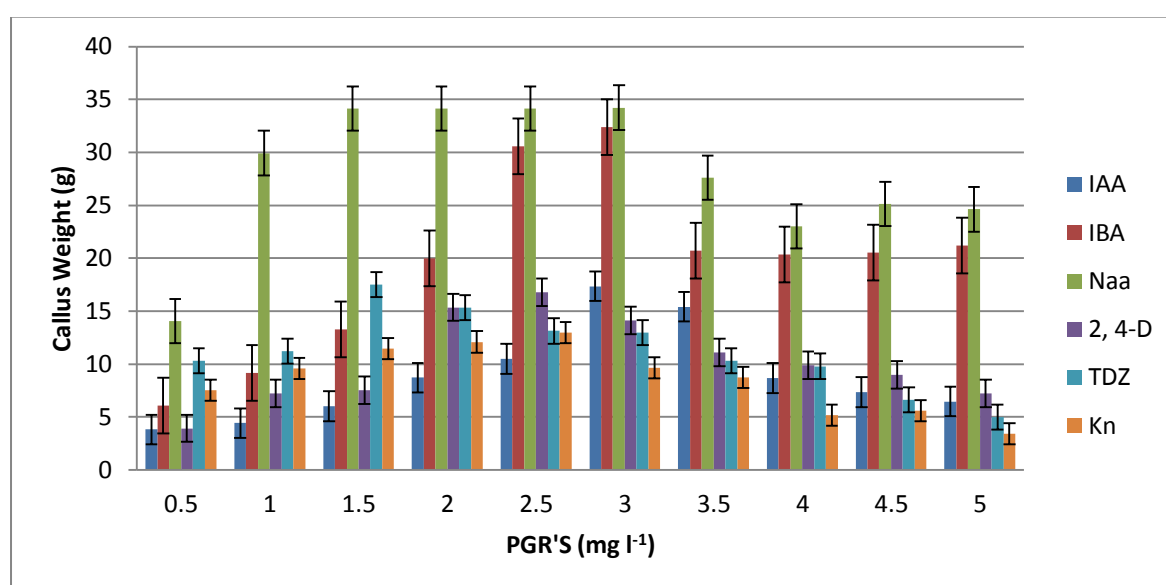
Figure 4.25: Fresh weight of callus in MS medium supplemented with various types of hormones and different concentrations

Callus fresh weight on MS medium was measured (14.04 g) on 0.5 mg l^{-1} of NAA and a high growth was observed (29.94 g) on 1.0 mg l^{-1} and rose to 34.13 g on 1.5 mg l^{-1}. Callus Fresh weight gradually increased to 34.23 g on 3.0 mg l^{-1} of NAA and dropped (24.26 g) on higher concentrations of this auxin from 3.0 to 5.0 mg l^{-1}.

NAA and IBA at 1.5 to 4 mg l^{-1} produced same amount of fresh weight on WPM medium. The minimum volume of fresh callus was weighed to be 21.2 and

22.1 g on 0.5 and 1.0 mg l^{-1} and these weights were kept in 28.7-30.0 g between 1.5 to 4.0 mg l^{-1} of NAA. Fresh callus on MS medium supplemented with IBA (0.5 mg l^{-1}) weighed 6.08 g. However, this weight was increased (9.17 to 20.0 g) on higher concentration of hormone (1 to 2 mg l^{-1}) and maximum weight of callus (30.5 and 32.4 g) was measured on 2.5 and 3 mg l^{-1} of IBA , this amount was kept on 20±2 g on other concentrations. Increasing weight of 10.0 and 16.64 g callus started on WPM medium from 0.5 and 1.0 mg l^{-1} of the IBA and showed slight changes on the weight (21.6 to 20.8) between 2.0 to 4.5 mg l^{-1} of the IBA. Based on reviewed literature, 2, 4-D is used for callus induction and embryogenesis, but based on data as shown in figures 4.24 and 4.26, this auxin gave lower result on fresh weight of callus on both media compared to others auxin. Minimum weight of fresh callus (3.9 g) was observed on 0.5 mg l^{-1} with a slight growth and maximum weights were demonstrated between 15.36, 16.76, and 14.10 g on 2.0, 2.5 and 3.0 mg l^{-1} concentrations of 2, 4- D supplemented to MS medium. Weight of callus on WPM medium supplemented with 2, 4-D from 9.4 g to 10.59 g at concentrations of 0.5 to 5.0 mg l^{-1} were varied. Maximum weight (14 g ± 0.2) of fresh callus was recorded between 2 until 3 mg l^{-1} of 2,4-D supplemented to WPM medium.

Two cytokinin (TDZ and Kn) gave the lowest weight of callus compared to other hormones on both MS and WPM media. The maximum weight of induced callus from MS (17.5 and 15.3 g) and WPM medium (9.7 and 9.08 g) were recorded between 1.5 – 2.0 mg l^{-1} TDZ. The weight of callus dropped with high concentrations of TDZ in MS (4.99 g) and WPM medium (4.65 g).

In addition, MS medium supplemented with 1.5, 2.0, and 2.5 mg l^{-1} kinetin showed optimum weight (11.46, 12.8, and 12.98 g) among another cytokinin and decreased (5.56 and 3.39) on higher concentrations of Kn (4.5 and 5.0 mg l^{-1}) on MS

medium. In contrast, this hormone supplemented to WPM medium showed similar weights (3.93-3.98 g) from 0.5 to 5 mg l^{-1}.

Figure 4.29 shows there are a close relationship between callus dry and fresh weight grown on both media supplemented with different auxin and cytokinin with various concentrations. However, the relationship between dry and fresh weight of callus is approximately linear.

Effect of NAA and IBA on promoting fresh and dry weight of callus supplemented in MS medium was reported by Chinnamadasamy *et al* (2010), Pant & Joshi (2009), Helgeson & Upper (1970) and one study on comparing of WPM and MS media was carried out by Behbahani *et al* (2011). These results were in agreement with the findings of Kende, (1989); Sayd *et al* (2010) and George *et al* (1993). Using NAA or IBA in the media, promoted fresh and dry weights values. The increasing effect of IBA, NAA on growth and callus formation might be attributed to auxin as it encourages the biosynthesis of ethylene by promoting the activity of 1-amino cylopropane-1-carboxylic acid (ACC) syntheses. 2, 4-D was the auxin choice for callus induction, but it gave the lowest values of both fresh and dry weights of callus. These results were in agreement with those obtained before (Sayd *et al.,* 2010; Abdel-Rahim *et al.,* 1998). Consequently, the higher fresh and dry weight of callus was recorded on both media supplemented with NAA as auxin and TDZ as cytokinin.
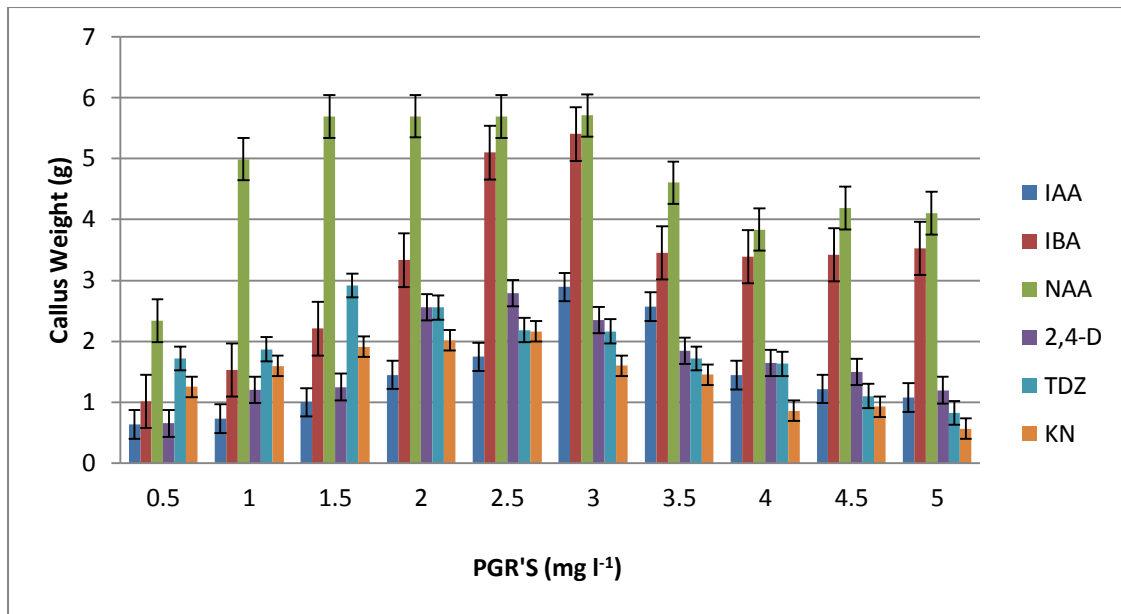
Figure 4.26: Dry weight of callus in MS medium supplemented with various type of hormones in different concentrations
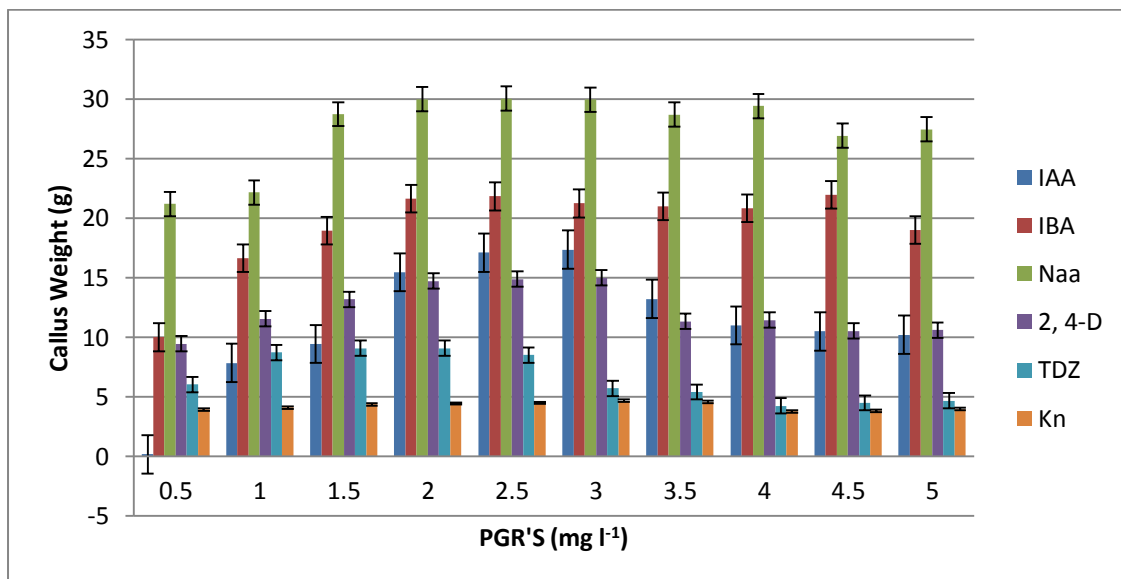

Figure 4.27: Fresh weight of callus in WPM medium supplemented with various type of hormones and different concentrations
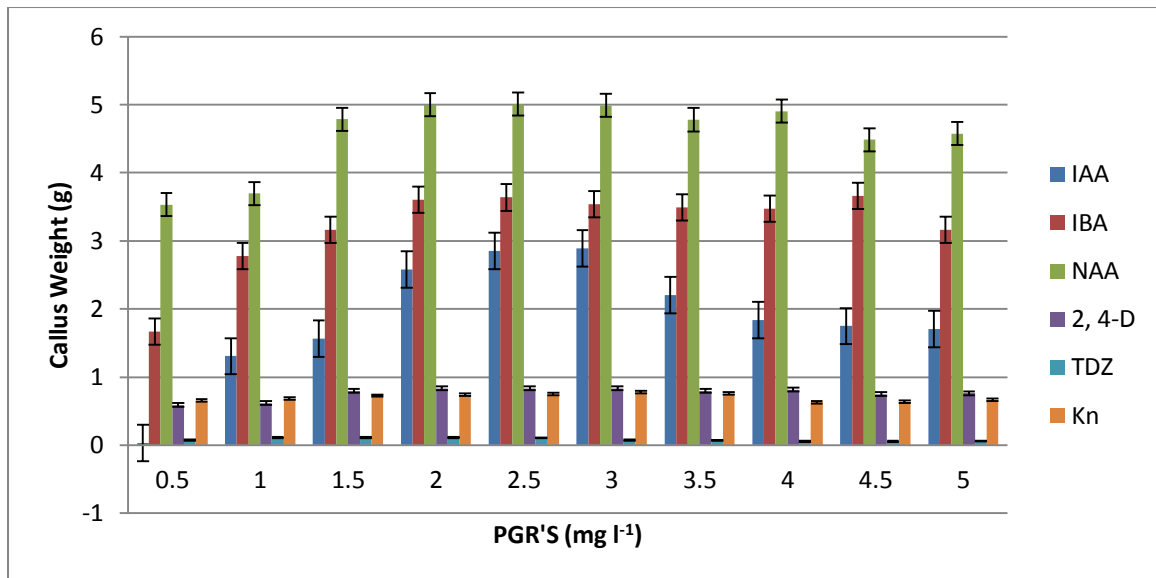
Figure 4.28: Dry weight of callus in WPM medium supplemented with various type of hormones in different concentrations
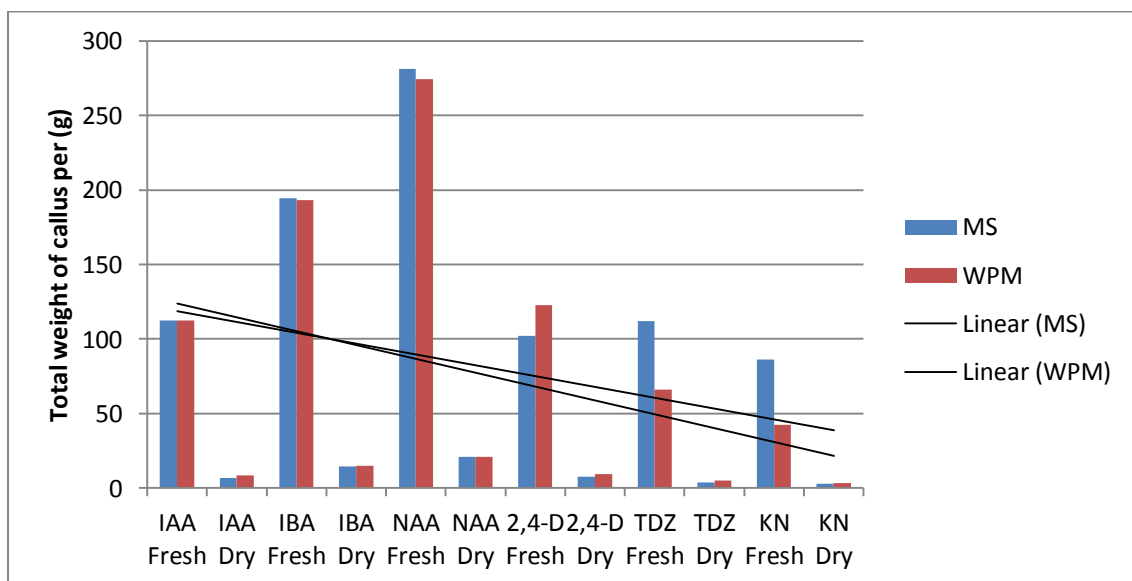

Figure 4.29: A comparison between dry and fresh weight of callus in WPM and MS media supplemented different withhormones

4.5   Antibacterial Assay

The results of different studies provide evidence that some medicinal plants might indeed be potential sources of new antibacterial agents even against some anti-biotic-resistant strains (Kone *et al.,* 2004). In this study, the disk diffusion method showed that extracts of *G. jasminoides* Ellis produced antibacterial activity against pathogenic bacteria. Among all *in vitro* and *in vivo* tested extracts, only callus on MS and WPM media supplemented with NAA showed inhibition zone to *Escherichia coli* and *Bacillus cereus*. However, the rest of the extracts showed no inhibition zone in the concentration of 100 mg/ml against tested pathogenic bacteria (Fig 4.30 and Fig 4.31).
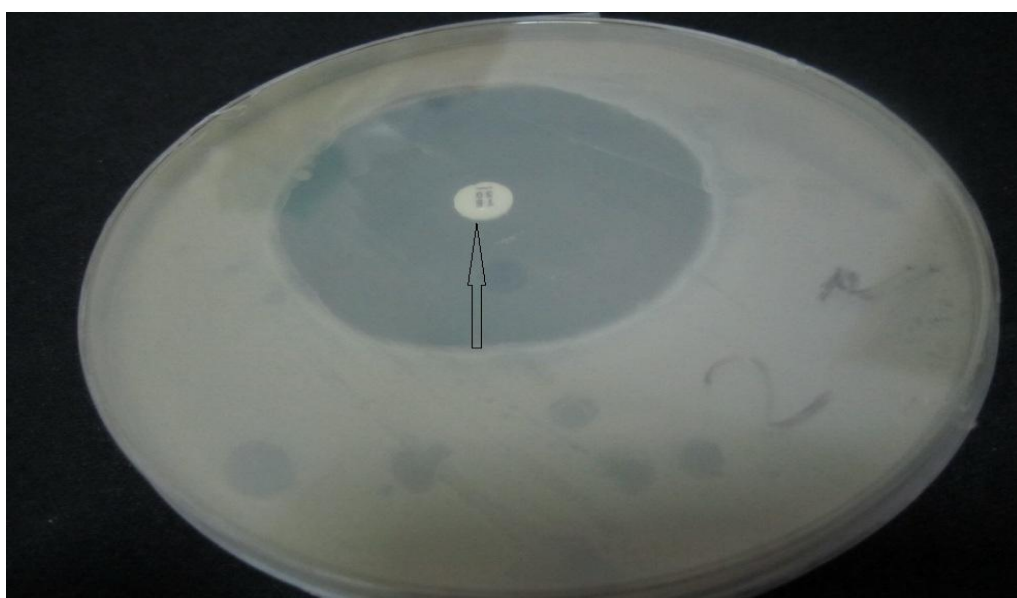

Figure 4.30: Inhibition zone in tetracycline as a control

Figure 4.31: Inhibition zone of *in vitro* extract grown on MS medium supplemented with NAA against *E. coli*

Only few reports on (Ragasa *et al.,* 2007 ; Ali *et al.,* 1995) a slightly antibacterial activity of fruit extracts of *G. jasminoides* against *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus*, and *Trichophyton mentagrophytes*; and inactive against *Bacillus subtilis* and *Aspergillus niger*.

Generally, plant extracts are usually more active against gram-positive bacteria than gram-negative bacteria (Basri & Fan, 2005). Abu-Shanab *et al* (2005) reported gram-negative bacteria are more resistant to plant extract compared to gram-positive bacteria. This may be due to the permeability barrier provided by the cell wall or to the membrane accumulation mechanism (Wei *et al.,* 2008).

Callus grown on both MS and WPM media supplemented with NAA showed antibacterial activity against *E. coli* and *B. cereus* as gram-negative and gram-positive

bacteria. The others *in vivo* and *in vitro* extracts showed no antibacterial zone against *E. coli*, *S. aureus*, *P. aeruginosa*, and *B. cereus* as pathogenic bacteria. This result is in agreement with those found previously (Ali *et al.,* 1995; Ragasa *et al.,* 2007). Møretrø *et al.,* (2006) and Wei *et al.,* (2008), also reported similar results.

Table 4.1: Inhibition zone from 100 g l^{-1} *in vivo* and *in vitro* leaf extracts of *Gardenia jasminoides* Ellis against the four pathogenic bacteria

Bacteria	Inhibition zone (mm)		
	In vitro (MS with NAA)	*In vitro* (MS with NAA)	**Tetracycline (30 µg)**
Escherichia coli	+	+	39 ± 3.04
Staphylococcus aureus	-	-	21 ± 1.98
Pseudomonas aeruginosa	-	-	11 ± 1.99
Bacillus cereus	+	+	41±3

Inhibition zone = +

None inhibition zone = -

4.6 Antioxidant Assay

In this study, the antioxidant activity of *G. jasminoides* Ellis was evaluated (Fig 4.32). The data were analyzed by two-way ANOVA and there are significant differences between *in vivo* and *in vitro* extracts. Leaf extract from intact plants as *in vivo*, showed the minimum rate of SOD-like in MS (10.84 Uml^{-1}) and WPM (10.93 Uml^{-1}) media. In contrast, there are no statistical differences between the two types of media supplemented with various hormones in antioxidant content (Debnath *et al.,* 2011). Screening of antioxidant compounds from callus extract of *G. jasminoides* was confirmed, that this plant could be considered as a good source of antioxidant activity.

Previous reports from fruit extracts by free radical scavenging activity method (Chen *et al.,* 2010; Chen *et al.,* 2008; Choi *et al.,* 2007) and Superoxide dismutase-like (SOD-like) activity and catalase activity of the extracts (Debnath *et al.,* 2011) proved that *G. jasminoides* Ellis is a plant with high antioxidant activity.

Furthermore, *in vivo* and *in vitro* extract of *G. jasminoides* Ellis by free radical scavenging activity method revealed high antioxidant properties as reported by Sayd *et al* in 2010. Superoxide dismutase (SOD) is a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. As such, they are an important antioxidant defense in nearly all cells exposed to oxygen. SOD is also a part of the defense system against oxidative stress in aerobic organisms. It catalyses superoxide anion (O_2) and hydrogen peroxide, which is then reduced to water (by hydrogen peroxide scavenging enzyme- Catalase). Therefore, Catalase and SOD are thought to limit the accumulation of reactive oxygen species.

Miszalski *et al.,* (2007), Dos Santos *et al.,* (2000), Shilpashree & Kumar, (2011) used SOD-kit like enzyme in a different study. However, there are other re-

ports of evaluating antioxidant from fruit extract of *G. jasminoides* Ellis by Fan *et al.,* (2011), Wei *et al.,* (2009), Lee *et al.,* (2006) and Pham *et al.,* (2000).
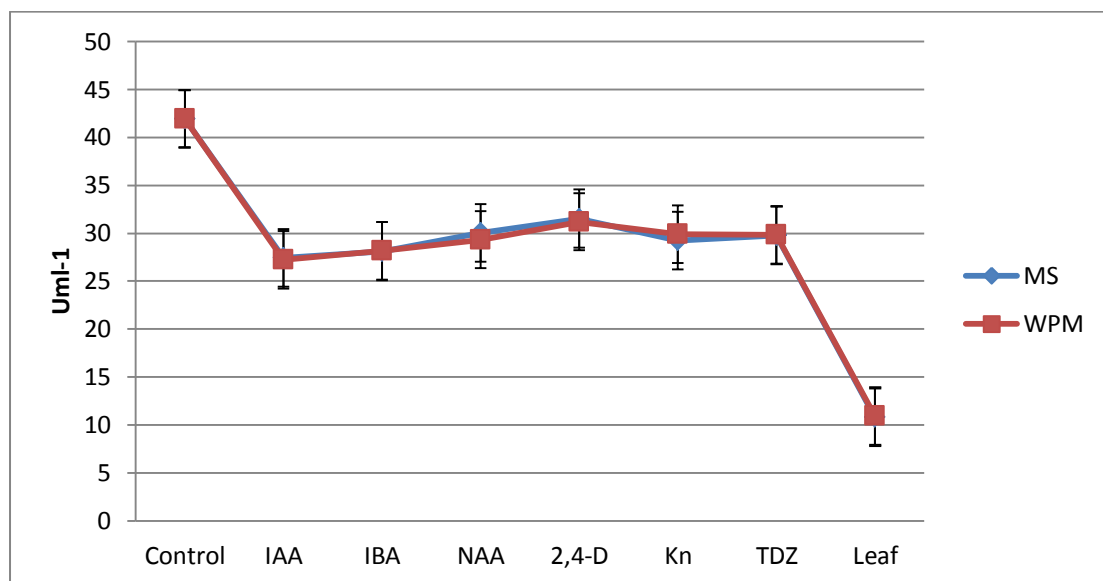
Figure 4.32: A comparison of SOD activity between *in vivo* (leaf of *G. jasminoides* Ellis) and *in vitro* from different media and various PGR's

CONCLUSION

The present study evaluated *in vitro* propagation and callus indication of *Gardenia jasminoides* Ellis by using two different media (MS and WPM) and various types of auxin and cytokinin. Callus extract were tested for broad antibacterial and antioxidant activities.

1. **Investigation of media effects on callus formation**

   WPM medium was observed as a better medium for callus formation compared to MS medium this could possibly be due to the various nutrients such as calcium or phosphate the major reason for this diffrence.

2. **Investigation of hormones effects on root induction**

   WPM medium supplemented with IAA, NAA, and IBA, respectively showed statistical differences on root induction. Furthermore, these differences were observed on MS medium when supplemented with NAA compare to IAA and IBA in terms of root elongation.

3. **Screening of antioxidant activity between intact plant and different callus grown on MS and WPM**

   The data analyzed indicated that the calluses from *in vitro*-grown tissues using NAA, IBA, TDZ, and Kn have antioxidant activities that are significantly different with intact plant. *G. jasminoides* Ellis is indeed a rich source of antioxidant.

4. **Screening of antibacterial activity different callus grown on MS and WPM and intact plant**

   Only callus grown on both media supplemented with NAA showed antibacterial activity against *E. coli* and *B. cereus*. The other *in vivo* and *in vitro* extracts showed no antibacterial zone.

This is the first study to do micropropagation of *Gardenia jasminoides* Ellis on woody plant media (WPM) and screen its callus for antibacterial activity.

Recommendations for future work

Alternative media for micropropagation of *Gardenia jasminoides* Ellis can be further carried out, which include SH, B5 and LS.

Anti-tumor and anti-microbial activities from the plant and callus can be investigated.

A study can be conducted to find the best organ with maximum antioxidant content.