ELICITATION STUDY ON TOTAL PHENOLIC AND FLAVONOID CONTENT, AND ANTIOXIDANT ACTIVITY OF *Gardenia jasminoides* CELL CULTURES

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ABSTRACT

Most of the plants are rich sources of bioactive compounds and being used in pharmaceutical industries. Hence, synthesis of secondary metabolites via plant breeding using plant *in vitro* technique is important. In this study, *Gardenia jasminoides* Ellis, an important medicinal plant, was used to evaluate its secondary metabolite activities via elicitation and callus culture. The explants were cultured on the WPM medium (Lloyd and McCown, 1980) supplemented with NAA and 2,4-D, and were manipulated with different abiotic elicitors such as gamma ray as a physical as well as methyl jasmonate and salicylic acid as hormonal elicitors at different doses and concentrations. The effect of callus age (35, 70 and 105 days), various elicitors, elicitor concentrations, and the interaction between time and elicitors on callus growth and secondary metabolite contents were studied. The combination of mercuric chloride and ethanol by a proper immersion time significantly increased the survival rates of the leaf explants. To determine the best auxin type and auxin concentrations for callus induction and growth, the leaf explants were cultured on the WPM medium containing 3% (v/v) sucrose, supplemented with 0.5, 1, 2 or 4 mg L^{-1} NAA or 2,4-D. The results showed that 2 mg L^{-1} of 2,4-D or NAA significantly enhanced callus induction (100 and 97.3%, respectively) and increased the fresh and dry weights of embryogenic calli. The leaf explants were cultured on WPM media supplemented with 2 mg L^{-1} of 2,4-D or NAA and were subjected to irradiation with gamma ray (0, 5, 10, 20, or 40 Gy) and the radiation free explants (0 Gy) were considered as the control. The callus growth measured as the fresh weight on the WPM supplemented with 2,4-D or NAA were significantly increased by the 10 Gy (14.09 ± 0.42 g) and 5 Gy (13.87 ± 0.39 g) irradiation after 105 days. However, the best dry weights were measured (0.79 ± 0.16 g, 0.87 ± 0.12 g) at 20 Gy radiation when harvested at 105 days, respectively. The significant values for total phenolic (5.56 ± 0.01, 5.49 ± 0.01), flavonoid (3.04 ± 0.01, 3.0 ± 0.01) contents and DPPH (91.32 ± 0.06, 90.71 ± 0.21) were

detected at first harvesting time in the WPM medium supplemented with 2,4-D or NAA at 20 Gy. Callus was induced by methyl jasmonate (0, 0.022, 0.22, 1.12, 2.24 and 4.50 mg L^{-1}) and the highest concentration (4.5 mg L^{-1}) was observed with a lethal effect. Methyl jasmonate (MeJA) at 0.22 mg L^{-1} significantly increased fresh (15.39 ± 0.42 g, 15.71 ± 0.45 g) and dry (0.90 ± 0.01 g, 0.93 ± 0.01 g) weight after 70 and 105 days of initial culture. The highest total phenolic (7.30 ± 0.03, 6.60 ± 0.01) was observed at 70 days when MeJA at 0.22 mg L^{-1} was applied. Furthermore, the highest contents of flavonoid (3.38 ± 0.00, 3.36 ± 0.00) and DPPH (100.21 ± 0.37, 99.33 ± 0.37) were obtained at 0.22 mg L^{-1} of MeJA, when harvested after the first month. Salicylic acid showed a significant effect on the callus, but lower concentrations increased fresh weight and dry weight. The highest fresh and dry weights were recorded as 2.5 mg L^{-1} (14.92 ± 0.14 g, 15.39 ± 0.12 g) and (0.88 ± 0.01g, 0.91 ± 0.01 g) when harvested at day 105. Contents of total phenolic (6.19 ± 0.04, 6.17 ± 0.06), flavonoid (3.44 ± 0.02, 3.49 ± 0.02), and DPPH (97.25 ± 0.16, 95.44 ± 0.11) at 2.5 mg L^{-1} of SA were measured in one month old callus. *Gardenia jasminoides* Ellis has been confirmed as a rich source of phenolic and antioxidants. There was no difference between NAA and 2,4-D on their effect on callus growth, total phenolic, flavonoid, and DPPH. The first harvesting period was detected as the most effective period on accumulation of secondary metabolites.

ABSTRAK

Kebanyakan tumbuhan kaya dengan sumber sebatian bioaktif dan digunakan dalam industri farmaseutikal. Oleh itu, pengeluaran metabolit sekunder melalui pembiakan tumbuhan menggunakan teknologi kultur tisu tumbuhan adalah penting. Dalam kajian ini, *Gerdenia jasminoides* Ellis, sejenis tumbuhan perubatan yang penting, telah digunakan untuk menilai aktiviti metabolit sekundernya melalui rangsangan dan kultur tisu. Eksplan telah dikultur dalam media WPM (Lloyd dan McCown, 1980) yang diperkaya dengan NAA dan 2,4-D, dan dimanipulasi dengan perangsang abiotik yang berbeza seperti sinar gamma sebagai perangsang fizikal dan juga metil jasmonat serta asid salisilik sebagai perangsang hormon pada dos dan kepekatan yang berbeza. Kesan usia kalus (35, 70 dan 105 hari): pelbagai perangsang, kepekatan perangsang, dan interaksi antara masa dan perangsang ke atas pertumbuhan kalus dan kandungan metabolit sekunder telah dikaji. Kombinasi antara merkuri klorida dan etanol dengan masa rendaman yang betul meningkatkan kadar keterusan hidup eksplan daun dengan signifikan. Untuk menentukan jenis auksin dan kepekatan auksin yang terbaik untuk pengiduksian dan pertumbuhan kalus, eksplan daun telah dikultur dalam media WPM yang mengandungi 3% (v/v) sukrosa, diperkaya dengan 0.5, 1, 2, atau 4 mg L^{-1} NAA atau 2,4-D. Keputusan menunjukkan bahawa 2 mg L^{-1} 2,4-D atau NAA meningkatkan induksi kalus dengan signifikan (masing-masing 100% dan 97.3%) dan meningkatkan berat segar dan kering kalus embriogenik. Eksplan daun telah dikultur dalam media WPM yang diperkaya dengan 2 mg L^{-1} 2,4-D atau NAA dan tertakluk kepada penyinaran dengan sinar gamma (0, 5, 10, 20 atau 40 Gy) dan eksplan yang bebas sinaran (0 Gy) dianggap sebagai kawalan. Pertumbuhan kalus yang diukur sebagai berat segar dalam media WPM yang diperkaya dengan 2,4-D atau NAA telah ditingkatkan dengan signifikan dengan penyinaran 10 Gy (14.09 ± 0.42 g) dan 5 Gy (13.87 ± 0.39 g) selepas 105 hari. Walau bagaimanapun, berat kering yang terbaik (0.79 ± 0.16 g, 0.87 ± 0.12 g) telah diukur pada

sinaran 20 Gy apabila dituai pada hari ke-105. Nilai signifikan untuk jumlah fenolik (5.56 ± 0.01, 5.49 ± 0.01): kandungan flavonoid (3.04 ± 0.01, 3.0 ±0.01): dan DPPH (91.32 ± 0.06, 90.71 ± 0.21) telah dikesan pada masa penuaian pertama dalam media WPM yang diperkaya dengan 2,4-D atau NAA pada 20 Gy. Kalus telah diinduksi dengan metil jasmonat (0, 0.022, 0.22, 1.12, 2.24 dan 4.5 mg L^{-1}) dan kepekatan tertinggi (4.5 mg L^{-1}) dilihat memberi kesan maut. Metil jasmonat (MeJA) pada 0.22 mg L^{-1} meningkatkan berat segar (15.39 ± 0.42 g, 15.71 ± 0.45 g) dan kering (0.90 ± 0.01 g, 0.93 ± 0.01 g) dengan signifikan selepas 70 dan 105 hari kultur permulaan. Jumlah fenolik yang tertinggi (7.30 ± 0.03, 6.60 ± 0.01) telah dilihat selepas 70 hari apabila MeJA pada 0.22 mg L^{-1} digunakan. Tambahan pula, kandungan tertinggi flavonoid (3.38 ± 0.00, 3.36 ± 0.00) dan DPPH (100.21 ± 0.37, 99.3 ± 0.37) telah diperoleh pada 0.22 mg L^{-1} apabila dituai selepas bulan pertama. Asid salisilik telah menunjukkan kesan yang signifikan ke atas kalus, namun kepekatan yang lebih rendah meningkatkan berat segar dan berat kering. Berat segar dan kering yang tertinggi telah dicatat pada 2.5 mg L^{-1} (14.92 ± 0.14 g, 15.39 ± 0.12 g) dan (0.88 ± 0.01 g, 0.91 ± 0.01 g) apabila dituai pada hari ke-105. Kandungan jumlah fenolik (6.19 ± 0.04, 6.17 ± 0.06), flavonoid (3.44 ± 0.02, 3.49 ± 0.02), dan DPPH (97.25 ± 0.16, 95.44 ± 0.11) pada 2.5 mg L^{-1} SA telah diukur dalam kalus berusia satu bulan. *Gardenia jasminoides* Ellis telah disahkan sebagai sumber yang kaya dengan fenolik dan antioksida. Tidak terdapat perbezaan antara NAA dan 2,4-D ke atas pertumbuhan kalus, jumlah fenolik, flavonoid, dan DPPH. Tempoh penuaian pertama dikesan sebagai tempoh yang paling efektif untuk pengumpulan metabolit sekunder.

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LIST OF SYMBOLS AND ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxy acetic acid
ANOVA	Analysis of variance
CGR	Callus growth rate
CRD	Completely Randomized Design
DMRT	Duncan's multiple range test
DW	Dry weight
FW	Fresh weight
GLM	General linear model
MeJA	Methyl jasmonate
MS	Murashige and Skoog (1962) medium
NAA	1-Naphthaleneacetic acid
PGR	Plant growth regulators
RNS	Reactive Nitrogen Species
RCBD	Randomized Complete Block Design
ROS	Reactive oxygen species
SA	Salicylic acid
TFC	Total flavonoid content
TPC	Total phenolic content
v/v	Volume by volume
w/v	Weight by volume
WPM	Lioyd and McCown (1981) medium

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CHAPTER 1 : INTRODUCTION

1.1 Background of the study

Plants have been employed as a valuable resource of medicine and preserve to serve as the key for pharmaceutical improvement. Although the modern pharmaceutical industry was embarked on botanical medicine, and this artificial approaches has led to facilitate development new drug in recent years. Most of botanical therapeutics are obtained from medicinal herbs that which have been cultivated for maximize the yields of bioactive components. Nowadays, a huge consideration was paid to the natural source from medicinal plants as a remedy for many diseases and illness. There are around 5000 identified medicinal plant for their medicinal characteristics, which many of these species were almost vanished because of over collections from their natural habitats for marketing purposes (Chow and Sato, 2013). Biotechnology has shown an incredible influence on features of human life, health and wealth. The aggregation of natural products in plant tissue culture has been established for a wide range of plants and a new assessment of metabolic engineering has initiated to enhancing secondary metabolites. The World Bank estimated trading of medicinal herbs will increase more than 60 billion U.S. dollars with 7% increasing rate annum (Veeresham, 2012).

The medicinal plants demand at the global level is increasing dietary and pharmaceuticals supplements and Malaysia with more than thousands of identified medicinal plants is one of the 12 mega-diversity in the world (Jamal et al., 2010). Elicitation is one of the most successful among different approach method can be exploited to increase the production of bioactive compounds in cell suspensions. The international companies of food, pharmaceutical, and academic centers are trying to identify, improve and obtain secondary metabolites as well as to maximum yield and production efficiency by *in vitro* method.

Standardized production of secondary products in *in vitro* condition has been developed for specific genotypes. Plant *in vitro* culture is one of the noteworthy methods to produce secondary metabolites under controlled conditions (Sato & Yamada, 2008). Applying biotic or abiotic elicitors have been successfully improved the synthesis of secondary metabolites in some medicinal plants (Gomez-Garela et al., 2007; Sarin, 2005). Based on the research and studies (Chow & Sato, 2013; Ramachandram & Ravishankar, 2002), several approaches have been applied to further improve in secondary metabolites yield such as: selection of high-yield lines and treatment with an elicitor, modification in the formulation of *in vitro* medium, changing in culture, environment condition cultivation strategies and using specialized techniques such as elicitation, genetic transformation, and metabolic or bioreactor. The plant defense system accelerates by a type of compounds, which is named "elicitor". This definition includes exogenous elicitors and endogenous elicitors. Moreover, based on the biological or non-biological origin, elicitors have been divided into biotic and abiotic, respectively. Gamma irradiation had widely drawn attention to the biological study of many crops. It is reported as one of the important factors in mutant plants and increase of genetic variability (Jan et al., 2013; Jen et al., 2011).

Gamma ray is entitled by various effects in cytological, genetically, biochemical, physiological, and morphogenetic alteration in cells and tissues in plants (Ikram et al., 2010). Based on Chung et al. (2006) gamma irradiation could improve production of plant secondary metabolites. Plant growth regulators, UV, gamma ray, methyl- jasmonate, and salicylic acid have also been reported to synthesize secondary metabolites in *in vitro* culture condition have. *Gardenia jasminoides* or commonly called Cape jasmine is a fragrant flowering and evergreen tropical plant. This plant with its shiny green leaves and fragrant white summer flowers originated in the tropical and subtropical regions of Africa, Southern Asia, Australia, and Oceania.

It has been cultivated in China for at least ten centuries (Keswick et al., 2003) and was presented to English gardeners in the middle of the 18th century. Some varieties with large flower and long stem have been bred for using in horticulture purpose. This plant was chosen for this research due to its medical uses for treating jaundice, hemorrhage, hepatitis, toothaches, wounds, sprains, and skin conditions (Choi et al., 2007; George et al., 1993; Lelono et al., 2009). Gardenia is perceived very effective as a hemostatic agent and a promising remedy for muscles injuries, joints, and tendons. A yellow silk dye has been found for centuries from the chemical compound crocetin, which is derived from the gardenia berry.

1.2 Objectives

The objectives of this research were:

1) To study the effect of gamma irradiation as a physical abiotic elicitor on the accumulation of secondary metabolites (including total phenolic content, total flavonoid content and antioxidant activity) in *G. jasminoides* Ellis.
2) To determine the effects of methyl jasmonate and salicylic acid as hormonal abiotic elicitors on the accumulating of secondary metabolites (total phenolic content, total flavonoid content and antioxidant activity) in *G. jasminoides* Ellis.
3) To examine the effect of callus age on production of secondary metabolites (i.e. total phenolic content, total flavonoid content and antioxidant activity) in *G. jasminoides* Ellis.

CHAPTER 2 : LITERATURE REVIEW

5.1 Medicinal plants

Medicinal plants are the primary health care resources and exploited for their numerous profits and usages. An excavated clay tablet related to 2500 B.C, in Iraq, described the usage of medicinal plants such as the poppy as the "the plant of joy" in Sumerian. The ancient evidence from India and China also exhibited using a variety of plant-based remedies (Judith, 2000). These show that humanity has been depending on plants for food and medicine. Plants are the most abundant and valuable source of medication to cure various types of diseases. Based on the World Health Organization (WHO, 2005) more than 80% of the new drug is made of herbs and have a considerable demand in markets. This has resulted in over-harvesting of a distinct species, and many medicinal plants are listed as endangered species.

In past decades, biotechnology has been improved to address regarding endangered plants and production of a consistent product for the market. There has been a significant growth in research activity in the natural science area (Clardy & Walsh, 2004; Joshee et al., 2013). The essential information regarding reproduction models of plants, growth condition, and medical uses have expanded. Moreover, it is reported that around 25% of current medications in the world contains plant compounds (Raskin et al., 2002; Schmidt et al., 2007). According to Harvey (2008) more than half of underwent clinical drugs in 2008 have had plant base.

5.2 Antioxidant

Transferring an electron from electron-rich to electron-deficient (oxidizer and oxidizing) unit is known as oxidation. Based on a classic description, an antioxidant is any compounds or systems that could significantly prevent the formation or interrupts the oxidisable substrate at low concentrations such as lipids, proteins, DNA or carbohydrates (Halliwell & Gutteridge, 2001; Tirzitis & Grzegorz, 2010). Antioxidant activity refers to the frequent reaction rate between the antioxidant and the oxidant species. However, the capacity of antioxidant states to the amount (in moles) of a specified free radical scavenged by a sample. Based on chelating agents, their mechanism of action is categorized into non-enzymatic, and antioxidant enzymes. They are known to respond the effect of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and to diffuse free radicals leading to the limited risk of oxidative stress (Swaran, 2009). Kumar and Kumar (2006) described some of the main categories (Table 2.1) as follows:

- Enzymes: they are present in plasma and transforming reactive oxygen and nitrogen into the more stable compounds such as superoxide dismutase (SOD) catalase (CAT) and glutathione peroxidase (GPx).
- High molecular weight compounds: they hamper the metal catalyzed free radicals formation (albumin, transferrin, and ceruloplasmin).
- Low molecular weight compounds: this group is divided into two lipids soluble (tocopherol, quinines, bilirubin, and some polyphenols) and water-soluble antioxidants (ascorbic acid, uric acid, and some polyphenols) categories.
- Minerals: selenium, copper, manganese, zinc, and chromium.
- Vitamins: vitamin A, C, and E.

Table 2.1: Alphabetical classification of antioxidants based on categories with few examples

Alphabetical by name	Categories of antioxidants	Examples
Antioxidant C	Carotenoids	β-carotene, lycopene, lutein
Antioxidant E	Enzymes	SOD, catalase, GPx
Antioxidant G	Glutathione	Glutathione
Antioxidant H	Hormones	Melatonin, oestrogen
Antioxidant L	Lipid-associated chemicals	Ubiquinol-10, N-acetyl cysteine, lipoic acid
Antioxidant M	Minerals	Zinc, selenium, copper
Antioxidant P	Phenolics	Quercetin, catechin
Antioxidant S	Saponins, steroids	Cortisone, estradiol, estriol
Antioxidant V	Vitamins	α-tocopherol, ascorbic acid

5.3 Secondary Metabolites

Plant has a wide range of low molecular weight compounds, which are known as secondary metabolites. These molecules have no vital role in plant life, but they play significant functions between the plant and its environment (Wink, 2003). Based on Namdeo et al. (2007) secondary metabolite compounds depend on the physiological and growth factors and commonly are less than 1% of dry weight. Research and development on secondary metabolites from herbs have been expanding over the last 90 years. Czapek at 1920 was the first person who defined these products could be derived from nitrogen metabolism by subsequent modifications such as deamination.

Primary metabolites are large molecule compounds such as common carbohydrates, fats, proteins and nucleic acids are essential to generate and life maintains. Characteristically, these molecules involved in the energy regulation, growth, and development of organs (Alseekh et al., 2015). Many herbs, rich of secondary metabolite, are employed in the pharmaceutical industry, such as morphine and codeine. Many pharmaceutical compounds are still isolated from plant and without synthetic replacements (Sabater-Jara et al., 2010).

5.4 Importance of Secondary Metabolites

A massive variety of secondary metabolites are resulting from shikimic acid pathway or aromatic amino acids. These compounds are believed to support plant ability by inhibiting pathogen attack or serving reproduction via providing pollinator attraction as coloration or floral scent. The role of secondary metabolites in defense may involve in prevent/anti-feedant activity, toxicity or performing as precursors to physical immune mechanism in plants. The defense-related compounds such as tannins, polyphenols, proteases and chitinases are widely distributed in plant species contain the major compounds. However, indirectly they are involved in plant reproduction (Rispail, 2005). These promising materials as the excessive resource for pharmaceutical research, however, structural complex in their biosynthesis (Yazaki, 2006). Some of the compounds as multi target agents are modifying the activity of proteins, nucleic acids and bio membranes and have detected in medicinal plant extracts. However, these compounds influence on the animal neuro system or appear as various drugs which can be applied to treat an extensive range of infections or diseases.

Plant secondary metabolites are involved in various biological events. Control of plant responses to biotic and abiotic stress condition in many of published literature has been proven. Communication and signaling between nodulation in legumes by flavonoid compounds and pollinators by volatile terpenoids prove their responsibility. Secondary metabolites are an extensive variety of capable compounds in response to stress (Hattenschwiler & Vitousek, 2000). Some species are recognized for their toxic or psychedelic properties which contain alkaloids, terpenoids or other compounds which associated in molecular target (Wink, 2015).

Table 2.2: Secondary metabolites classification (Bourgaud et al., 2001)

Class	Compound	Example Sources	Some Effects and Uses
NITROGEN-CONTAINING			
Alkaloids	Nicotine	Tobacco	Interfere with neurotransmission
	Cocaine	Coca plant	Block enzyme action
	Theobromine	Chocolate (Cocoa)	Treat high blood pressure
NITROGEN AND SULFUR CONTAINING			
Glucosinolates	Sinigrin	Brassica family	Antitumor effects
TERPENOIDS			
Monoterpenes	Menthol	Mint oils	Interfere with neurotransmission
	Linalool	Cinnamon Spp, etc.	Block ion transport, Anesthetic
Sesquiterpenes	Parthenolide	Parthenium and Asteraceae	Anti-inflammatory and Anti-hyperalgesia
Diterpenes	Gossypol	Cotton	Block phosphorylation
Triterpenes, Cardiac glycosides	Digitogenin	Digitalis (foxglove)	Stimulate heart muscle
			Alter ion transport
Tetraterpenoids	Carotene	*Nerium Oleander*	Antioxidant
Terpene polymers	Rubber	Hevea, Dandelion	Gum up insects; Airplane tire
Sterols	Spinasterol	Spinach	Interfere with animal hormone action
PHENOLICS			
Phenolic acids	Caffeic	Most of the plants	Anticancer
	Chlorogenic		Antioxidant
Coumarins	Umbelliferon	Carrots, Parsnip	Cross-link DNA, Block cell division
Lignans	Podophyllin	Mayapple	Cathartic, Vomiting
	Urushiol	*Poison ivy*	Allergic dermatitis
Flavonoids	Anthocyanin,	Most of the plants	Inhibit enzymes, Antioxidants
	Catechinalmost		
Tannins	Gallo tannin	Oak, Legumes	Bind to Proteins, Antioxidants
	Condensed tannin		
Lignin	Lignin	Most of the land plants	Structure, Toughness, Fiber

Based on Table 2.2, some of the secondary metabolite compounds have been categorized as:

- Chemical structure (presence of rings, sugar).
- Composition (may contain nitrogen)
- Solubility
- Synthesis Pathway

The pathway of synthesis classification contains three groups:

- The terpenes, contains of mevalonic acid, full of hydrogen and carbon.
- The phenolic, this group have a simple sugar, benzene rings, hydrogen or oxygen.
- The nitrogen-containing compounds which are impressively varied.

Phenolics are one of the common metabolite families which molecules are involved in lignin synthesis that can be found in most of the plants. The other complexes such as alkaloids specifically are to identify plant genus and species and infrequently distributed in the plant (Bourgaud et al., 2001).

2.1 Alkaloids

The alkaloids are a class of secondary metabolites containing nitrogen-based atoms. most of them are extractable by acid-base and also are toxic to other living organisms. It is produced by a large variety of wildlife, such as bacteria, fungi, animals and mostly plants as secondary metabolites. They have diverse pharmacological effects with an old history in medication (Aniszewski, 2007). The edge between alkaloids and other natural nitrogen-based compounds is not unique (Giweli et al., 2013). They are formed from amino acids such as tyrosine (Dewick, 2009).

The morphine biosynthesis that contains a phenol coupling reaction containing a benzylisoquinoline alkaloid could be a good example. Furthermore, some compounds such as amino acids, proteins, peptides, nucleotides, nucleic acid, and amines are not usually reported as alkaloids. Alkaloids do not identified with a uniform classification in comparison with other natural compounds, and are categorized by a great structural diversity. Some of the most well-known of alkaloids have been listed in Table 2.3 (Kabera et al., 2014). The First classification which combined alkaloids by the common natural source is outdated. More recent classifications are based on similarity of the carbon skeleton or biochemical precursor (Savithramma et al., 2011).

Table 2.3: Some of the well-known alkaloid, source, and effects (Kabera et al., 2014).

Alkaloid name	Source	Pharmacological activity
Atropine	*Atropa belladonna, Datura stramonium, Mandragora officinarum*	Competitive antagonist of muscarinic acetylcholine receptors, anti-cholinergic, anti-myopia affects
Berberine	Berberis species, Hydrastis, Canadensis, *Xanthorhiza simplices sima*, *Phellodendron amurense*, *Coptis chinensis, Tinospora cardi folia, Argemone mexicana* and *Eschscholzia california*	Anti-inflammatory, anti-bacterial/viral, recently experiments showed antidiabetic and beneficial effects on the cardiovascular system and anti-cancer and other disorders such as intestinal
Codeine	*Papaver somniferum*	Analgesic, antitussive, anti-diarrheal, antidepressant, sedative and hypnotic properties
Coniine	*Conium macularum*, *Sarracenia flave*	Neurotoxin, poisonous
Cytisine (baptitoxine, sophorine)	Cytisus of the Fabaceae family, most extracted from seeds of *C. laburnum*	Acetylcholine agonist, smoking cessation drug
Morphine	*P. somniferum* and poppy derivatives	Act on CNS (central nervous system), on myenteric plexus, acute pulmonary edema and reduce the shortness of breath
Nicotine	Solanaceae	Stimulant, ant herbivore, insecticide, anti-inflammatory
Quinine	*Cinchona succirubra*, *C. calisya, C. ledgeriana*, Rubiaceae family	Antimalarial, antipyretic, analgesic, anti-inflammatory, antiarrhythmic, bacteriostatic
Solanine	*Solanum tuberosum*, *S. lycopersiam*, *S. igrum,* plants of Solanaceae family	Antifungal, ant pesticide, sedative, anticonvulsant, ant carcinogenic, anti-inflammatory
Strychnine	*Strychnos nux-vomica*, Loganiaceae	Pesticide, strong poisonous, Convulsant
Thebaine (paramorphine)	*P. bracteatum*	Analgesic, not therapeutically used
Tomatine	Green parts of tomato plants	Immune effects, anticancer, antifungal, poisonous

2.2 Phenolic compounds

Phenolic compounds are derivatives of the shikimate, pentose phosphate, and phenylpropanoid pathways in plants. This compound is a huge category of metabolites, and new classifications splits the wide category of phenolic into polyphenols and simple phenols. Phenolic compounds with one carboxylic acid have a notable role in many of specialized physiological such as normal growth, development and defense mechanisms of plants (Balasundram et al., 2006; Maisuthisakul, 2012), also with influencing towards the color and physical features of fruits and vegetables (Alasalvar et al., 2001). However, they can be categorized into several classes (Harborne, 1999; Harborne, 1989). As shown in Table 2.4, phenolic acids, flavonoids, and tannins are regarded as the main nutritional phenolic compounds (Peia & Huang, 2016). The hydroxycinnamic and hydroxybenzoic are two simple phenolic acids with the same skeletons and difference in the hydroxyl groups and conjugation (Stalikas, 2007). Hydroxybenzoic acids in common have a one-carbon ($C_6 – C_1$) structure (Gallic, ρ-hydroxybenzoic, protocatechuic, vanillic and syringic acids). Hydroxycinnamic are known as aromatic compounds (Caffeine, Ferulic, ρ-coumaric and Sinapic acids) with three-carbon ($C_6 – C_3$) side chain (Bravo, 1998). Phenolic acids contribute to plant cell wall defense system (deAscensao & Dubery, 2003) supplementary for antioxidant properties, color, and physical qualities and nutritious in foods (Lorenzo & Munekata, 2016).

Table 2.4: Classes of phenolic compounds in plants (Harborne, 1999)

Class	Structure
Simple phenolics, Benzoquinones	C_6
Hydroxybenzoic acids	C_6–C_1
Acetophenone, Phenylacetic Acids	C_6–C_2
Hydroxycinnamic acids, Phenylpropanoids (Coumarins, Isocoumarins, Chromones, Chromenes)	C_6–C_3
Naphthoquinones	C_6–C_4
Xanthones	C_6–C_1–C_6
Stilbenes, Anthraquinones	C_6–C_2–C_6
Flavonoids, Isoflavonoids	C_6–C_3–C_6
Lignans, Neolignans	$(C_6$–$C_3)_2$
Bioflavonoids	$(C_6$–C_3–$C_6)_2$
Lignins	$(C_6$–$C_3)_n$
Condensed tannins (Proanthocyanidins or Flavolans)	$(C_6$–C_3–$C_6)_n$

2.3 Flavonoids

The flavonoid chemical structure comprises a C_6 - C_3 - C_6 carbon structure. These compounds are benzo-ϒ-pyrone consisting of phenolic and pyrene rings, which are different from hydroxylation and /or methylation flavonoids due to three-ring systems (Raaman, 2006). The high molecular weight and a third significant group of the phenolic compound are tannins that subdivided into hydrolysable and condensed (Porter, 1989). Flavonoids from polyphenolic groups could be found in higher plants. These compounds with more than 4000 different, variously identified sub-groups, have been shown anti-allergic, anti-mutagenic, anti-inflammatory, and anti-cancer activities and have been sourced in traditional medicine (Middleton & Kandaswami, 1992; Musarrat et al., 2006). These compounds are the main components of polyphenolic in food which including anthocyanins, proanthocyanidins, flavonols, and catechins.

Flavonoids have been assigned to their intrinsic reducing capabilities and garnered particular attention as potential protective agents against human disorders in the past few years (El-Far & Taie, 2009; Fiedor & Burda, 2014). They play an essential role in the improvement of the endothelial function, inhibition of angiogenesis and cell proliferation and are involved in biological activities such as antioxidant, anti-apoptosis, anti-aging, anti-carcinogen, anti-inflammation, anti-atherosclerosis and cardiovascular protection. Flavonoids are responsible for yellow, orange, and red flower, fruit, and leaf pigments (Patel, 2008). The origination of their biosynthesis detected from phenylalanine and proceeds through trans-cinnamic acid and p-coumaric acid (Vogt, 2010). They perform as antioxidants and act against some diseases and disorders such as viruses, inflammation, arthritis, osteoporosis and diarrhea (Patel, 2008). Based on the position of the aromatic ring to the benzopyran (chromano) moiety, Kumar and Pandey (2013) have been classified them into six groups: Flavanol, Flavone, Flavonol, Flavanone and Isoflavone (Table 2.5). However, some of the flavonoid contents have been summarized in Table 2.6.

Table 2.5: Classification, structure, and food sources of some dietary flavonoids

Class	Flavonoid	Dietary source
Flavanol	(+)-Catechin (−)-Epicatechin Epigallocatechin	Tea
Flavone	Chrysin, Apigenin Rutin, Luteolin, luteolin glucosides	Fruit skins, Red Wine, Buckwheat, Red Pepper, Tomato Skin
Flavonol	Kaempferol, Quercetin, Myricetin, and Tamarixetin	Onion, Red Wine, Olive oil, Berries, Grapefruit.
Flavanone	Naringin, Naringenin, Taxifolin, hesperidin	Citrus fruits, Grapefruits, Lemons, Oranges
Isoflavone	Genistin, Daidzin	Soybean
Anthocyanidin	Apigenidin, Cyanidin	Cherry, Raspberry, Strawberry

Table 2.6: Medicinal plants rich in flavonoids contents (Kumar & Pandey, 2013)

Plant	Family	Flavonoid
Aloe vera	Asphodelaceae	Luteolin
Acalypha indica	Euphorbiaceae	Kaempferol glycosides
Azadirachta indica	Meliaceae	Quercetin
Andrographis paniculata	Acanthaceae	5-hydroxy-7, 8-dimethoxyflavone
Bacopa moneirra	Scrophulariaceae	Luteolin
Betula pendula	Betulaceae	Quercetrin
Butea monospermea	Fabaceae	Genistein
Bauhinia monandra	Fabaceae	Quercetin-3-O-rutinoside
Brysonima crassa	Malphigaceae	(+)-catechin
Calendula officinalis	Compositae	isorhamnetin
Cannabis sativa	Compositae	Quercetin
Citrus medica	Rutaceae	hesperidin
Clerodendrum phlomidis	Verbenaceae	Pectolinarigenin,
Clitoria ternatea	Fabaceae	kaempferol-3-neohesperidoside
Glyccheriza glabra	Leguminosae	Liquiritin,
Mimosa pudica	Mimosoideae	Isoquercetin
Limnophila indica	Scrophulariaceae	3,4-methlenedioxyflavone
Mentha longifolia	Lamiaceae	Luteolin-7-O-glycoside
Momordica charantia	Curcurbitaceae	Luteolin
Oroxylum indicum	Bignoniaceaea	Chrysin
Passiflora incarnate	Passifloraceae	Vitexin
Pongamia pinnata	Fabaceae	Pongaflavonol
Tephrosia purpurea	Fabaceae	Purpurin
Tilia cordata	Tiliaceae	hyperoside

5.5 Major metabolic pathways

Biosynthetic responses are a replica of common organic replies, which are dependent on altered conditions. The clarification of biosynthetic trail in plants for production of different metabolites has been broadly studied. The secondary metabolism is related to specific environmental or developmental phases. Hence, the producing of secondary metabolites in plants is the opposing response to environmental or developmental conditions. Starch in plants and glycogen of animals is known as carbohydrate storage, which produces energy by involving a method to pyruvate and then acetate. In this process, acetyl coenzyme A, would passing into the tricarboxylic acid cycle. Because of this, the energy-rich carbohydrate is oxidized to water and carbon dioxide. The coenzymes are transferred, released hydrogen into the cytochrome system and the possible formation of ATP, ADP, and inorganic phosphate followed with the energy.

A glucose pathway is a primary metabolism in living tissues. This pathway contains compounds from photosynthesis cycle and Embden Meyerhoff system of glycolysis. The glucose reaction with regard to ADP and ATP is:

$$\boldsymbol{C6H12O6 + 6CO2 + 38\,ADP + 38P\,(inorganic) \rightarrow 6H2O + 6CO2 + 38\,ATP}$$

The various metabolite activities (primary and secondary) are obtained from carbon metabolism are demonestrated in figure 2.1. The association of biosynthetic trails heading to of secondary metabolites development is displayed in figure 2.2. The malonic acid system is an imperative source in fungi and bacteria for phenolic secondary products (Varzakas et al., 2016).

The shikimic acid pathway is engaged in the synthesis of many phenolic compounds in most plants, fungi, and even bacteria. This pathway is converting a simple carbohydrate resulted from glycolysis and the pentose phosphate pathway to the aromatic amino acids, phenylalanine and tryptophan (Mandal et al., 2010).

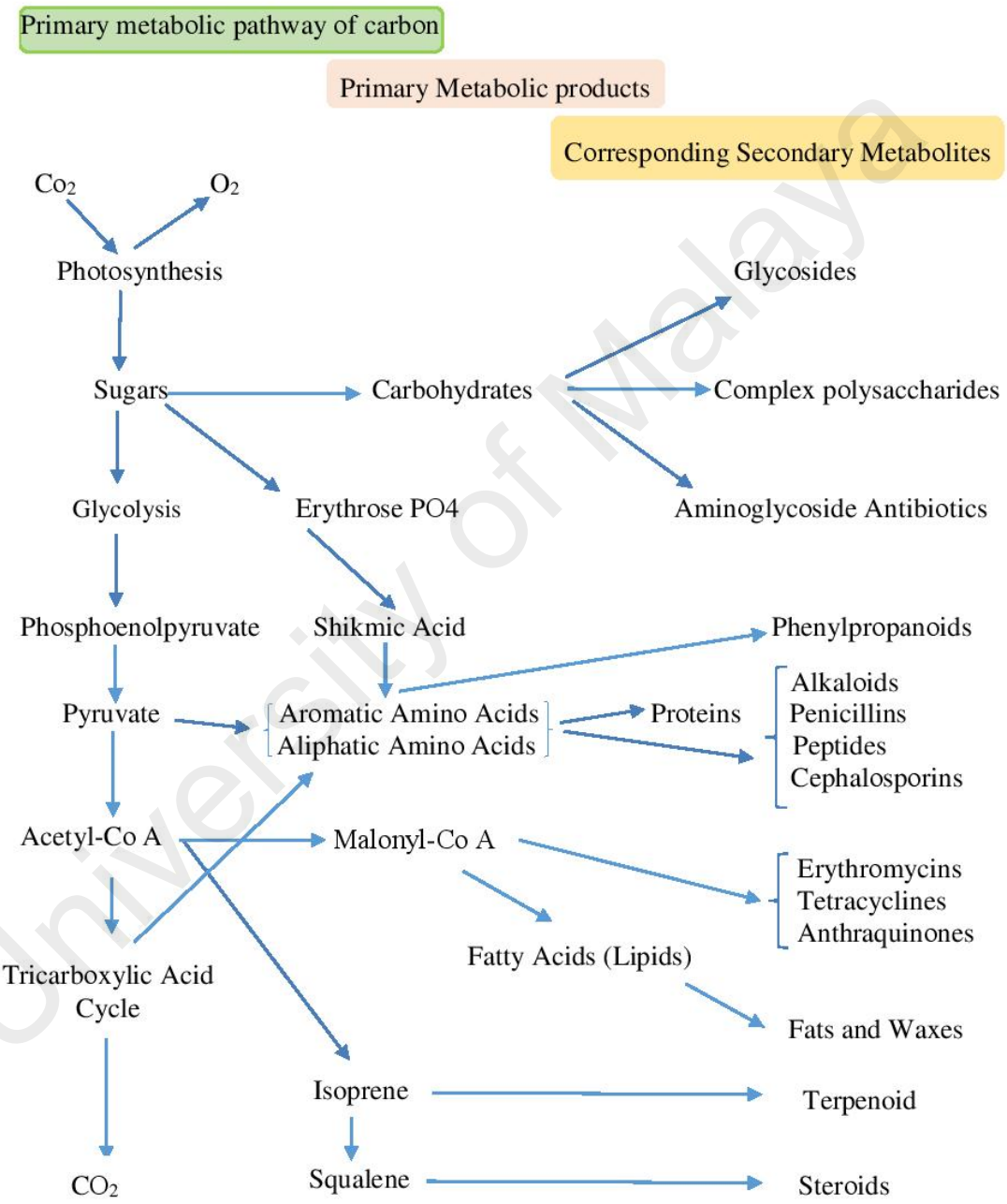


Figure 2.1: Inter relationships of biosynthetic pathways leading to secondary constituents in plants (Khanam, 2007)

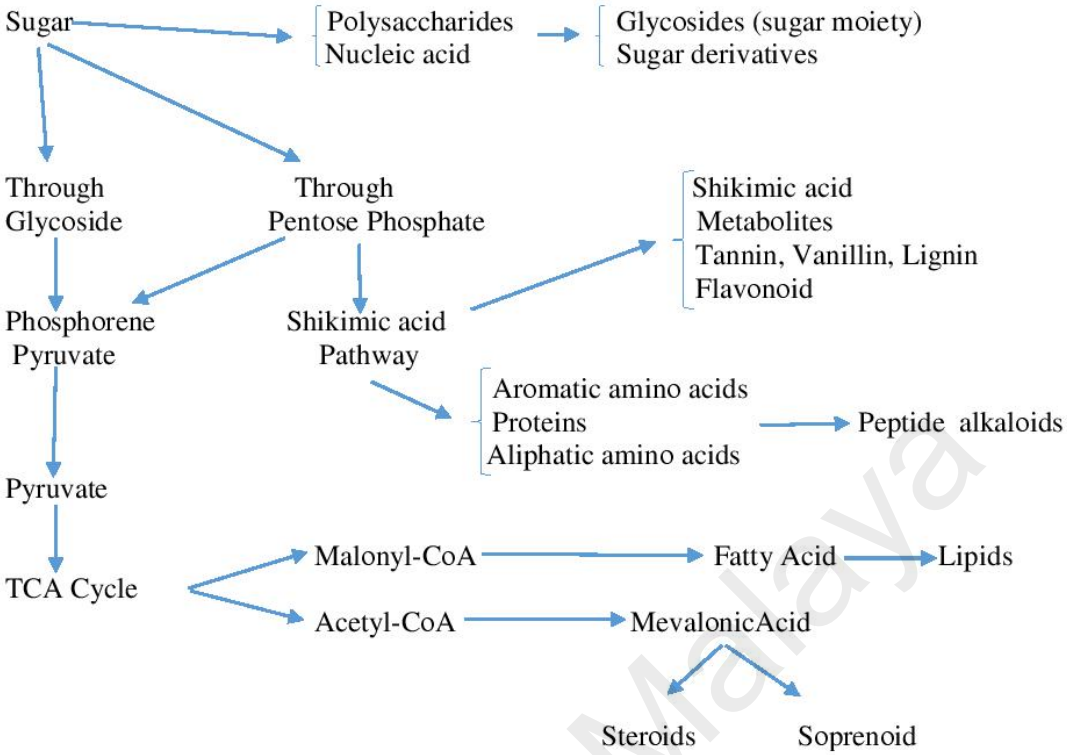


Figure 2.2: Malonic acid and pentose phosphate pathway (Khanam, 2007)

5.6 Botanical characteristics of *Gardenia jasminoides* Ellis

Gardenia jasminoides Ellis an evergreen tree that belongs to the Rubiaceae family, is cultivated in multiple areas in Southern Asia. Some botanical characteristics are:

- Leaves: Opposite, thick, lanceolate to ovate shape, dark green color, around 6-10 cm length.
- Flower: Terminal, sweetly fragrant, five green fascinated teeth in calyx, corolla with five to nine white waxy petals and six whorls, 8-10 cm across in size (Fig 2.3). Sterile flowers are due to transforming of stamens and pistil(s), the various issues affect flower induction and development. The conventional method of propagation is stem cutting after flowering (Nower & Hamza, 2013).

Figure 2.3: Leaf and flower of *Gardenia jasminoides* Ellis

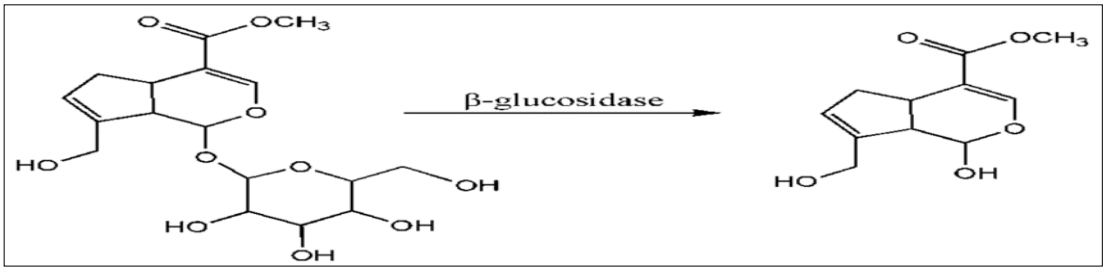
5.7 Benefits of Gardenia jasminoides Ellis

Apart from using as a cut flower, landscape designs, oil & scent (flower) in Traditional Chinese Medicine, this plant is applied as fried and charred to stanch the bleeding and the husk (fruit without seeds), flower clears heat from the lungs. The simple dried fruit (Fig 2.4), commonly is applied to heat/fire signs such as irritability, restlessness, insomnia, delirious speech and a stifling sensation in the chest.

Figure 2.4: Dry and fresh fruit of *Gardenia jasminoides* Ellis

There are many subjects on this plant based on Traditional Chinese Medicine (TCM), Gardenia seed clears internal heat and removes heart vexation, eliminating 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). Mortensen (2006) revealed natural colorants from pigments of Gardenia fruit. The chemopreventive agent in Alzheimer have been reported from *G. jasminoides* extract (Choi et al., 2007). This plant showed eco-friendly antifungal activity (Lelono et al., 2009). The fleshy fruit is a diuretic, energizing, an emetic; however, has a beneficial effect for lung, jaundice and kidney disorders (George et al., 1993). Geniposide and related constituents as major colorless iridoids in Gardenia fruits, convert into blue and red pigments (Fig 2.5).

Polar crocetin as a key element of Gardenia fruit has been known as a substantial factor in industries due to coloring properties and water-soluble behavior (Bergonzi et al., 2012; Mortensen, 2006; Yamauchi et al., 2011). The carotenoids responsible for a broad range of pharmacological effects, such as preventing cardiovascular diseases and as the main supplier to the antioxidant ability of the plant (Shu-Ying et al., 2005; Xiang-Chun & Qian, 2006). There are many reports of crocetin, Kuratsune et al. (2010) reported that crocetin can improve sleeping problem; however it could use as a remedy for the liver disease (Kotoky & Das, 2008), inhibiting tumor cell gowth (Magesh et al., 2006), and hepatocytes (Tseng et al., 1995).



Geniposide Genipin

Figure 2.5: Structure of Genioposide (R-Glucose) and Genipin (R-H)

5.8 Secondary metabolites in Gardenia

Geniposide, crocin, and crocetin are the major metabolite compounds in the Gardenia fruit (Machida et al., 2000). Geniposide is a preeminent iridoid compound in *Gardenia jasminoides* Ellis fruits which revealed a wide range of pharmacological activities such as the hepatoprotective effect (Ma et al., 2011), hypoglycemic effect (Wu et al., 2009), insulin resistance-alleviating effect (Kojima et al., 2011), anti-proliferation effect (Kim et al., 2003), antioxidant effect (Yin et al., 2010), neuroprotective effect (Guo et al., 2009), and a cross-linker to making polymeric material in biomedical applications. A variety of pharmacological effects from crocin and crocetin such as anti-oxidant (Kanakis et al., 2007), anti-hyperlipidemia (Sun et al., 2011), anti-atherosclerotic (Zheng et al., 2006), anti-inflammatory (Kazi & Qian, 2009), anti-proliferation (Dhar et al., 2009), neuroprotective effects (Mehri et al., 2012), insulin resistance improvement (Sheng et al., 2008), positive effects on sleep (Kuratsune et al., 2010), have been revealed. Some of the identified compounds from *G. jasminoides* have been listed in Table 2.7.

Table 2.7: Isolated compounds from *Gardenia jasminoides*

Species	Compound (s)	References
Gardenia jasminoides	**Coumarone**: ferrulic acid; skimmin; uracil; 5,8-di-(3-methyl-2,3-dihydroxy-butyloxypsoralen); 3-o-α-d-glucopyranosyl-(1→4)-β-d-glucopyranosyloxypeucedanin	(Moon et al., 2002)
	Iridoids: genipin 1-o-β-d-d-isomaltoside; 1,10-di-o-β-d-glucopyranoside; genipin 1-o-β-d-gentiobioside; geniposide; scandoside methyl ester; deacetylasperulosidic acid methyl ester; 6-o-methyldeacetylasperulosidic acid methyl ester; gardenoside	(Chen et al., 2009)
	Iridoid: 8-epi-apodantheroside; 7β,8β-epoxy-8α-dihydrogeniposide	(Machida et al., 2003)
	Iridoids: 6′-o-[(E)-sinapoyl] gardoside; 4″-o-[(E)-p-coumaroyl]-gentiobiosylgenipin; 6′-o-[(E)-caffeoyl]-deacetylasperulosidic acid methyl ester	(Fu et al., 2008)
	Iridoid: 6-o-sinapoylgeniposide	(Zhou et al., 2007b)
	Monoterpenes: gardenone; gardendiol	(Zhao et al., 1994)
	Carotenoids: crocetin; crocetin mono (β-d-glucosyl) ester; crocetin di-(β-d-glucosyl) ester; crocetin mono-(β-gentiobiosyl) ester; crocetin (β-d-glucosyl)-(β-gentiobiosyl) ester; crocin [crocetin-di-(β-gentiobiosyl)ester]; crocetin (β-gentiobiosyl)-(β-neapolitanosyl) ester; crocetin-di-(β-neapolitanosyl) ester	(Pfister et al., 1996)
	Monoterpenes: jasminosides J–K; 6′-o-trans-sinapoyljasminoside B; 6′-O-trans-sinapoyljasminoside L; jasminosides M–P; jasminoside C; jasminol E; sacranoside B	(Yang et al., 2013)
	Flavonoid: luteolin-7-o-β-d-glucopyranoside **Triterpenes**: ursolic acid; oleanolic acid; methyl 3,4-di-o-caffeoylquinate; methyl 5-o-caffeoyl-3-o-sinapoylquinate; methyl 3,5-di-o-caffeoyl-4-o-(3-hydroxy-3-methyl)glutaroylquinate; methyl 5-o-caffeoyl-4-o-sinapoylquinate **Glycosides**: 2-methyl-l-erythritol-4-o-(6-o-trans-sinapoyl)-β-d-glucopyranoside; 2-methyl-l-erythritol-1-o-(6-o-trans-sinapoyl)-β-d-glucopyranoside	(Yang et al., 2007)
	Iridoids: 6′-o-trans-p-coumaroyl geniposidic acid; 11-(6-o-trans-sinapoyl glucopyranosyl)-**gardendiol**; 10-(6-o-trans-sinapoyl glucopyranosyl) gardendiol; 6″-o-trans-sinapoylgenipin **gentiobioside**; 6″-o-trans-cinnamoylgenipin gentiobioside; 10-o-succinoylgeniposide; 6′-o-acetylgeniposide; 6″-o-trans-p-coumaroylgenipin gentiobioside	(Yu et al., 2009)

Table 2.7, continued: Isolated compounds from *Gardenia jasminoides*

	Iridoids: gardaloside	(Martins & Veronica Nunez, 2015)
	Iridoids: garjasmine; dunnisin; α-gardiol; β-gardiol; diffusoside A diffusoside B; genameside C; deacetylasperulosidic acid	(Song et al., 2014)
	Iridoid glycoside: 6″-o-trans-feruloylgenipin gentiobioside; 2′-o-trans-p-coumaroylgardoside; 2′-o-trans-feruloylgardoside	(Qin et al., 2013)

5.9 *In vitro* culture of medicinal and aromatic plants

In vitro micropropagation is crucial for some herbs such as *Catharanthus roseus* (L.) or (Madagascar periwinkle). This group of plants is required in vast amounts, but offer in small quantities owing to costly yield. Only one gram of alkaloid drove from two tons of the leaves as a treatment for a leukemia patient. This pattern is observed for *Taxus brevifolia* Nutt (Pacific yew), bark from two hundred years of age is required for one ovarian cancer patient (Chaturvedi, 2007). Plenty of famous Chinese traditional medicinal plants have been successfully micropropagated. Each plant has a special group of bioactive compounds. Taxol (complex diterpene alkaloid) from the Taxus tree bark is known as an anti-cancer (Chaturvedi, 2007). However, the latex of *Papaver somniferum* or *Opium poppy* is a source for codeine, analgesics, and morphine. The maximum codeine and morphine concentrations were measured 3.0 mg dry weight and 2.5 mg dry weight, respectively (Mulabagal et al., 2004; Siah & Doran, 1991). Ginsenosides (primary components) from Ginseng are categorized as triterpenoid saponins (Kim et al., 2012; Zhou et al., 2007a) and Berberine (isoquinoline alkaloid) from the roots of *Coptis japonica* (Lee et al., 2005) could be considered as another example. However, diosgenin from *D. deltoidea* as one of an early known compound of steroidal drugs possesses tremendous (Mulabagal et al., 2004).

Effect an extract compound of *Withania somnifera* (Saritha & Naidu, 2007) and camptothecin from *Camptotheca acuminata* (Li & Liu, 2001) as antitumor has been described. Hypocotyl cultures of *Ruta graveolens* L. uses as sources of the pharmacologically active compound has been carried out (Asgarpanah & Roghaieh, 2012). Micropropagation of *Paederia foetida* L. as an important Asian pharmaceutical plant (Amin et al., 2003), tissue culture of *Crocus sativus* (Mir et al., 2010), *Gingko Biloba* (Tolyat et al., 2009), *Clitoria ternatea* L. or Aparajita as Indian medicinal plant (Mukhtar et al., 2012) and *Curculigo latifolia* as aromatic and medicinal plant (Farzinebrahimi et al., 2016) were reported. *In vitro* propagation via root explants in many of medicinal plants have been studied by Cheruvathur et al. (2015), Guillon et al. (2006), Khalafalla et al. (2009), Maheswari et al. (2011), Sevón and Oksman-Caldentey (2002), Sharma et al. (2013), Srivastava and Srivastava (2007) and Zhang et al. (2014). However, leaf as an appropriate explant part is applied in tissue culture. Leaf explant of medicinal plants reported by Filipović et al. (2015) on *Centaurium erythraea*, Cantelmo et al. (2013) on *Petiveria alliacea*, Chung et al. (2007) on Dendrobium, Sen et al. (2014) on *Achyranthes aspera*, Karataş et al. (2013) on *Hygrophila polysperma*, Sahoo and Rout (2014) on *Aloe barbadensis* and Pathak and Joshi (2015) on *Hemidesmus indicus*. Sivakumar et al. (2006) applied shoot tip as the explants from *Centella asiatica,* Huang et al. (2000) from *Limonium wrightii*, Moharami et al. (2014) on *Agastache foeniculum*, Sasidharan and Jayachitra (2017) on *Enicostema axillare.* Shoot tip was cultured successfully by Ahmed and Anis (2014) on *Vitex trifolia* and also by Xu et al. (2004) on *Aconitum coreanum.*

5.10 Micropropagation of *Gardenia jasminoides* Ellis

2.3.1 Explant

Micropropagation via shoot tips (Economou & Spanoudaki, 1986; Sayd et al., 2010; Serret et al., 1996) and microshooting explants (Hatzilazarou et al., 2006) from *G. jasminoides* Ellis were reported. George et al. (1993) recommend the root explant does not as a well established *in vitro* method for shoot regeneration. Chuenboonngarm et al. (2001) applied the young shoot as explants and Al-Juboory et al. (1998), Mizukami et al. (1987) applied leaf explant. In addition, ovary and immature ovary portion culture for callus initiation was reported (George et al., 1993). Single nodes followed with shoot tip explants applied by Duhoky and Rasheed (2010).

2.3.2 Plant growth hormone

According to Sayd et al. (2010), high proliferation of *G. jasminoides* was observed on media supplemented with benzylaminopurine (BAP) compared to 2iP and kinetin. However, Hatzilazarou et al., (2006) reported that indole-3-butyric acid (IBA) was induced the high percentages of root on *in vitro* and *ex vitro* conditions from micro cuttings of cape jasmine. A successful combination of IAA (0.1 mg L^{-1}) and BAP (1 mg L^{-1}) for chlorophyll extract, and an optimum range of shoot proliferation via BAP on *G. jasminoides* Ellis achieved (Chuenboonngarm et al., 2001). Furthermore, raising length, number and improving the quality of the shoots in *G. jasminoides* was studied by Economou & Spanoudaki (1986). There are two reports on the effect of 2iP in the Cape jasmine; Pontikis (1983) reported high-quality in the long shoot, but Chuenboonngarm et al. (2001) revealed chimeric plants. Furthermore, using of BA was encouraged axillary buds (Economou & Spanoudaki, 1986). Al-Juboory et al. (1998) applied a combination of TDZ (1.7 mg L^{-1}) and IAA (1 mg L^{-1}) and obtained adventitious shoots.

However, the addition of BAP and NAA displayed high production compared to kinetin and 2ip (Sayd et al., 2010). Indolic-3-butyric acid (IBA) in micro cuttings of *G. jasminoides* formed higher rate of roots in *in vitro* and *ex vitro* condition (Hatzilazarou et al., 2006; Pontikis, 1983). Mizukami et al. (1987) and Kubota et al. (1995) reported a combination of Kinetin and 2, 4-D (0.2 mg L^{-1}) on suspension culture to gain salicin from salicyl alcohol.

5.11 Secondary metabolite in Rubiaceae

This family has a great potential to synthesis pharmacological metabolite compounds. The distribution of these compounds in the Rubiaceae family follows the patterns that could be applied as a method to classify among genera and subfamilies (Barreiro, 1990; Farias, 2006). This family offers a huge variety of constituents with an emphasis on production of bioactive alkaloids such as iridoids, indole alkaloids, anthraquinones, terpenoids (diterpenes and triterpenes), flavonoids and other phenolic derivatives (Table 2.8). The total products, organic range, and pharmacological manners reported from different species of Rubiaceae proven this family to be a capable source of new secondary metabolite contents. Most parts of this species have been used extensively in folk medicine with proven effect of anti-inflammatory, analgesic, antibacterial, mutagenic, antiviral and antioxidant (Table 2.9). The caffeine from *Coffea arabica* (genus Coffea, Ixoroideae subfamily), known as coffee acts as energizing in the central nervous system, as well as a vasoconstrictor, bronchodilator and diuretic, besides being one of the constituents of migraine medications (Simões et al., 2004).

More than 180 years, the quinine from Cinchona species (Cinchonoideae subfamily) known as the only constituent against malaria (Pelletier & Caventou, 2007). There are some reports that alkaloids from *Uncaria tomentosa* (Uncaria species) have immunostimulant and antitumor activity (Goncalves et al., 2005; Lemaire et al., 1999).

C. ipecacuanha from Cephaelis genus is a major source of emetine, an alkaloid with emetic, anthelminthic and expectorant effects (Di Stasi & Hiruma-Lima, 2008).

Table 2.8: Classification of Rubiaceae family (Martins & Veronica Nunez, 2015)

Rubiaceae		
Cinchonoideae	**Subfamily** **Rubioideae**	**Ixorideae**
Chiococceae	Anthospermeae	Alberteae
Cinchoneae	Argostemmateae	Bertiereae
Guettardeae	Coussareeae	Coffeeae
Hamelieae	Craterispermeae	Condamineeae
Hillieae	Danaideae	Cremasporeae
Hymenodictyeae	Gaertnereae	Gardenieae
Isertieae	Knoxieae	Ixoreae
Naucleeae	Lasiantheae	Mussaendeae
Rondeletieae	Morindeae	Octotropideae
	Ophiorrhizeae	Pavetteae
	Paederieae	Posoquerieae
	Psychotrieae	Retiniphylleae
	Putorieae	Sabiceeae
	Rubieae	Sipaneeae
	Schradereae	Vanguerieae
	Spermacoceae	
	Theligoneae	
	Urophylleae	

Table 2.9: Some well-known metabolites isolated from Rubiaceae family (Heitzman et al., 2005)

Genera	Class	Substance
Cephaelis	Alkaloid	Emetine
	Lactone	Chelidonic acid
	Alkaloid	Cephalin
		Psycotrin
Cinchona		Quinine
	Triterpene	Cincholic acid
		Quinovic acid
	Alkaloid	Quinidine
		Cinchonine
		Cinchonidine
Coffea	Methyl xantine	Caffeine
	Diterpene	Cafestol
	Anthraquinone	Galiosin
		Copareolatin
		Munjistin
Corynanthe	Alkaloid	Yohimbine
Galium	Iridoide	Macedonine
Gardenia		Geniposide
	Triterpene	Crocin
Genipa	Monoterpene	Genipin
Hedyotis	Anthraquinone	Alizarin
Landerbergia	Alkaloid	Quinidine
		Cinchonine
		Cinchonidine
Morinda	Anthraquinone	Alizarin
Mussaenda	Triterpene	Arjunolic acid
Oldenlandia	Anthraquinone	Alizarin
Psychotria	Alkaloid	Psycotrin
		Cephalin
Relbunium	Anthraquinone	Purpurin
Remijia	Alkaloid	Quinidine
		Cinchonine
		Cinchonidine
Rubia	Anthraquinone	Purpurin
		Alizarin

5.12 Biotechnology for production of secondary metabolite

Producing secondary metabolite compound in *in vitro* system is a desirable method. These produced compounds can be more efficient, when compared with intact plants from field grown. Tissue and cell cultures are a defined standard enormous source of phytochemicals in large volumes (Naik & Al–Khayri, 2016). However, artificial environment of *in vitro* technique may increase growth rate and phytochemicals. Some methods and factors are influencing on the specific plant bioactive metabolite production.

2.3.3 Manipulation of cultural conditions

Manipulation of physical features, nutritional elements and plant hormones in a medium of culture is the major optimization approach in secondary metabolite productivity. Biosynthesis of *Clavine ergot* and quinocitrinines in the medium supplemented with various carbon and nitrogen was increased (Koslovskiĭ et al., 2010). An optimizing in nitrate, ammonium and phosphate ions with a various concentration of sucrose raised the biosynthesis of galanthamine in shoot culture of *Leucojum aestivum* (Georgiev et al., 2009). Behera et al. (2009) reported optimize the culture conditions in *G. awasthi* was increased biomass and total phenolic compound. Karuppusamy (2009) indicated that various concentration of sucrose with BA in micropropagation of turmeric was enhanced biomass and phytochemical biosynthesis. Moreover, Jedinák et al. (2004) optimized growth and isoflavonoid production media in Genista species; the best growth and highest isoflavone production was obtained on SH basal medium containing 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin and 3% (w/v) of sucrose. The influence of medium factors such as carbon (Mulabagal & Hsin-Sheng, 2004), nitrogen (Franklin & Dixon, 1994), phosphate (Zhang & Zhong, 1997), potassium ion (Furuya, 1988) and plant growth hormones to increase the biosynthesis of ginsenosides were reported.

The yield of berberine was improved when nutrients in cell culture media with plant growth regulators were optimized (Guern et al., 2012; Patil et al., 2012; Sato & Yamada, 1984; Shad & Deepa, 2015). Plant growth regulators are essential factors in secondary metabolite biosynthesis in *in vitro*. Modifications or optimization of auxin or cytokinin, as well as the in combination ratio has intense effects on metabolite synthesis and growth in plants (Jeong et al., 2007).

Callus cultures offer an appropriate biological system in *in vitro* condition which maintaining and regulating by growth regulators in the nutrient medium causes a rapid production of plant metabolites (Arumugam et al., 2011). The auxin effects on growth control and roots morphology and its effect on biosynthesis of secondary compounds in *in vitro* condition are proven (Arvy et al., 1994). The plant growth regulation (PGR) have described the crucial role of phytohormones on the secondary metabolites biosynthesis, but the biosynthetic pathways still are researchable. Additionally, the concentration of some PGR able to promote or prevent the biosynthesis of specific secondary compounds depending on *in vitro* cultivated species (Narashimhan & Nair, 2004).

Bota and Deliu (2015) reported that flavonoid contents increased with the age of harvested callus. Callus age of *P. pulture* enhanced biosynthesis of secondary compounds (Danaee et al., 2015a). Tour et al. (2015) and Tsay and Agrawal (2005) revealed the accumulation of crypto tanshinone yield in different callus age in *Salvia multiorrhza* was varied and increased along callus age. The same pattern has been observed in the phytochemical compound at various callus growth cycles on *Morinda citrifolia* (Hagendoorn et al., 1997), and on *Momordica charantia* (Agarwal & Kamal, 2007).

2.3.4 Organ cultures

Plant organ culture offers a fascinating substitute to *in vitro* technique for the secondary plant biosynthesis. The hairy roots (Fig 2.6) and shoot cultures are counted for this objective (Table 2.10). Organ cultures show a minor sensitivity to the stress with a high degree of heterogeneity in producing biomass. The culturing of large-scale and high cost are the main problem of organ cultures. Shoot cultures have been considered for production of phytochemicals (Bourgaud et al., 2001; Nogueira & Romano, 2002; Smith et al., 2002).

It usually creates somaclonal variation and selecting high compound yielding clones (Dhawan, 2003). However, shoot culture in similar condition compared to the intact plants may result in the improvement of the specific compounds. Saito and Mizukami (2002) reported the enhancement in menthol production due to variation in photoperiod of shoot cultures in *Mentha arvensis*. Improvement of secondary metabolite compounds via shoot culture reported by Karalija and Parić (2011), Dias et al. (1998) and Budzianowski et al. (2002).

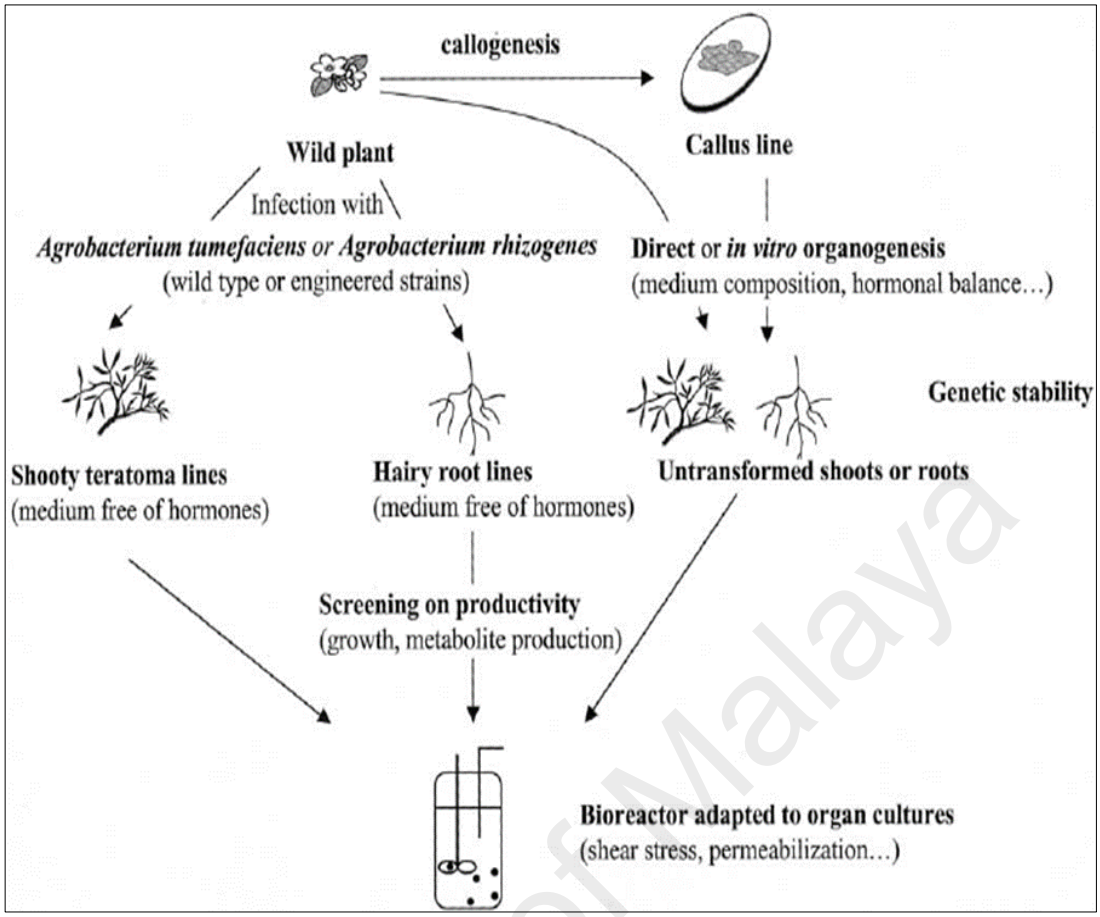


Figure 2.6: Schematic procedure production of biological activities from plant organ cultures (Bourgaud et al., 2001)

Table 2.10: Secondary metabolites produced from various organ cultures in different conditions (Ahmad et al., 2013)

Plant name	Family	Phytoconstituents	Explant/ media/conditions
Catharanthus roseus ①	Apocynaceae	Catharanthine, ajmalicine	Leaf, stem/Liq MS+NAA (2 mg L^{-1}), IM (2 mg L^{-1}), kin (0.1 mg L^{-1})/ shaker at 23±2 °C in darkness
Catharanthus roseus ②	Apocynaceae	Canthinone alkaloids and ajmalicine. Vindoline, vincristine, vinblastine	Petiole from seedlings/ Solid MS+NAA (0.1, 5, 10, 20 ppm), Kin (0.1,5,10,20 ppm)/ Only the first 2 weeks in dark/temp 35 °C
Leucojum aestivum ②	Amaryllidaceae	Galanthamine	Bulb, shoot/Solid MS+BAP, NAA/Dark, temp 25°C
Nandina domestia ②	Berberidaceae	Protoberberine alkaloids, dehydrodiscretamine, berberine, jatorhizine	Fruits/Solid MS+2,4-D (1.0 mg L^{-1}), kin (0.1 mg L^{-1})/ light, temp 25±2 °C
Pinellia temata ①	Araceae	Guanosine. inosine, trigonelline	Tubercles, shoots/Solid MS+NAA (0.5 ppm)BA (1.0 ppm); NAA (02 ppm) BA (1.0 ppm)/light, temp 25 °C
Przewalskia tangutica ③	Solanaceae	Tropane alkaloids	leaves from *in vitro* seedlings/liquid MS+AS (100 µmol L^{-1})/25±0.5 °C in the dark
Rauwolfia serpentine ①	Apocynaceae	Reserpine	Leaves/MS+BA (1 ppm)+ NAA (4 ppm)/16/8 light and dark cycle temp 25±2 °C
Schizanthus hooken ②	Solanaceae	Tiglic acid, seneciocic acid and methyl mesaconic acid, tropane alkaloids	Roots, callus /Solid MS+ NAA (2.69 µM), BA (2.22 µM);BA (4.44 µM)+NAA (0.54 µM)/temp 22±1 °C, light
Solanum tuberosurn ①	Solanaceae	Glycoalkaloids	Tubers/ Solid MS+BA (0.5 ppm), IAA (2 ppm)/temp 26 °C+ light
Taxus globose ①	Taxaceae	Baccatin III, paclitaxel	Stem, intemodes, leaves, meristmatic tissues/ Solid modified gamborg B5/Temp 25 ± 1 °C dark
Metabolite produced by: Suspension culture ①, Callus culture ②, Root culture ③ and Shoot culture ④			

2.3.5 Hairy root cultures

Secondary metabolites in some species are only produced from organ (hairy root) or shooty teratoma (tumor-like) cultures. In addition, transformed root or hairy root culture is also applied to produce secondary compounds. The word of 'hairy root' is obtained from the transformation with bacterium *Agrobacterium rhizogenes* (contains Ri plasmids). The term of "hairy root" was introduced in the literature at 1900 (Mehrotra et al., 2015). *Agrobacterium rhizogenes* is a plant disease agent and could be induced through genetic transformation. The disease is known as a hairy-root syndrome. The phenolic compounds from the wounded part of the plant are attracted *A. rhizogenes*, and caused the infection. This infection is a new way of secondary production metabolites from plant species using the natural vector system *A. rhizogenes*. Bhattacharyya and Bhattacharya (2004) reported the hairy root in *P. amarus* was inhibited (85%) in binding of hepatitis B surface antigen. Hairy roots have a plagiotropic, rapid and extremely induced shoots on medium free of hormone (Hu & Du, 2006).

They have been applied widely to produce the secondary metabolites without losing genetic or biosynthetic stability (Giri & Narasu, 2000). Hairy root cultures of *L. erythrorhizon* and *H. procumbens* by Ludwig-Müller (2011), adventitious roots of *P. ginseng* reported by Murthy and Paek (2016) and *S. parviflora* by Min et al. (2007) to obtain shikonin, harpagide, ginsenosides and alkaloids were studied, respectively. Hairy root culture is an interesting method for production of secondary metabolite and have been widely applied in various plant species (Table 2.11).

Table 2.11: Secondary metabolite production from hairy root cultures obtained from Giri & Narasu, (2000)

Plant Name	Secondary Metabolite
Aconitum heterophyllum	Aconites
Ajuga replans var. atropurpurea	Phytoecdysteroids
Ambrosia spp.	Polyacetylenes and Thiophenes
Anisodus luridus	Tropane alkaloids
Armoracia laphthifolia	Peroxidase, Isoperoxidase, Fusicoccin
Astragalus mongholicus	Cycloartane saponin
Brugmansia candida	Tropane alkaloids
Calystegia sepium	Cuscohygrine
Campanula medium	Polyacetylenes
Carthamus	Thiophenes
Cassia obtusifolia	Anthraquinone
Catharanthus tricophyllus	Indole alkaloids
Centranthus ruber	Valepotriates
Chaenatis douglasis	Thiarubrins
Coleus forskohlii	Forskolin
Coreopsis	Polyacetylene
Daucus carota	Flavonoids, Anthocyanin
Echinacea purpurea	Alkamides
Fagra zanthoxyloids Lam.	Benzophenanthridine
Furoquinoline	Alanine
Fagopyrum	Flavanol
Fragaria	Polyphenol
Geranium thubergee	Tannins
Glycyrrhiza glabra	Flavonoids
Hyoscyamus albus	Alkaloids, Phytoalexins
Hyoscyamus muticus	Tropane, Alkaloids
Lactuca virosa	Sesquiterpene lactones
Leontopodium alpinum	Anthocyanins & Essential oil
Lobelia inflata	Lobeline, Polyacetylene
Lotus corniculatus	Condensed tannins
Panax Hybrid	Ginsenosides
Papaver somniferum	Codeine
Perezia cuernavcana	Sesquiterpene quinone
Rubia peregrina	Anthraquinones
Rubia tinctorum	Anthroquinone
Rudbeckia sps.	Polyacetylenes &thiophenes
Scopolia japanica	Hyoscyamine
Solanum aculeatissi	Steroidal saponins
Solanum aviculare	Steroidal alkaloids
Swainsona galegifolia	Swainsonine
Swertia japonica	Xanthons
Tagetus patula	Thiophenes

2.3.6 Callus culture

Culturing of dedifferentiated plant cells on media which usually supplemented with a specific amount of auxin alone or in combination with cytokinin *in vitro* conditions known as callus culture, which it contained an embryogenic or non-embryogenic cell. Embryogenic callus contains capable cells to regenerate complete plants; this process is called somatic embryogenesis (Ptak et al., 2013). Non-embryogenic callus whis is containing more or less homogenous masses of dedifferentiated cells is utilized for secondary metabolite production. Basic of somatic embryogenesis is the development of somatic cells into somatic embryos through appropriate embryological developments without gamete fertilization (von Arnold et al., 2002).

Somatic embryogenesis due to high production of regenerates, lower frequency of chimeras and incidence of somaclonal variation is a reliable micropropagation method (Ahloowalia & Maluszynski, 2001) and also can be induced to occur directly or indirectly by modulating tissue culture conditions *in vitro* (Namasivayam, 2007). Direct or indirect somatic embryogenesis can be achieved in a plant species by manipulating the plant growth regulators and explant types (Ali et al., 2007b; Siong et al., 2011). The study on the proceedings of biosynthetic pathways in the plant is difficult or not possible on the whole plant level, this action, conveyed at a certain time, cells and within a particular organ. Therefore, the cell culture metabolism is higher than intact differentiated plants due to the beginning of cell growth in a culture that leads to a fast proliferation of cell mass and a reduced biosynthetic cycle (Fig 2.7). It could be one of the most significant advantages for the study of biosynthetic pathways, as secondary metabolite formation during a short cultivation time (about 2-4 weeks) (Dörnenburg & Knorr, 1995). Researchers have successfully applied several methods for *in vitro* production of secondary metabolite (Table 2.12).

In vitro propagation of medicinal plants with improved bioactive values and cell culture methods for selective metabolite production is found to be extremely beneficial for commercial production of secondary metabolite. Plant tissue cultures development may provide new definition of commercial production from rare and exotic plants. The substantial progress in production of biological activities from plant *in vitro* technique has been achieved within last few years. (Hussain et al., 2012b).

The effects of some chemical and physical parameters such as media, PGRs, pH, temperature, O_2, Co_2, agitation and light on culture growth condition have been examined with various plant cells. The influence of the physical features and nutritional elements in the culture media is the optimization method for *in vitro* culture productivity. The nourishing forerunner to cell cultures could be rising directly or indirectly through degradative metabolic pathways in the biosynthesis of specific secondary metabolites (DiCosmo & Misawa, 1995). Mulabagal and Hsin-Sheng (2004) obtained tropane and indole alkaloids by added amino acids to cell suspension culture.

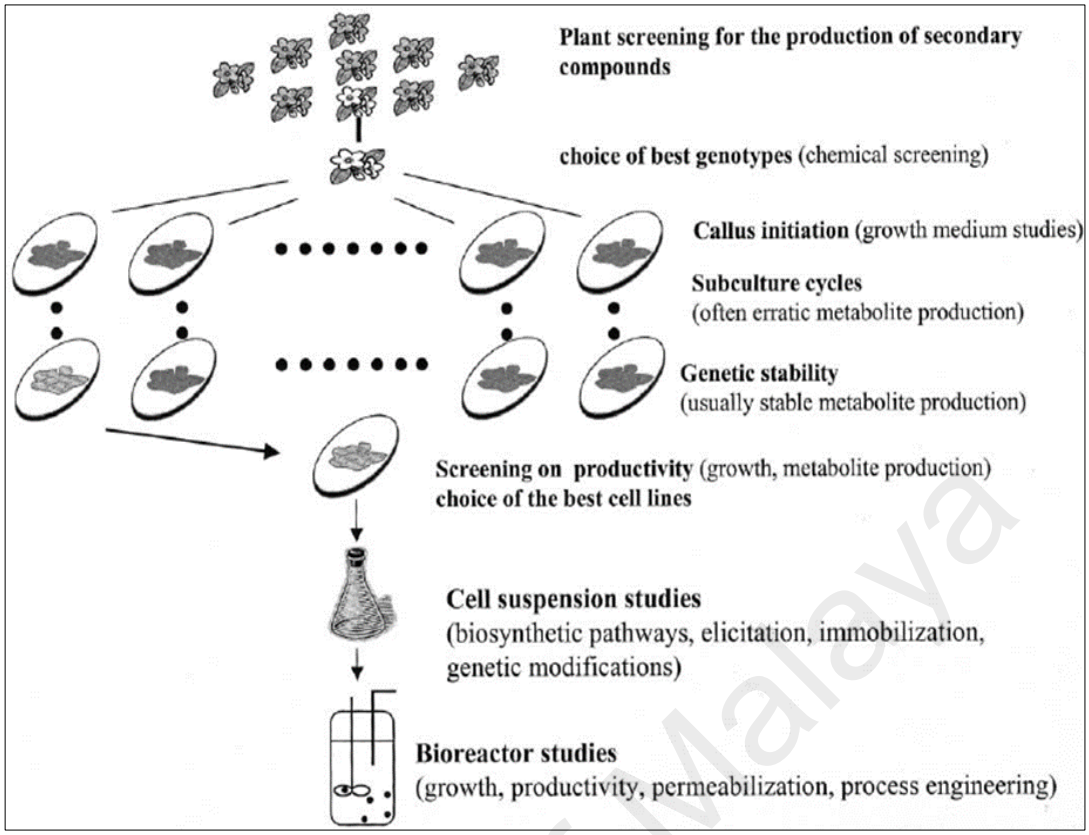


Figure 2.7: Schematic procedure production of secondary metabolites from plant cell culture (Bourgaud et al., 2001)

Table 2.122: Bioactive secondary metabolites from plant cell culture (Mulabagal & Hsin-Sheng, 2004)

Plant Name	Secondary Compound	Culture type
Agave amaniensis	Saponins	Callus
Ailanthus altissima	Alkaloids	Suspension
Ailanthus altissima	Canthinone alkaloids	Suspension
Allium sativum L.	Alliin	Callus
Ambrosia tenuifolia	Altamisine	Callus
Anchusa officinalis	Rosmarinic acid	Suspension
Brucea javanica L. Merr.	Canthinone alkaloids	Suspension
Camellia sinensis	γ-glutamyl derivatives	Suspension
Canavalia ensiformis	L-Canavanine	Callus
Capsicum annuum L.	Capsaicin	Suspension
Cassia acutifolia	Anthraquinones	Suspension
Catharanthus roseus	Indole alkaloids	Suspension
Catharanthus roseus	Catharanthine	Suspension
Cinchona L.	Alkaloids	Suspension
Cinchona robusta	Robustaquinones	Suspension
Cinchona spec.	Anthraquinones	Suspension
Cinchona succirubra	Anthraquinones	Suspension
Citrus sp.	Limonin	Callus
Coffea arabica L.	Caffeine	Callus
Corydalis ophiocarpa	Isoquinoline alkaloids	Callus
Croton sublyratus Kurz	Plaunotol	Suspension
Cruciata glabra	Anthraquinones	Suspension
Digitalis purpurea L.	Cardenolides	Suspension
Dioscorea deltoidea	Diosgenin	Suspension
Dioscorea doryophora Hance	Diosgenin	Suspension
Ephedra spp.	L- Ephedrine,	Suspension
Eriobotrya japonica	Triterpenes	Callus
Eucalyptus tereticornis SM.	Sterols and Phenolic	Callus
Fumaria capreolata	Isoquinoline alkaloids	Suspension
Gentiana sp.	Secoiridoid glucosides	Callus
Ginkgo biloba	Ginkgolide	Suspension
Glehnia littoralis	Furanocoumarin	Suspension
Glycyrrhiza echinata	Flavanoids	Callus
Hyoscyamus niger	Tropane alkaloids	Callus
Isoplexis isabellina	Anthraquinones	Suspension
Linum flavum L.	5-Methoxypodophyllotoxin	Suspension
Lithospermum erythrorhizon	Shikonin derivatives	Suspension
Mentha arvensis	Terpenoid	Suspension
Morinda citrifolia	Anthraquinones	Suspension
Mucuna pruriens	L-DOPA	Suspension
Nandina domestica	Alkaloids	Callus
Nicotiana rustica	Alkaloids	Callus
Nicotiana tabacum L.	Nicotine	Suspension
Ophiorrhiza pumila	Camptothecin related alkaloids	Callus
Panax ginseng	Saponins and Sapogenins	Callus
Panax notoginseng	Ginsenosides	Suspension
Papaver bracteatum	Thebaine	Callus

Table 2.13, continued: Bioactive secondary metabolites from plant cell culture (Mulabagal & Hsin-Sheng, 2004)

Plant Name	Secondary Compound	Culture
Papaver somniferum L.	Morphine, Codeine	Callus/suspension
Peganum harmala L.	β-Carboline alkaloids	Suspension
Phytolacca americana	Betacyanin	Suspension
Picrasma quassioides B	Quassin	Suspension
Podophyllum hexandrum	Podophyllotoxin	Suspension
Polygala amarella	Saponins	Callus
Polygonum hydropiper	Flavanoids	Suspension
Portulaca grandiflora	Betacyanin	Callus
Rauwolfia sellowii	Alkaloids	Suspension
Rauwolfia serpentina B	Reserpine	Suspension
Salvia miltiorrhiza	Rosmarinicacid	Callus
Scopolia parviflora	Alkaloids	Callus
Scutellaria columnae	Phenolics	Callus
Solanum laciniatum Ait	Solasodine	Suspension
Solanum paludosum	Solamargine	Suspension
Tabernaemontana divaricata	Alkaloids	Suspension
Taxus spp.	Taxol	Suspension
Taxus baccata	Taxol baccatin III	Suspension
Thalictrum minus	Berberin	Suspension

5.13 Elicitors and improvement of biosynthesis of secondary metabolites

The plant and their nutritious value have been investigated for decades. Furthermore, aside of primary metabolites such as carbohydrates, lipids and amino acids, the higher plants capable of synthesizing secondary metabolites. Several biotechnological approaches such as high yielding cell line, media manipulation, precursor feeding, elicitation, large-scale cultivation in the bioreactor system, hairy root culture, plant cell immobilization, biotransformation have been offered for the improvement of secondary metabolite production (Namdeo et al., 2007). However, the low yield in the production and productivity of natural compounds by cell culture technology still is one of the obstacles. In plant tissue culture technique, many methods have been applied to fix this problem which elicitor was appeared as an efficient way to improve production of bioactive metabolites. Elicitors which encouraging defense system in the plant (Siddiqui et al., 2013) are stable compounds with microbial or nonbiological origin, which is considered as a signal molecule and could be known by specific receptors on the plasma membrane of plant cells (Zhao et al., 2005).

Elicitors should be accepted by plant receptors localized to the plasma membrane or the cytoplasm to initiate defence system. This compound directly or indirectly activate the consistent effectors such as G-proteins, lipases and kinases, which then transduce the elicitor signal to downstream defence responses. The defence reaction contains synthesis of pathogenesis associated proteins or natural compounds (Goyal et al., 2012). The subsequent binding events activate a signal-transduction cascade leading to the activation and expression of genes related with the biosynthesis of secondary metabolites. Biotic stress is expressed as any condition that ensues as a result of damaging to plants by other living organisms and the abiotic stress is descibe as any disorder caused by environmental factors (Fujita et al., 2006).

These stresses are recognized as indicators of various reactions in plants and encourage reorganization of metabolic fluxes between pathway and an elicitor. These difference reflects an important feature of elicitor induction of plant natural compounds or secondary metabolite activity. In many cases, secondary metabolite production can be improved by the dealing of undifferentiated or differentiated cells with elicitors, amino acids and precursors such as methyl jasmonate, salicylic acid, chitosan, yeast and fungal extracts, tyrosine and heavy metals (Angelova et al., 2006; Radman et al., 2003). Elicitors caused signals that generate the formation of phytoalexins. The elicitation of cultured tissues and intact plants rises the amount of natural products, such as taxol in *Taxus chinensis* (Wang et al., 2001), tropane alkaloids in *Datura stramonium* (Chandra & Chandra, 2011) and indole alkaloids in *Catharanthus roseus* (Almagro et al., 2014). Elicitors are non-specific, as it might increase the levels of certain enzymes and decrease others.

In most cases, elicitors imitated the pathogen defenses or wound responses in plants, and activate the enzymeencoding genes which are responsible for phytoalexin biosynthesis. Most of the secondary metabolites are associated with these responses, in order to support the plant defense system. Some success in inspiring metabolite production has also been achieved by adding protein synthesis inhibitors and precursors (Siibak et al., 2009), altering hormone levels and lowering the incubation temperature (Hussain et al., 2012a). Mandal (2010) has been stated that elicitors are capable molecules for creating the pathogen perception via plant, which causing an initiation of synthetic defense response in crops by rising of phytochemicals production. Based on elicitors source, structure, and type, they are categorized in two biotic and abiotic groups (Bi et al., 2011). Abiotic elicitors are physically or chemically originated in nature. These elicitors contains of ultraviolet radiations, alkalinity, temperature, osmotic pressure and heavy metal ions; whereas biotic elicitors are mostly fungal homogenates or bacterial fractions. Fungal elicitors such as pathogenic (Phytophthora, Botrytis and Verticillium) and non pathogenic

(Aspergilus, Micromucor and Rhodotorula) microbes were used to stimulate the production of gymnemic acid in embryogenic suspension cultures of *Gymnema sylvestre* (Tiwari et al., 2014). Abiotic elicitors were generally available as ordinary chemicals; while biotic elicitors were commonly attained from fermentations, which directed to cost ineffectiveness. However, the optimum usage of an elicitor depended upon factors such as specificity of elicitor, concentration, exposed time of elicitor, growth stage, PGR, and the nutrient in culture media (Purohit & Rocke, 2003). For instance, the elicitation on field-grown crops was found to be expensive, incompetent, and difficult to maintain in the field (Pérez-Montaño et al., 2014).

The elicitors usage as a supportable method for metabolite production has been successfully described in the formation of catharanthine in periwinkle (*Catharanthus roseus*) cell cultures, saponin in Panax, ginseng cultures and sanguinarine formation in *Papaver somniferum* culture (Yue et al., 2016). Applying of methyl jasmonate elicitation improved the yield of chlorogenic acid and reserpine in embryogenic suspension culture of *Eleutherococcus senticosus* (Shohael et al., 2007) and *Rauwolfia serpentine* (Raj et al., 2008). Samuelsson et al. (2004) has been proven effective of this approach in increasing the metabolite production by 10-20 folds in many woody plant species. However, the secondary metabolites production significantly depends on the physiological and developmental stage of plant species. Furthermore, elicitation of tissue cultures has yet to be utilized for the production of these natural products. Future research is needed in order to optimize the culture condition and elicitation condition for optimum production of antioxidant and flavonoid compounds.

2.4 Classification of elicitors

The elicitors based on nature can be categorized into abiotic (non-biological) and biotic groups (biological). The biotic elicitors include polysaccharides derived from plant cell walls e.g. chitin, pectin, and cellulose and microorganisms are labeled based on their originated outside or inside the cell into exogenous (polysaccharides, polyamines, and fatty acids) and endogenous (galacturonide or hepta-β-glucosides) (Table 2.14). This group acts by triggering or inactivating of some enzymes or ion channels. Abiotic elicitors are grouped in physical (UV/gamma radiation, ethylene, fungicides, antibiotics, light, osmotic stress, salinity, drought, and thermal stress), chemical (Heavy metals), and hormonal factors (JA or Jasmonic acid, SA or Salicylic acid and GA or Gibberellin) (Table 2.15). The inappropriate concentration of elicitor or wrong combination with a medium, lead to unsuccessful elicitation with the very challenging process and demanding intense calibrations (Namdeo et al., 2007). Ramirez-Estrada et al. (2016) and Ahmed and Baig (2014) reported the elicitor performing with high biomass secondary metabolite production yields at the last logarithmic growth phase. Most of the elicitors presented immediate surge in the biological activities while suppressing the biomass (Zhao et al., 2005).

Table 2.134: Biotic elicitors and production for secondary metabolites (Namdeo et al., 2007)

Plant species	Natural Compound	Biotic Elicitor
Arabidopsis thaliana	Camalexin, indole glucosinolates	Erwinia, carotovora
Bidens pilosa	Phenylheptaryn	Fungal,culture, filtrate
Brugmansia candida	Hyosujamine	Hemicellulase
Capsicum annuum	Capsidol	Cellulase
Catharanthus roseus	Indole alkaloids	Fungal elicitor
Catharanthus roseus	N-acetyl-tryptamine	Pythium aphanidermatum
Catharanthus roseus	Ajmalicine	Trichoderma viride
Carthamus tinctorius	Polyacetylenes	Fungal polysaccharide
Cicer arientium	Medicarpin Maackiain	Ascochyta rabiei
Cupressus lusitanica	Beta-thujaplicin	Fungal elicitor
Datura stramonium	Lubimin	Fungal spores
Dioscorea deltoidea	Steroid (Diosgenin)	Fungal mycelia
Glycine max	Glyceollin	Fungal glucan
Lithospermum erythrorhizon	Shikonin	Agaropectin
Medicago sativa	Isoflavonoid	Collectotrichum indemuthianum
Medicago sativa	Phytoalexins	Fungal elicitor
Medicago truncatula	Beta-amyrin	Yeast elicitor
Papaver somniferum	Morphine, Codeine	Vertcillium dahliae
Petroselinum crispum	Enzymes	Fungal Elicitor
Phaseolus vulgaris	Krevitone	Fungal polysaccharide
Ruta graveolens	Rutacridone epoxide	Chitosan
Salvia miltiorrhiza	Diterpenoid tanshinones	Yeast elicitor
Silybum marianum	Silymarin	Yeast extract
Various plant cells	Enzymes and secondary metabolites	Erwinia carotovora

Table 2.145: Abiotic elicitors and production for secondary metabolites (Namdeo et al., 2007)

Plant species	Natural Compound	Abiotic Elicitor
Arabidopsis thaliana	Camalexin	Oxidative stress, amino acid starvation
Atropa belladona	Tropane alkaloids	Cu^{2+}, Cd^{2+}
Catharanthus roseus	Indole alkaloids	Diethyl amino ethyl dichloro phenyl ether
Brassica rapa	Alkaloid	Gamma ray
Catharanthus roseus	Catharanthine	Vanadium sulphate
Capsicum annuum	Capsidiol Rishitin	Arachidonic acid
Capsicum frutescens	Capsaicin	Curdlan, Xanthan
Coleus blumei	Rosmarinic acid	Methyl jasmonate (MeJA)
Coleus forskolin	Forskolin	Methyl jasmonate (MeJA)
Cupressus lusitanica	Beta-thujaplicin	Methyl jasmonate (MeJA)
Datura stramonium	Metal ions: Al^{3+}, Cr^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}	Sesquiterpenoids
Daucus carota	Chitinase	Salicylic acid
Glycyrrhiza echinata	Echinatin	Na-alginate
Hyoscyamus albus	Phytoalexin	Copper sulphate
Lithospermum erythrorhizon	Benzoquinone	Activated carbon
Medicago truncatula	Triterpene 2-amyrin	Methyl jasmonate (MeJA), UV light
Panax ginseng	Saponins	Low-energy ultrasound
Rauvolfia canescens	Secondary metabolites	Jasmonic acid
Silybum marianum	Silymarin	Methyl jasmonate (MeJA)
T. canadensis, T. cuspidata	Taxoids	Methyl jasmonate (MeJA)
T. wallichiana	Taxanes	Methyl jasmonate (MeJA)
Taxus chinensis	Taxuyunnanine C (Tc)	Trifluoroethyl salicylate (TFESA)
Valeriana wallichii	Valepotriates	Colchicine
T. wallichiana	Taxanes	Methyl jasmonate (MeJA)
Valeriana wallichii	Valepotriates	Colchicine

5.14 Mechanism of elicitation in plant cells

The plant's reaction to elicitor-induced stress usually initiates at the cell plasma membrane. Substantial effort has been devoted in isolating elicitor signal molecules and recognizing the corresponding receptors. Among several identified elicitor-binding sites on cell plasma membranes for a range of elicitors of different chemical structures, *r* (resistance) and *avr* (avirulence) gene products play a key role in this step (Garcia-Brugger et al., 2006). The transduction of the elicitor signal perceived by the receptors involves the action of second messengers, which further amplify the signal for other downstream reactions (Bel & Mithoefer, 1998).

The consecutively arise events in elicitor-induced immune responses can be summarized as follows (Fig 2.8). Elicitor perception by the receptor; reversible phosphorylation and dephosphorylation of plasma membrane proteins and cytosolic proteins; Cytosolic [Ca2+] enhancement; Cl- and K+ efflux/H+ influx, extracellular alkalinization and cytoplasmic acidification; mitogen-activated protein kinase (MAPK) activation; NADPH oxidase activation and production of reactive oxygen and nitrogen species (ROS and RNS) (Zhao et al., 2005); early defense gene expression; jasmonate production; late defense response gene expression, and secondary metabolite accumulation (Ferrari, 2010; Zhang et al., 2012). Ferrari (2010) believed that systemic responses by plants resulted to synthesis of some compounds such as phytoalexins or pathogenesis-related proteins which playing a key role in defence system. Elicitor perception is capable to surge plant resistance level against future pathogen attack. Therefore, elicitor signal transduction is multiple component networks that find an efficient defense by various sequential reactions. These multiple components consist of parallel or cross-linking signaling pathways leading to different target responses.

An elicitor-signaling pathway may vary in the perception of elicitor signals or target defense responses. Hypertensive responses can also take place, characterized by rapid cell death near the point of exposure to the pathogen (Zhang et al., 2012), as well as the formation of structural defensive barriers, such as lignin deposition to reinforce cell walls. Considerable research is being devoted to the elucidation of the mechanism of action of the main elicitors currently applied in plant biotechnology. Although the receptors, secondary messengers, transduction pathways and responsive genes have been determined in some cases, in general, the data are very incomplete.

The inconsistency of these mechanisms of action involves a broad range of metabolic responses. Due to this complexity, most of the studies about the enhancing effect of elicitation on secondary compound production in plant cell cultures have been empirical, without exploring the cellular response at a molecular level. The components signaling are reported to be involved in elicitor signaling pathways towards PSM production. Studies are increasingly revealing that a defensive cellular process is usually regulated by two or more signaling pathways working in collaboration. This cross-talk among multiple signaling pathways is an important mechanism in plant signal transduction networks (Zhao et al., 2005), enabling plants to regulate different sets of genes temporally and spatially in a range of situations against many types of stress.

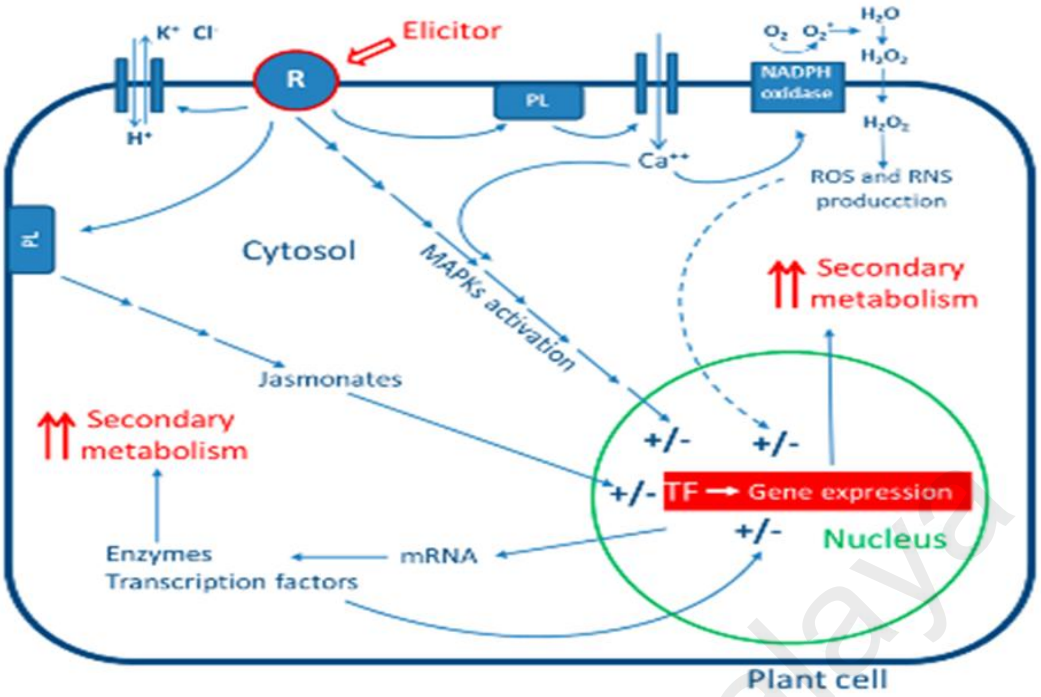


Figure 2.8: Representation diagram of the cells responses to elicitation (Ramirez-Estrada et al., 2016)

R: **Receptor**; PL: **Phospholipase**; MAPKs: **Mitogen Activated Protein Kinases**; ROS: **Reactive Oxygen Species**; RNS: **Reactive Nitrogen Species**; TF: **Transcription Factors**

2.4.1 Gamma ray

Electromagnetic radiation (EM) including are gamma rays, X-rays, visible light, and UV. EM radiation varies in energy and hence in frequency. In the last decade, radiation of gamma-ray had drawn consideration as a fast and new improvement techniques to qualitative and the quantitative nature of plants. Gamma rays with an energy level of around 10 ke V to several hundred-kilo electron volts are more energetic than other radiation such as alpha and beta rays (Kovacs & Keresztes, 2002). This kind of radiations belongs to ionizing radiation and by interaction with other atoms or molecules produce free radicals that can damage or modify essential components of plant cells. They have been reported to involve different the morphology, anatomy, biochemistry, and physiology of plants depending on the irradiation level.

These influences containing of cytological, physiological and genetic inducing in cells and tissue, also changing in cellular structure or metabolism, such as increase of thylakoid membranes, modification in photosynthesis, a variation of the antioxidative system, and gathering of phenolic compounds (Kim et al., 2004; Kovacs & Keresztes, 2002; Wi et al., 2005). Chakravarty and Sen (2001) reported that irradiation of low-dose ionizing on some plants and microorganisms accelerated the propagation, growth and germination rates, activity enzyme, stress resistance, and yields. As an accepted rule, the water radiolysis produces reactive oxygen species (ROS) e.g. hydrogen peroxide (H_2O_2), superoxide anion ($O_2.^-$), hydroxyl radicals (.OH) and singlet oxygen (Kovacs & Keresztes, 2002; Piao et al., 2013). When the concentrations of hydrogen peroxide raised up due to environmental stresses and ionizing radiation, this type of reactive oxygen can leads to cell death (Halliwell, 1974).

There are some reports on the effects of irradiation against fresh vegetables and fruits for finding the ripening delay, control of the pathogen and pests (Niemira et al., 2001; Song et al., 2006). Fan et al. (2003) reported that free generated radicals during irradiation, employing as a stress signal and trigger responses in lettuce, and was increased secondary metabolite synthesis. Kim et al. (2004) reported 10% of survival rate in plants after applying a higher dose of gamma ray (more than the 80 Gy) but the plants died on the fifteenth week. The plant growth was induced at the first gamma-irradiated at 2 to 8 Gy in red pepper seed. Ling et al. (2008) found that plant growth was inhibited at radiation levels above 10 Gy. The gamma ray irradiation increased the total shikonin yields to 400% at 16 Gy (Chung et al., 2006). El-Beltagi et al. (2011) reported the various antioxidant defense enzymes positively correlated with the increased of gamma irradiation at low doses in *Rosmarinus officinalis*.

Biosynthesis of shikonin significantly stimulated the in *Lithospermum erythrorhizon* cell cultures by gamma (Chung et al., 2006). Moreover, cerium per hydroxide in different tissues of pumpkin extremely rose due to gamma irradiation (Wi et al., 2007).

2.4.2 Methyl jasmonate

The family of cyclopentanone compounds which able to control a broad variety of plant responses has two effective elicitors called jasmonic acid (JA) and methyl jasmonate (MeJA) (Thaler et al., 2004; Wasternack, 2007). Methyl jasmonate is a signal transduction molecule and its more active derivative MeJA (Fig 2.9) can induce a wide range production of secondary metabolites such as rosmarinic acid, terpenoid indole alkaloid, plumbagin, flavonoids, and alkaloids in various cell cultures (Almagro et al., 2014; Krzyzanowska et al., 2012; Silja et al., 2014). Jasmonic acid and methyl jasmonate can be produced in response to abiotic stresses in plants. They have been applied as elicitors for biosynthesis of stilbene in *V. vinifera* foliar cuttings (Gangopadhyay et al., 2011) and *V. rotundifolia* hairy root cultures (Belhadj et al., 2006). Supplementing of MeJA to *V. vinifera* on cell culture was improved the synthesis of anthocyanin (Tassoni et al., 2012) and when supplemented to hairy root culture of *Salvia miltiorrhiza* was highly increased tanshinones production (Xiaolong et al., 2015). The production of paclitaxel in *Taxus canadensis* and *T. cuspidate* were stimulated by MeJA (Tassoni et al., 2012) also MeJA elicitation was increased the accumulation of raspberry ketone benzal acetone in *Rubus idaeus* cell culture media (Pedapudi et al., 2000).

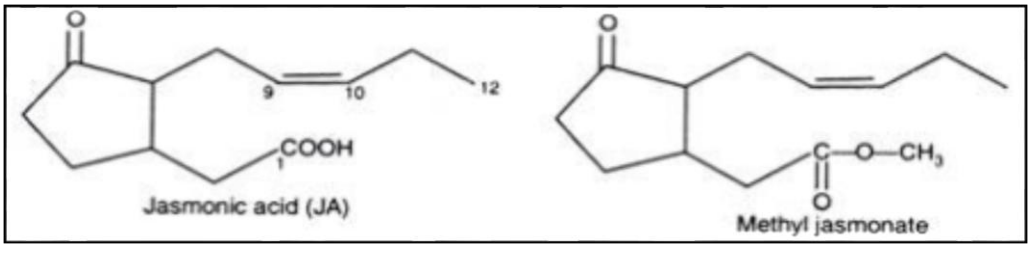


Figure 2.9: Structures of Methyl jasmonate and Jasmonic acid (Kang et al., 2013)

2.4.3 Salicylic acid

Salicylic acid or SA is a mono hydroxybenzoic groups, belongs to phenolic acids category. This small molecule with a key role in plant defense regulatory systems is known to agitated systemic developed resistance (SAR) to many pathogens (Hayat et al., 2010) (Fig 2.10). When SA is utilized as an elicitor to synthesis of secondary metabolism, a quick accretion of SA in the infection site initiates a hypersensitive reaction, during the plant-pathogen interaction. In order to stimulate an extensive range of defense responses, this signal extents to other parts of the plant (Dučaiová et al., 2013; Namdeo et al., 2007). SA is one of the endogenous signals and is involved in several plant defense responses (Sudha & Ravishankar, 2003). It stimulates specific variations in leaf anatomy and chloroplast structure and increased production of secondary metabolite (Sivanandhan et al., 2013). There are many reports of SA elicitation to secondary metabolite production. Treated cell suspension cultures with 20 mg L^{-1} of SA was induced paclitaxel production from *T. chinensis* (Wang et al., 2007). Khosroushahi et al. (2006) reported that different concentrations of SA was improved taxane yields in cell suspension culture of *T. baccata*. SA-elicited was increased production of podophyllotoxin in *Linum album* (Yousefzadi et al., 2010), ginsenoside production in *P. ginseng* via adventitious and hairy roots culture at low doses (Tewari & Paek, 2011) and yield improvement of tanshinones in *S. miltiorrhiza* by hairy roots technique (Xiaolong et al., 2015). Salicylic acid treatment was enhanced alkaloids production such as vincristine and vinblastine in periwinkle (Idrees et al., 2011), the tropane alkaloid scopolamine from *Brugmansia candida* (Pitta-Alvarez et al., 2000), and pilocarpine in jaborandi leaf culture (Avancini et al., 2003). Bulgakov et al. (2002) was reported that SA elicitation was improved the anthraquinone production in *Rubia cordifolia*.

It encouraged accumulation of the triterpenoids ginsenosides in ginseng and glycyrrhizin in licorice (Ali et al., 2006). Shabani et al. (2009) revealed that proper concentrations of SA may promote the monoterpene production.

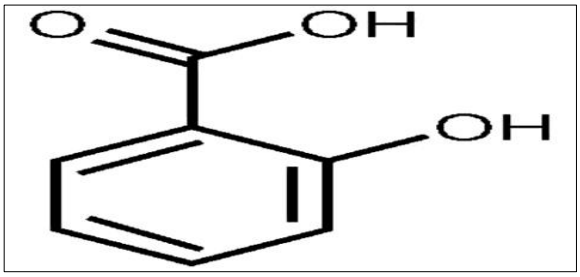


Figure 2.10: Structure of salicylic acid (Bektas and Eulgem, 2014)

CHAPTER 3 : MATERIALS AND METHODS

3.1 Optimization of sterilization procedure and auxin concentrations for callus induction of *Gardenia jasminoides* Ellis leaf explants

3.1.1 Plant materials

The 2-year-old grown field plants of *G. jasminoides* were selected from the UM botanical garden and nurseries. The young and green leaves were excised from healthy, plant for initiation of callus cultures. To remove dust or soil residual, the samples were washed under running tap water, followed by washing in liquid detergent (Teepol, Sigma-Aldrich brand) for 30 minutes.

3.1.2 Optimization of sterilization procedure

Explants were immersed in 70 % (w/v) ethanol for 1 minute. The sterilization was conducted based on Chuenboonngarm et al. (2001) and Sayd et al. (2010). The leaves were disinfected with 25, 50 and 75 % (v/v) sodium hypochlorite (Clorox) plus one drop of Tween-20 per 100 ml (a surfactant) for 10, 15 and 20 minutes. The leaf explants were surface sanitized by dipping in 0.1% (w/v) mercuric chloride ($HgCl_2$) for 1, 3 and 5 minutes. In the final step, explants were rinsed 3 times with sterile distilled water inside the laminar flow bench.

3.1.3 Callus induction study using different auxin concentrations

The sterilized leaf explants were placed into sterile containers (70 ml) containing 30 ml WPM (Lloyd & Mc Cown, 1980) sterilized basal media. The culture media was supplemented with different concentrations (0.5, 1, 2 and 4 mg L^{-1}) of 2,4-D or NAA followed by 30 g L^{-1} sucrose and 3 g L^{-1} Gelrite. The medium was adjusted to pH 5.8 prior to autoclaving. Samples were kept in 16/8 hour light and dark condition at 25 °C. The

light source was a fluorescent tube (40 watts) with 1000 Lux intensity. The callus were monitored daily to record contaminated and the survival explants rate. Percentage of contaminated and survived explants were calculated. Explants with induced callus were counted and fresh weight was recorded.

3.1.4 Experimental design and data analysis

The sterilization and callus induction experiments were carried out as a Completely Randomized Design (CRD). Data were analyzed using one sample Kolmogorov-Smirnov test, to check normality of data prior to data analysis. A factorial experiment with three factors including the concentration of Clorox® (25%, 50% and 75 %), Clorox immersion time (10, 15 and 20 min) and immersion time in 0.1 g L^{-1} mercuric chloride (1, 3 and 5 min) based on a Completely Randomized Design (CRD) was arranged. This experiment consisted of 10 replications with three experimental units. The callus induction experiments were arranged as RCBD (Randomized Completely Block Design) with four different concentrations of 2,4-D and NAA (0.5, 1, 2 and 4 mg L^{-1}), with ten replications and three explants were placed in each container.

3.2 Elicitor effects on accumulation of secondary metabolite (total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity) of *Gardenia jasminoides* Ellis cell culture

This part was aimed to study the effect of various elicitors and harvesting time on callus growth, TPC, TFC, and antioxidant activity in *G. jasminoides* cell culture. Three different harvesting times (35, 70 and 105 days) from irradiated callus by five doses of gamma ray (0, 5, 10, 20, and 40 Gy), seven concentrations of salicylic acid (0, 0.5, 2.5, 5.0, 7.5, 12.5 and 20.0 mg L^{-1}) and six concentrations of methyl jasmonate (0, 0.022, 0.220, 1.120, 2.240, and 4.50 mg L^{-1}) were studied in WPM media supplemented with NAA or 2,4-D (2 mg L^{-1}).

3.2.1 Plant materials and Elicitation

The 2-year-old grown field plants of *G. jasminoides* were selected from the UM botanical garden and nurseries.

3.2.2 Media preparation

Two series of solid WPM media, supplemented with 30 g L^{-1} sucrose, 3 g L^{-1} Gelrite and optimum growth callus concentration of NAA (2 mg L^{-1}) and 2,4-D (2 mg L^{-1}) were prepared (Table 3.1). The media were sterilized by autoclaving at 121°C with a pressure of 15 psi for 15 min. The pH was adjusted to 5.7 prior autoclaving. Two series of WPM media supplemented with NAA and 2,4-D (2 mg L^{-1}) were dispensed into pre-sterilized containers under laminar air flow with the aseptic condition.

3.2.3 Gamma irradiation

Gamma radiation was provided from ^{60}Cobalt (0026 Pool Irradiator with isotope model) located at Physics Department, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia. The amount of radiation energy is measured in SI unit known as Gray (Gy), equal to one joule per Kg (Agency-IAEA, 1990). The dose rate is the quantity of radiation absorbed per unit time, which was calculated in Appendix 1. Gamma radiation used for this experiment was fixed at doses of 5, 10, 20 and 40 Gray (Gy). The dose rate was calculated on 0.0505 Gy $second^{-1}$ (based on the time the experiment at December 2013). Therefore, each exposure of the gamma radiation was fixed at 99, 198, 396, and 792 seconds. The leave explants as described in plant materials section (3.2.1), were packed in paper bags and were irradiated separately at 1.5 min (5 Gy), 3.3 min (10 Gy), 6.6 min (20 Gy), and 13.2 min (40 Gy). The radiated leaves were carefully wrapped in paper and aluminum foil and immediately were transferred to a tissue culture laboratory for sterilization step.

The four packs of gamma radiated leaves in various dosages followed by one pack control (non-radiated) were washed with tap water and sterilized based on the described method in the sterilization technique (section 3.1.2) and were cultured on WPM media supplemented with 2,4-D (2 mg L^{-1}), or NAA (2 mg L^{-1}). The sterilized radiated and non-radiated leaf explants was excised to 0.5 × 0.5 cm and placed into sterile containers (70 ml), containing 30 ml WPM (Lloyd & Mc Cown, 1980) supplemented with different concentrations of 2,4-D (2 mg L^{-1}) and NAA (2 mg L^{-1}) followed by 30 g L^{-1} sucrose and 3 g L^{-1} Gelrite. The pH of the medium was adjusted to 5.8 prior to autoclaving. Samples were kept in 16/8 hour light and dark condition at 25 °C. The light source was a fluorescent tube (40 watts) with 1000 Lux intensity. The daily monitoring to record contaminated and survival explants was carried out. The callus was separated and subcultured every 40 days into the same media for further experiments. After the third passage, fresh *in vitro* callus from each group of radiated and control explants were excised into small pieces (30 × 30 mm) of equal size (20 ± 1 mg) and transferred into 150 ml jam jar containing 40 ml of the same media and plant growth regulators concentrations. The cultures were grown at 25°C for a period of 8 h dark and 16 h light in a controlled culture room. Callus were harvested at 35, 70 and 105 days for further studies.

3.2.4 Methyl jasmonate elicitation

According to the results of callus growth experiment, WPM media supplemented with 2,4-D (2 mg L^{-1}) or NAA (2 mg L^{-1}), Gelrite (3 g L^{-1}) and 3% of sucrose was selected. The medium was adjusted to pH 5.8 and sterilized by autoclaving at 121°C with a pressure of 15 psi for 15 min. Methyl jasmonate (MeJA) was purchased from Sigma-Aldrich, (USA) and the stock solution was prepared at 10 mg L^{-1} concentration in ethanol. It was sterilized by passing through a 0.22 µm filter before addition to the autoclaved culture medium in six various concentrations (0, 0.022, 0.220, 1.120, 2.240 and 4.500 mg L^{-1})

under an aseptic condition in laminar air flow cabinet. Fresh *in vitro* callus with equal size (20 ± 10 mg) from non-irradiation leaf explants were cut into small pieces (30 × 30 mm) and transferred into 150 ml jam jar containing 40 ml of WPM media supplemented with 2 mg L^{-1} 2,4-D or 2 mg L^{-1} NAA and various concentrations of MeJA. The cultures were grown at 25 °C for a period of 8 h dark and 16 h light in a controlled culture room. Callus were harvested at 35, 70 and 105 days for further studies. The fresh weight of callus at culturing time was recorded to remove its effect from the final weight and growth as a covariate.

3.2.5 Salicylic acid elicitation

A stock solution of salicylic acid or SA as 10 g L^{-1} (Sigma-Aldrich, USA) was prepared by dissolving SA in 20% ethanol and the pH was adjusted with 10 N NaOH to 5.8. The solution was sterilized by passing through a 0.22 µm filter and added to the media in seven different concentrations (0, 0.50 mg L^{-1}, 2.50 mg L^{-1}, 5.00 mg L^{-1}, 7.50 mg L^{-1}, 12.50 mg L^{-1} and 20.00 mg L^{-1}) under aseptic condition in laminar air flow cabinet. Fresh *in vitro* callus with equal size (20 ± 10 mg) from non-irradiation leaf explants were cut into small pieces (30 × 30 mm) and transferred into 150 ml jam jar containing 40 ml of WPM media supplemented with 2 mg L^{-1} 2,4-D or 2 mg L^{-1} NAA and various concentrations of SA. The cultures were grown at 25°C for a period of 8 h dark and 16 h light in a controlled culture room. Callus were harvested at 35, 70 and 105 days for further studies. The fresh weight of callus at culturing time was recorded to remove its effect from the final weight and growth as a covariate.

Table 3: Various doses and concentrations of gamma ray, MeJA and SA

Gamma ray (Gy)	MeJA (mg L^{-1})	SA (mg L^{-1})
0	0.000	0.00
5	0.022	0.50
10	0.220	2.50
20	1.120	5.00
40	2.240	7.50
	4.500	12.50
		20.00

3.3.6 Determining callus growth rate

The explants with inducing callus were counted. Drying callus was carried out in the oven at 50 °C (Hussein et al., 2010; Sahraroo et al., 2014) to determine callus dry weight. The callus growth rate (CGR) was calculated using the following equation:

$$CGR = \frac{(W2 - W1)}{d}$$

Where: W_1 is the weight at culture time, W_2 is the fresh weight at harvest time, and d is the culture period.

3.3 Experimental design and data analysis

3.3.1 Gamma ray elicitation experiment design

This part of experiment with five various doses of gamma ray (0 or control, 5 Gy, 10 Gy, 20 Gy and 20 Gy), two type of auxin (2 mg L^{-1} of NAA and 2 mg L^{-1} of 2, 4-D) and three harvesting time (35, 70, and 105 days) was arranged as a factorial (with three factors and thirty treatments) in Randomized Complete Block Design (RCBD) with four replications; four explants were placed in each container.

3.3.2 Methyl jasmonate elicitation experiment design

This study comprised six various different concentrations of MeJA (0 or control, 0.022, 0.220, 1.120, 2.240, and 4.500 mg L^{-1}), 2 mg L^{-1} of NAA or 2 mg L^{-1} of 2,4-D and three harvesting time (35, 70, and 105 days) was arranged as a factorial (with 3 factors and 36 treatments) with 4 replications in RCBD design; four explants were placed in each container.

3.3.3 Salicylic acid elicitation experiment design

This study comprised seven various different concentrations of SA (0 or control, 0.5 mg L^{-1}, 2.5 mg L^{-1}, 5 mg L^{-1}, 7.5 mg L^{-1}, 12.5 mg L^{-1} and 20 mg L^{-1}) two types of auxin (2 mg L^{-1} of NAA or 2 mg L^{-1} of 2,4-D) and three harvesting times (35, 70, and 105 days) was arranged as a factorial (with 3 factors and 42 treatments) in RCBD design with 4 replications; four explants were placed in each container.

3.3.4 Statistical analysis

The data were subjected to one sample Kolmogorov-Smirnov test to check normality of prior to data analysis. The arcsine transformation was applied for percentage and proportion data. Analysis of variance (ANOVA), post hoc test (Duncan multiple range test), and Pearson correlation coefficient were evaluated using SAS version 9.1.

3.4 Cell culture extraction

The dried callus from different treatments was separately ground using mortar and pestle. The water as a solvent was enhanced secondary metabolites in *G. jasminoides* Ellis extract (Oniszczuk & Hawry, 2010; Wang et al., 2012; Wang et al., 2015; Xu et al., 2016). A stock solution of extracts (100 g L^{-1}) was prepared using 1 g of finely powdered callus from each group in 10 mL distilled water.

The soaked material was shaken at 90 rpm overnight, and extracts solutions were filtrated using Whatman filter papers no. 2 after 24 h at room temperature. The callus extracts (100 g L^{-1}) of irradiated samples were kept in covered containers and stored at 4ºC until further experimental analysis.

3.5 Total phenolic content

The total phenolic content (TPC) assay of water extract was determined by Folin-Ciocalteu method (Lewis, 2012) and Gallic acid was set as standard. From each extract samples, standard sample, and distilled water as blank 100 µ L were poured into 2 ml microtubes. The amount of 200 µ L of F-C reagent 10% (v/v) was added to all tubes and vortexed thoroughly. After 2 minutes, an 800 µL of the 700 mM sodium carbonate (Na_2CO_3) was added to each tube and mixed well. The assay tubes were incubated at room temperature for 2 hours and then the absorbance of the resulting blue color was measured at 765 nm using UV-VIS Spectrophotometer Model Libra SII. The data were recorded based on three replications. The total phenolic content was expressed as mg Gallic Acid Equivalent or GAE (standard curve was prepared using concentrations of 0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mg L^{-1}) (Lewis, 2012) (Appendix 2).

3.6 Total flavonoid content

The total flavonoid content (TFC) via the aluminum chloride colorimetric protocol (Zhishen et al., 1999) and using Rutin as a standard on the same extract concentrations was evaluated. An aliquot (1 ml) of each extract and standard solutions were added to 10 ml volumetric flask containing 4 ml of distilled water and 0.3 ml 5% $NaNO_2$ was added to flasks. After five minutes, 0.3 ml 10 % AlCl3 was added. The aliquot was incubated five minutes and 2 ml 1 M NaOH was added. The volume was made up to 10 ml with distilled water.

The solutions were mixed, and absorbance was measured against the blank at 510 nm using UV-VIS Spectrophotometer Model Libra SII. The total flavonoid content was expressed as mg Rutin Equivalents (RE). A standard curve was prepared using concentrations of 0, 0.01, 0.05, 0.10, 0.20, 0.40, 0.80 and 0.10 mg L^{-1} (Appendix 3).

3.7 DPPH radical scavenging assay

The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) has been widely applied to free radical scavenging capability of antioxidants evaluation. The free radical DPPH (purple color) is reduced to the corresponding hydrazine (yellow color) when it responds to the hydrogen donor. This method can make stable free radicals in aqueous solution. Furthermore, it is possible to determine the antiradical power of an antioxidant activity by measurement of the decrease in the absorbance of DPPH. The stable 1,1-diphenyl-2-picryl hydroxyl radical (DPPH) was used for determination of free radical-scavenging activity of the same concentration of callus samples from irradiated leaf extracts (Ebrahimzadeh & Bahramian, 2009). An aliquot of different extract (0.1 g L^{-1}) from different extracts and groups were prepared and added at an equal volume to a methanolic solution of DPPH (0.150 mM). An aliquot (1 ml) of DPPH and solvent were used as standard controls for each observation. The absorbance was recorded at 517 nm after 15 min incubation in dark at room temperature. The experiment was repeated three times. The antioxidant activity (AA) was determined using the following formula:

$$AA\% = (Abs_0 - Abs_1) * 100$$

Where Abs_1 is the absorption of the sample extracted with DPPH, and Abs_0 is the absorption of control. Distilled water was used as a blank for calibration. Spectrophotometric measurements were performed using UV-VIS spectrophotometer Model Libra SII.

CHAPTER 4 : RESULTS

4.1 Sterilization and optimization of auxin concentration for callus induction

4.1.1 Sterilization protocol

This study was conducted to determine the survival rate of explants and optimize the sterilization protocol. The data were collected after 45 days and mean comparison was carried out using ANOVA followed by Duncan's multiple range test (DMRT). The one sample Kolmogorov-Smirnov test prior to data analysis was applied to check the normal distribution of the data. Arcsine transformation to analyze the percentage of callus induction was used. Based on this study, more than 90% of treated explants by 25%, 50%, and 75% of sodium hypochlorite at 15 and 20 min immersion time turned brown and died. However, only sterilized leaf explants at 10 min immersion time in sodium hypochlorite were survived and analyzed. Arcsin function was transformed the percentage of contaminated and survival explants before analysis of variance.

The sodium hypochlorite concentrations, exposure time of mercuric chloride and the interaction between these factors showed a significant variation on leaf explants sterilization (Appendix 4). The highest contamination rate (95%) was observed when leaf explants were dipped in Clorox 25% (10 min) and then was immersed by 0.1 g L^{-1} mercuric chloride (1 minutes). However, the lowest contamination rate (2%) was recorded when explants were submerging in Clorox 75% (10 min) and were sinking in 0.1 g L^{-1} mercuric chloride (5 minutes). The sterilization revealed that 10 min immersion in Clorox 75% followed by 3 minute soaking in $HgCl_2$ (0.1 g L^{-1}) resulted in lower contamination rate (8%) and showed the highest survival rate of explants (73%); therefore this protocol has been selected as sterilization method for further experiment (Fig 4.1).

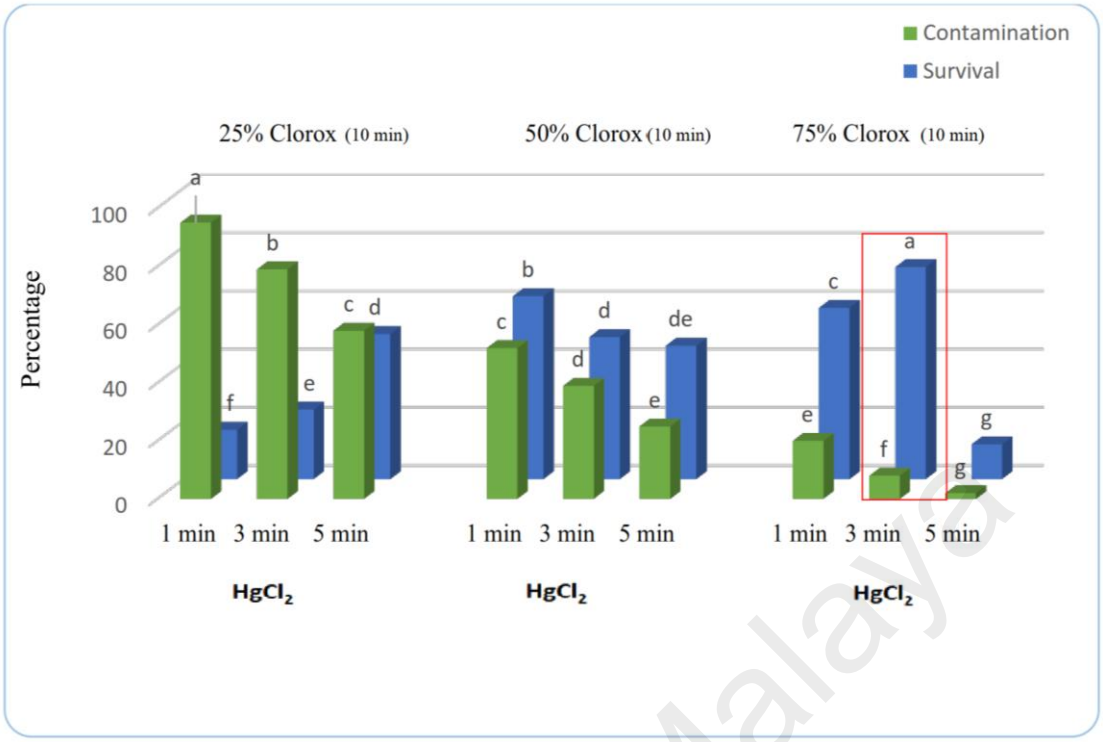


Figure 4.1: Survival and the contamination rate of sterilized leaf explants of *G. jasminoides* using various concentrations of Clorox in combination with different times of $HgCl_2$. Means with the same letter are not significantly different at 0.01 levels

4.1.2 Optimization of auxin concentrations

The leaf explants of *G. jasminoides* did not grown callus in WPM hormone-free media. As shown in figure 4.2, callus was formed at the explants edge after 20 and 23 days of culture on WPM media supplemented with NAA (2 mg L^{-1}) or 2,4-D (2 mg L^{-1}), respectively.

Figure 4.2: Callus induction on NAA (Left) and 2,4-D (right)

Based on the mean comparison results, 100% and 97% callus induction on 2 mg L^{-1} of 2,4-D and 2 mg L^{-1} of NAA was significantly differenced with other concentrations (Fig 4.3, Fig 4.4). To check embryogenic cells in induced callus, double staining test was applied. The results revealed embryogenic cells in callus growth on WPM supplemented with NAA or 2,4-D (Fig 4.5).The number of leaves that induced callus and the weight of excised callus was recorded. The data analysis showed significant difference on the callus formation and the fresh weight among different concentrations of 2,4-D (Fig 4.6) and NAA (Fig 4.7). However, no difference was found between NAA and 2,4-D (Appendix5-7).

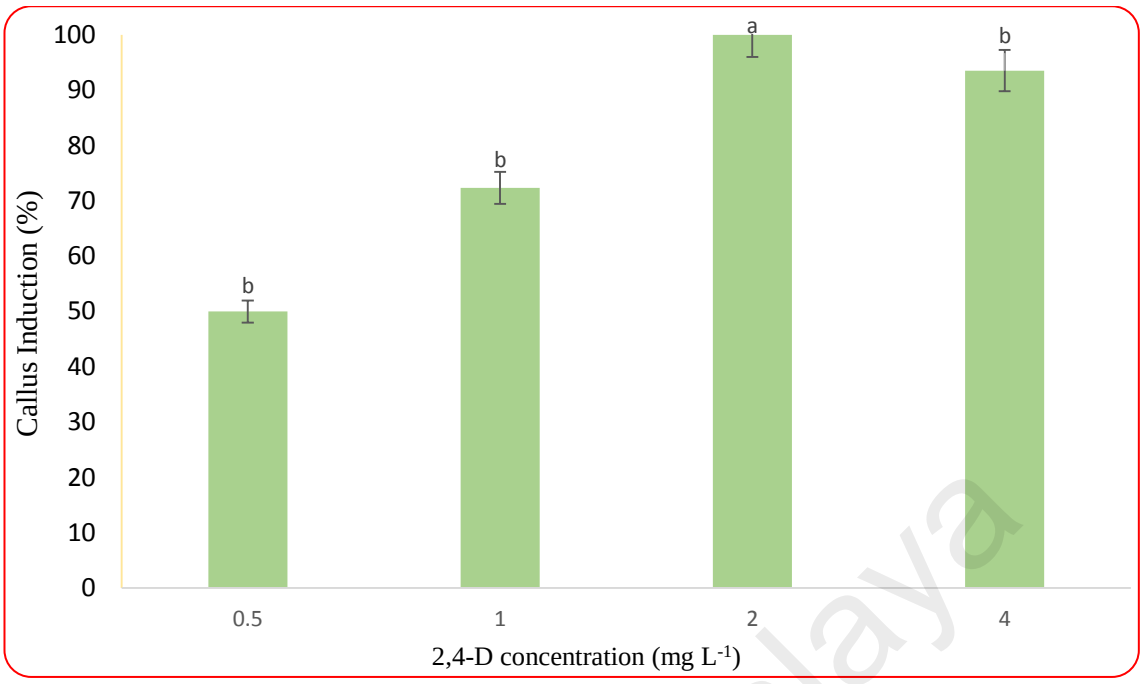


Figure 4.3: Percentage of callus induction in leaf explants of *G. jasminoides* using different concentrations of 2,4-D addition in WPM media. Means with the same letter are not significantly different at 0.01 levels

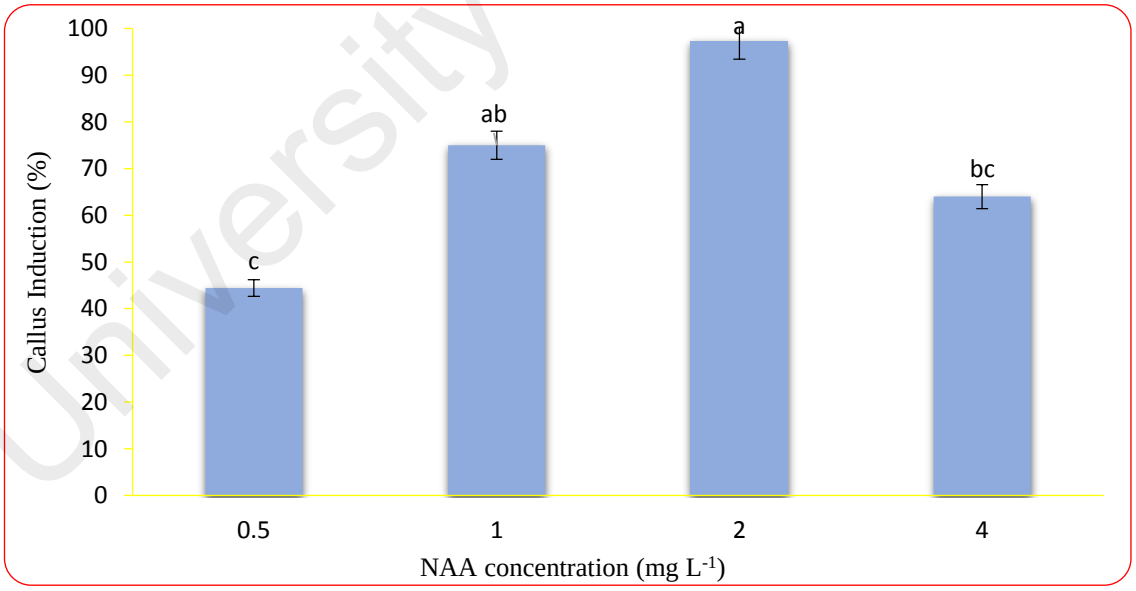


Figure 4.4: Percentage of callus induction in leaf explants of *G. jasminoides* using different concentrations of NAA addition in WPM media. Means with the same letter are not significantly different at 0.01 levels.

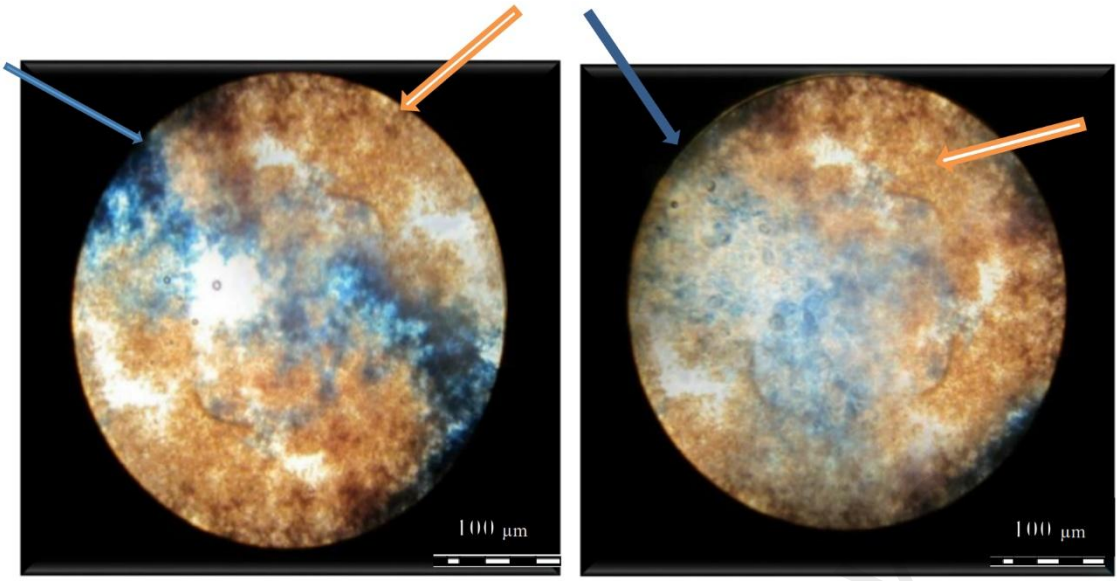


Figure 4.5: Early embryos stage from growth callus on WPM supplemented with 2,4-D (left) and NAA (right) after double staining test, embryonal heads stained orange (acetocarmine) and suspensors stained blue (Evan's blue).

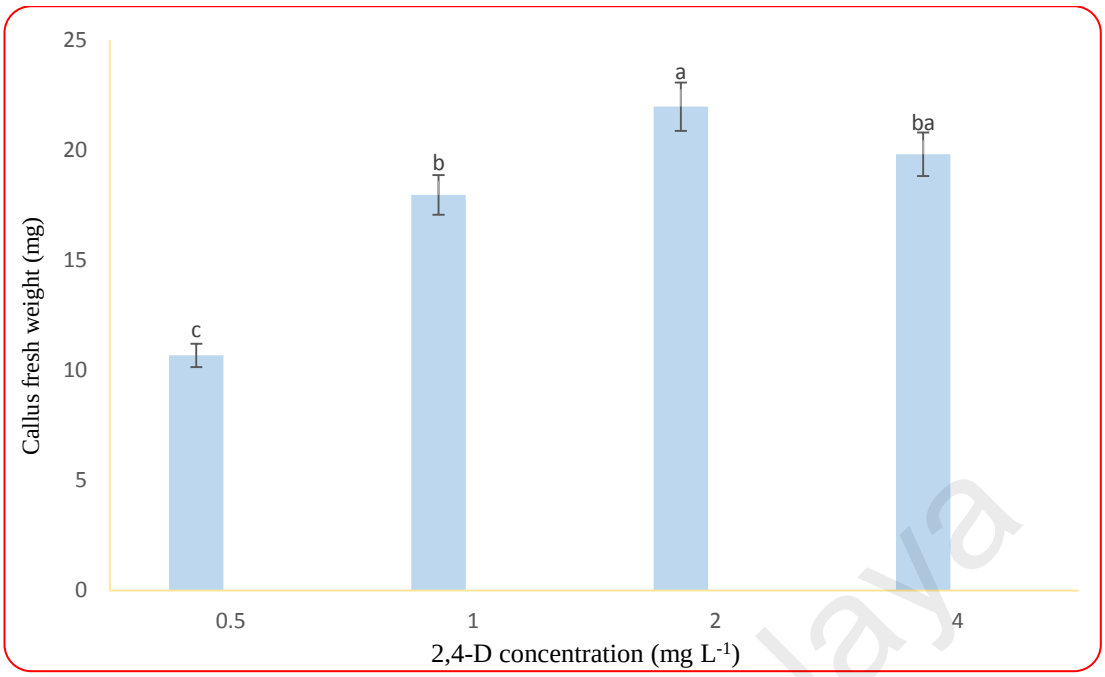


Figure 4.6: Callus fresh weight leaf explants of *G. jasminoides* induced on WPM media supplemented with various concentrations of 2, 4-D. Means with the same letter are not significantly different at 0.01 levels.

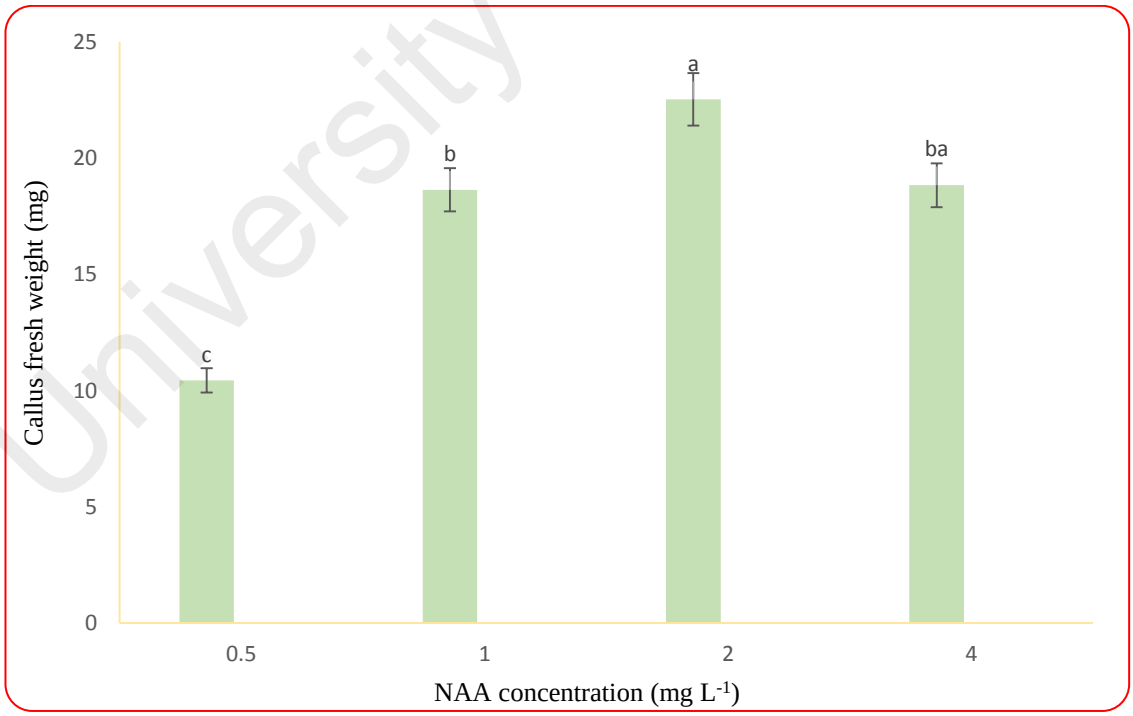


Figure 4.7: Callus fresh weight leaf explants of *G. jasminoides* induced on WPM media supplemented with various concentrations of NAA. Means with the same letter are not significantly different at 0.01 levels

4.2 Effect of gamma ray on callogenesis and callus growth in different auxin from leaf explants of *Gardenia jasminoides* Ellis

The friable and pale yellow callus was formed at lower and higher dosages of gamma ray (Fig 4.8). Based on the one-sample Kolmogorov-Smirnov test all the data were normally distributed. Some greenish color was observed on leaf explants subjected to 5 Gy (Fig 4.9). The primary weight of callus was considered as a covariate to prevent its possible effect in the result due to unequal callus weight at the induction time.

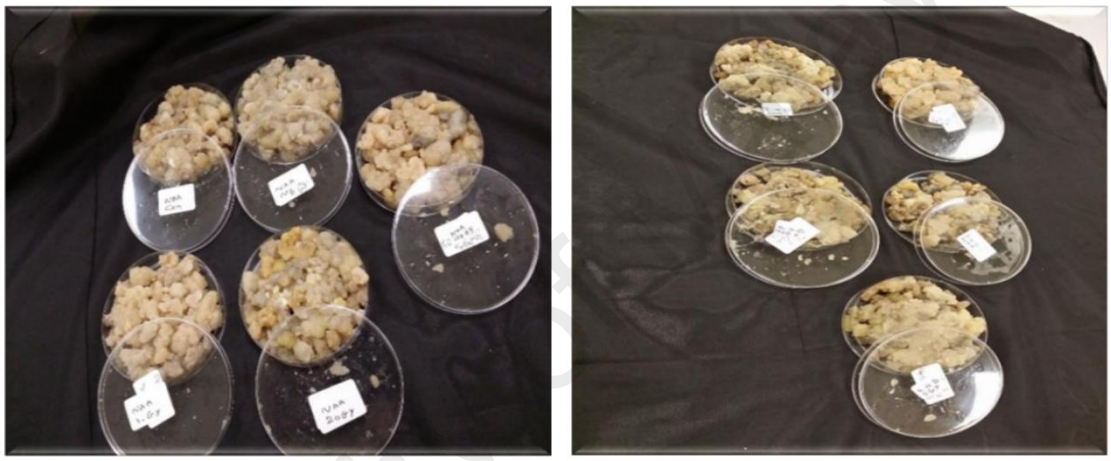


Figure 4.8: Callus induction from irradiation leaf explants of *G. jasminoides* Ellis on WPM Supplemented with 2,4-D (right) and NAA (left)

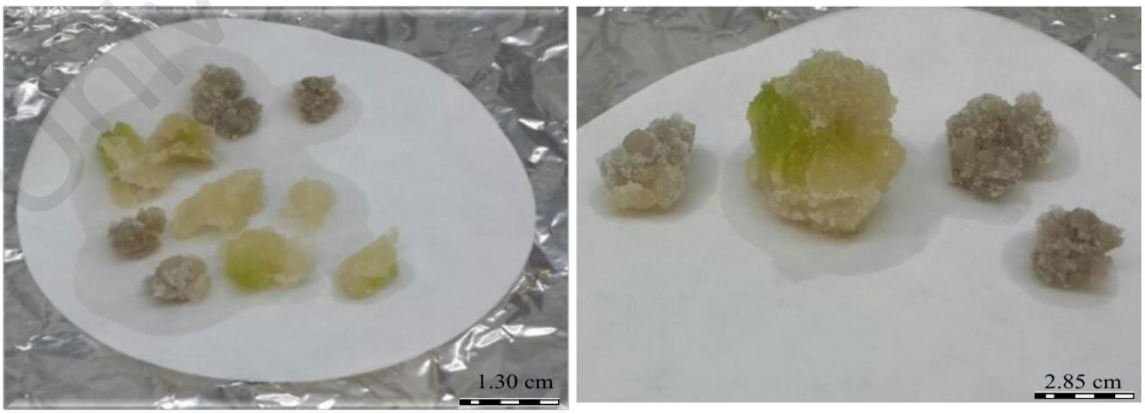
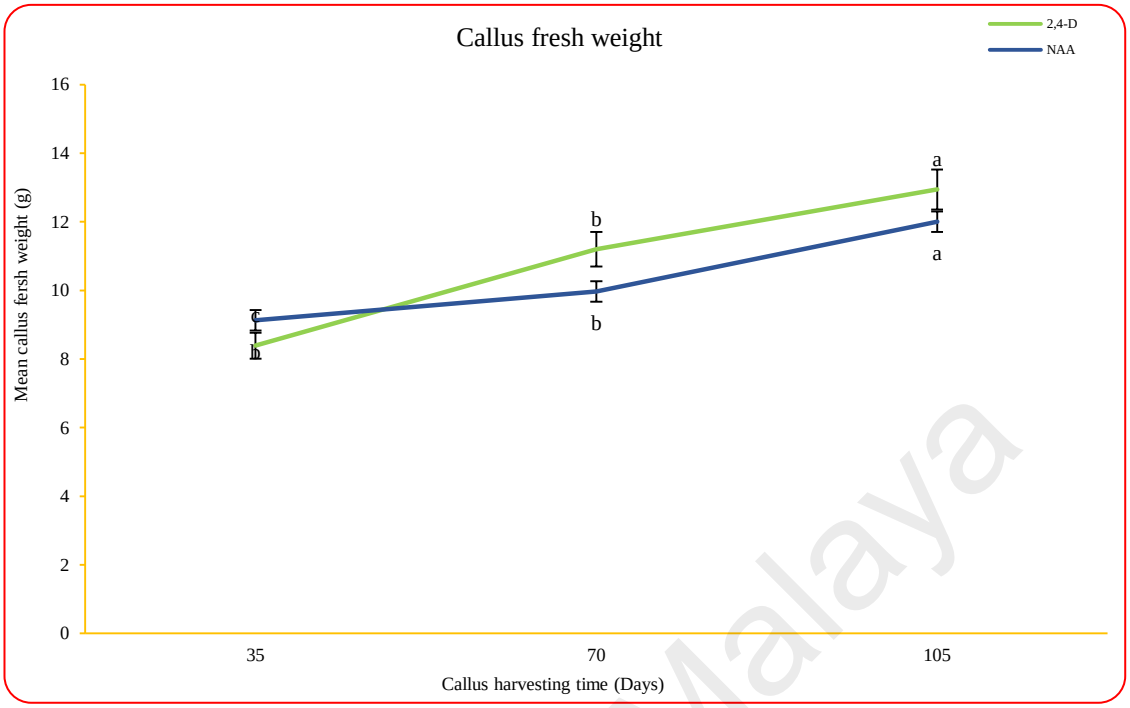


Figure 4.9: Greenish callus from leaf explants of *G. jasminoides* Ellis exposed to 5 Gy ofgamma ray, cultured on WPM supplemented with 2 mg L^{-1} 2,4-D (right) and 2 mg L^{-1} NAA (Left).

The leaf explants subjected to various doses of gamma and callus harvesting times showed a significant difference in growth. Based on collected data and callus development pattern, irradiation explants with high dose (40 Gy) and non-irradiation groups (Control) of gamma were inhibited growth cycle. Furthermore, none of the gamma doses have been displayed with a lethal power on callus culture of this species. Analysis of variance showed harvesting time significantly affected by callus fresh weight (FW), dry weight (DW) and callus growth rate (CGR) on gathered calli from 2,4-D and NAA at 0.01 and 0.05. Apart from the control treatments where its explants were not irradiated, the other explants were subjected to four doses of irradiation (5, 10, 20 and 40 Gy). All the explants were survived after irradiation until 105 days. Callus induction rate and callus fresh weight were increased in irradiated explants at 5, 10 and 20 Gy compared to non-irradiation (control), but these rates were dropped at higher radiation (40 Gy).

The higher doses of gamma ray may have given other alterations that initiated necrosis on the callus. The harvesting times (35, 70 and 105 days) of callus from radiated leaf explants by gamma ray (0, 5, 10, 20 and 40 Gy) cultured on WPM media supplemented with 2,4-D or NAA, showed various callus growth rate (CGR), fresh and dry weight at 0.05 and 0.01 levels. As displayed in figure 4.10, callus age was significantly affected on fresh and dry weight of callus. However, callus fresh weight from irradiation explants on 2,4-D showed almost sigmoid curve but grown irradiated callus on NAA revealed no difference in fresh weight on 35 and 70 days.

a)



b)

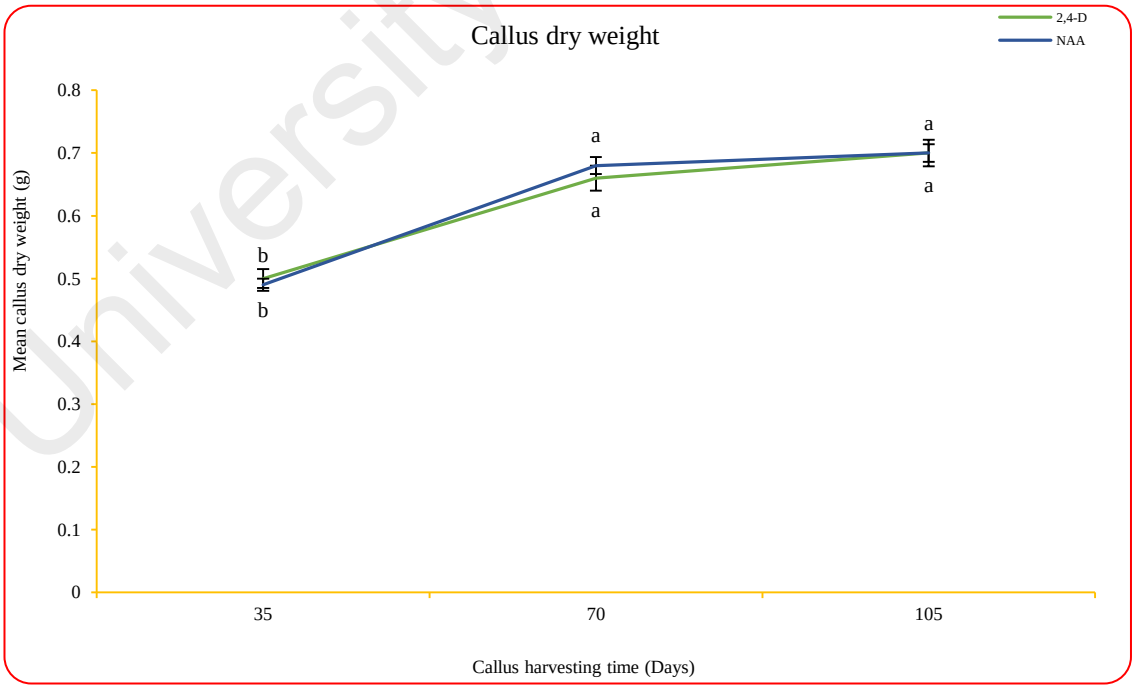
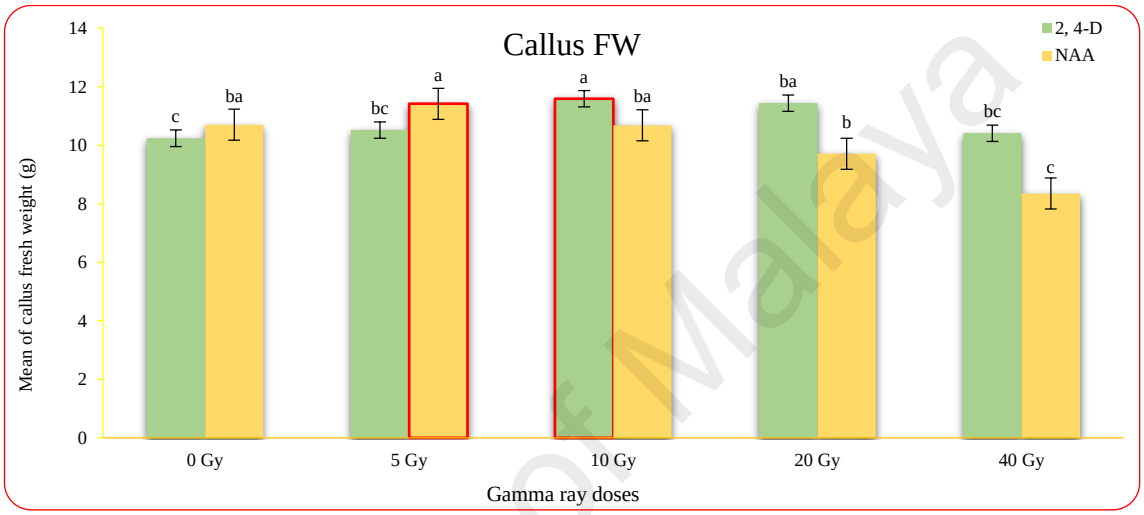


Figure 4.10: Effect of harvesting time on fresh (a) and dry (b) weight of irradiated leaf explant of *G. jasminoides* induced on WPM media supplemented with 2,4-D and NAA. Means with the same letter are not significantly different.

Callus dry weight also showed similar pattern on the obtained callus from 2,4-D and NAA. The difference was detected between first harvesting stage (35 days) and the other collection times (70 and 105 days). The significant differences in various doses of gamma ray on fresh and dry weight of callus on both auxin was observed (Fig 4.11).

a)



b)

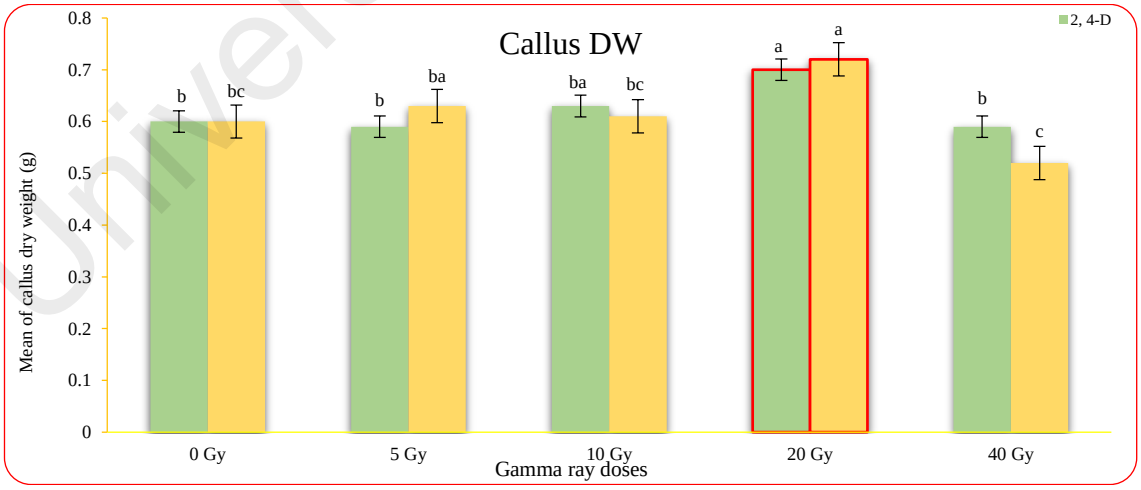


Figure 4.11: Effect of gamma ray on a fresh weight (a) and dry weight (b) of irradiated leaf explant of *G. jasminoides* induced on WPM media supplemented with 2,4-D and NAA. Means with the same letter are not significantly different

The amounts of adjusted R^2 showed the high goodness of fit for the models. (Table 4.1, Table 4.2). According to ANOVA, no differences were observed from the interaction between gamma irradiation and harvesting time on the fresh and dry weight of callus on 2,4-D and NAA (Appendix 8, Appendix 9).

Table 4.1: Summary of ANOVA for fresh weight, dry weight, and callus growth rate of callus from irradiated leaf explants of *G. jasminoides* with different gamma ray doses, growth in WPM medium supplemented with 2,4-D (2 mg L^{-1})

Source	FW	DW	CGR
Time	184.85^{**}	0.42^{**}	0.08^{**}
Gamma ray	8.30^{*}	0.05^{*}	0.02^{*}
Time × Gamma ray	2.50^{ns}	0.01^{ns}	0.01^{ns}
CV%	14.77	22.00	17.00
R^2	0.88	0.93	0.96

^{ns} Not significant * significant at 0.05 level ** significant at 0.01 level

Table 4.2: Summary of ANOVA for fresh weight, dry weight, and callus growth rate of callus from irradiated leaf explants of *G. jasminoides* with different gamma ray doses, growth in WPM medium supplemented with NAA (2 mg L^{-1})

Source	FW	DW	CGR
Time	88.61^{**}	0.47^{**}	0.18^{**}
Gamma ray	29.50^{**}	0.11^{**}	0.01^{*}
Time × Gamma ray	6.80^{*}	0.01^{ns}	0.04^{*}
CV%	16.41	24.50	20.21
R^2	0.83	0.96	0.95

^{ns} Not significant * significant at 0.05 level ** significant at 0.01 level

In order to determine the significant difference between treatments, analysis of variance by SAS version 9.1 was applied. The variation between means were calculated and compared by DMRT. Data are reported as means ± standard deviations (Table 4.3, Table 4.4). The callus age affected on callus fresh weight (FW) and the highest mean was recorded at the last stage of the harvesting period (105 days) on the WPM media by adding 2 mg L^{-1} 2,4-D or 2 mg L^{-1} NAA. The fresh weight with a significant difference were weighed out (14.09 ± 0.42, 13.87 ± 0.39 g) from exposed explants to gamma ray at doses 10 and 5 Gy on 2,4-D and NAA, respectively. The higher dose of gamma ray (40 Gy) was dramatically dropped FW to 8.05 ± 0.58 and 7.22 ± 0.67 g at the early stage of 35 days from 2,4-D and NAA. The highest dry weight from exposed explants at 20 Gy was collected on 2,4-D (0.79 ± 0.16) and NAA (0.87 ± 0.12) with a statistical difference at the days of 105, respectively. In addition, no difference was recorded between non-irradiate as control and irradiated explants (5, 10 and 20 Gy) on 2,4-D harvested after 35 days.

Dry weight was dropped on those treatments subjected to 40 Gy and harvested at 105 days on 2,4-D and NAA. The lowest DW was recorded in subjected explants to 40 Gy gamma ray when harvested at days 35, on 2,4-D (0.43 ± 0.03) and NAA (0.45 ± 0.03 g). Based on Duncan's multiple range test, no differences were observed in callus dry weigh among all non-radiation (control) and irradiation doses, at the day of 70.

Table 4.3: Mean comparison of callus fresh (g) weight from irradiated leaf explants of *G. jasminoides* by various doses of gamma ray cultured on solid WPM medium supplemented with 2 mg L^{-1} 2,4-D or NAA harvested at different times.

Time (Days)	**Gamma ray** (Gy)	2,4-D	NAA
35	0	8.53 ± 0.56^{de}	8.53 ± 0.25^{de}
	5	8.74 ± 0.23^{de}	8.94 ± 0.50^{de}
	10	8.91 ± 0.42^{de}	8.89 ± 0.36^{de}
	20	9.10 ± 0.49^{de}	9.90 ± 0.71^{bcd}
	40	8.05 ± 0.58^{e}	7.22 ± 0.67^{e}
70	0	10.45 ± 0.54^{cd}	10.74 ± 0.41^{cd}
	5	10.57 ± 0.31^{cd}	10.43 ± 1.16^{cd}
	10	12.59 ± 0.38^{ab}	10.99 ± 1.16^{cd}
	20	12.00 ± 0.68^{bc}	11.68 ± 0.99^{bc}
	40	10.38 ± 0.88^{cd}	10.73 ± 0.99^{cd}
105	0	12.25 ± 0.86^{abc}	11.84 ± 0.32^{abc}
	5	12.24 ± 0.42^{abc}	$\mathbf{13.87 \pm 0.39^{a}}$
	10	$\mathbf{14.09 \pm 0.42^{a}}$	12.65 ± 0.35^{bc}
	20	13.22 ± 0.60^{b}	12.53 ± 0.24^{ab}
	40	12.90 ± 1.21^{ab}	11.11 ± 0.39^{bc}

Means with the same letter are not significantly different.

Table 4.4: Mean comparison of callus dry weight (g) from irradiated leaf explants of *G. jasminoides* by various doses of gamma ray cultured on solid WPM medium supplemented with 2 mg L^{-1} 2,4-D or NAA harvested at different times.

Time (Days)	**Gamma ray** (Gy)	2,4-D	NAA
35	0	0.49 ± 0.03^{ef}	0.47 ± 0.03^{ef}
	5	0.51 ± 0.02^{def}	0.48 ± 0.02^{def}
	10	0.48 ± 0.03^{ef}	0.49 ± 0.02^{def}
	20	0.56 ± 0.03^{cdef}	0.56 ± 0.03^{bcdef}
	40	0.45 ± 0.03^{f}	0.43 ± 0.03^{f}
70	0	0.61 ± 0.05^{abdef}	0.62 ± 0.06^{bcd}
	5	0.62 ± 0.02^{bcde}	0.70 ± 0.08^{abc}
	10	0.70 ± 0.04^{abc}	0.66 ± 0.05^{bcde}
	20	0.75 ± 0.05^{ab}	0.74 ± 0.09^{ab}
	40	0.61 ± 0.02^{abdef}	0.60 ± 0.09^{bcdef}
105	0	0.66 ± 0.03^{abcd}	0.68 ± 0.02^{bc}
	5	0.63 ± 0.04^{abcde}	0.71 ± 0.02^{abc}
	10	0.71 ± 0.04^{abc}	0.70 ± 0.05^{abc}
	20	$\mathbf{0.79 \pm 0.16^{a}}$	$\mathbf{0.87 \pm 0.12^{a}}$
	40	0.72 ± 0.03^{abc}	0.54 ± 0.03^{cdef}

Means with the same letter are not significantly different.

4.3 Effect of gamma irradiation on secondary metabolite activities (total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity) from leaf explants of *Gardenia jasminoides* Ellis

This part of the research was carried out to study total flavonoids, phenolic contents and antioxidant activity which derived from exposed leaves of *Gardenia jasminoides* Ellis to gamma ray as an abiotic elicitor. The Kolmogorov-Smirnov test showed all variables were normally distributed and the amount of R-squared showed a high goodness of fit for the models (Table 4.5, Table 4.6).

Aside from no difference between interaction of gamma ray and callus age in TPC amount from grown callus on the 2,4-D, the other related variables showed significant difference at 0.01 levels (Appendix 10, Appendix 11). To find the differences between main effects of three harvesting times and various gamma doses, DMRT was applied. Based on the results in Appendix 12-a, three harvesting times showed individual effects on TPC, TFC and antioxidant activities. Gathering callus at the first stage (35 days) was displayed with significant difference compared to 70 and 105 days on 2,4-D and NAA. However, the subjected explants to various doses of gamma ray showed individual responses. Among the different doses of gamma ray, irradiation at 20 Gy showed the higher effect on TFC, TPC, and antioxidant activities on 2,4-D and NAA. The differences between means of secondary metabolite activities from induced callus on WPM media supplemented with 2,4-D (Table 4.7) and NAA (Table 4.8) were analyzed and compared by DMRT.

Table 4.5: Summary of ANOVA for total phenolic, total flavonoid contents and antioxidant activity in water extract of callus from irradiated leaf explants of *G.jasminoides* grown in WPM supplemented with 2 mg L^{-1} 2,4-D and harvested in different time

Source	TPC	TFC	Antioxidant
Time	1.5**	4.428**	1057.82**
Gamma ray	0.41**	0.41**	954.83**
Time × Gamma ray	0.023ns	0.02**	6.84**
CV%	2.77	0.81	0.92
R^2	0.90	0.999	0.998

ns Not significant, **Significant at 0.01 level

Table 4.6: Summary of ANOVA for total phenolic, total flavonoid contents and antioxidant activity in water extract of callus from irradiated leaf explants of *G.jasminoides* grown in WPM supplemented with 2 mg L^{-1} NAA and harvested in different time

Source	TPC	TFC	Antioxidant
Time	1.7**	3.94**	1301.38**
Gamma ray	0.63**	0.37**	973.98**
Time × Gamma ray	0.021**	0.025**	6.62**
CV%	0.41	0.52	1.05
R^2	0.998	0.999	0.998

**Significant at 0.05 level

4.3.1 Total phenolic content (TPC)

Total phenolic content was calculated to equivalent mg of gallic acid in 1 g dry weight. Based on the ANOVA (Appendix 10-a and Appendix 11-a), the interaction between gamma ray and callus age was not significantly affected TPC on 2,4-D grown callus (Table 4.7); however, Duncan's multiple range test showed significant difference of TPC level from grown callus on NAA (Table 4.8). Total phenolic at the day 35 showed higher level on all treatments subjected to various doses of gamma ray.

The higher amount of TPC was recorded at 85.23 and 83.67% on 2,4-D (5.56 ± 0.06 Eq GA g) and NAA (5.49 ± 0.06 Eq GA g) when exposed at 20 Gy gamma ray and harvested in the early stage (35 days). Control explants was revealed at 80% on both 2,4-D and NAA. Based on the results, a lower amount of TPC (50%) was obtained from irradiation by higher dose (40 Gy) and its callus harvesting at the final stage (105 days). In addition, the irradiation treatments by all doses of gamma ray displayed various responses for TPC compared to control (Fig 4.12). Uddin et al. (2014) was indicated that *Gardenia jasminoides* leaves extract possess antioxidant activities which are comparable to the standard vitamin C. In this study, TPC from the non-irradiation samples showed a higher amount when harvested at 35 days, but this value was slightly reduced at 70 and 105 days. TPC in irradiated explants at 40 Gy gamma ray was dropped from 4.82 ± 0.13 to 4.25 ± 0.01 on 2,4-D and 4.65 ± 0.01 to 4.24 ± 0.03 Eq GA g on NAA.

Table 4.7: A mean comparison on various doses of gamma irradiation and callus age on total phenolic, flavonoid content and antioxidant activity of *G. jasminoides* cultured on solid WPM media supplemented with 2,4-D (2 mg L^{-1})

Time (Days)	**Gamma ray** (Gy)	TPC	TFC	Antioxidant
35 Days	0	5.33 ± 0.01^{b}	2.45 ± 0.01^{e}	77.11 ± 0.26^{c}
	5	5.05 ± 0.00^{cd}	2.76 ± 0.01^{c}	74.91 ± 0.31^{d}
	10	4.96 ± 0.01^{d}	2.90 ± 0.01^{b}	69.71 ± 0.41^{e}
	20	$\mathbf{5.56 \pm 0.01^{a}}$	$\mathbf{3.04 \pm 0.01^{a}}$	$\mathbf{91.32 \pm 0.06^{a}}$
	40	4.84 ± 0.01^{de}	2.70 ± 0.02^{cd}	66.47 ± 0.93^{g}
70 Days	0	5.03 ± 0.07^{cd}	2.04 ± 0.01^{g}	68.35 ± 0.27^{f}
	5	4.92 ± 0.20^{d}	2.22 ± 0.01^{f}	65.0 ± 0.27^{h}
	10	4.83 ± 0.04^{ed}	2.47 ± 0.00^{e}	60.41 ± 0.30^{i}
	20	5.24 ± 0.00^{cb}	2.56 ± 0.01^{d}	84.83 ± 0.07^{b}
	40	4.82 ± 0.13^{de}	2.01 ± 0.01^{g}	58.10 ± 0.10^{j}
105 days	0	4.60 ± 0.1^{ef}	1.80 ± 0.01^{h}	61.31 ± 0.27^{i}
	5	4.64 ± 0.13^{ef}	1.67 ± 0.01^{j}	59.12 ± 0.16^{j}
	10	4.44 ± 0.03^{gf}	1.82 ± 0.012^{i}	53.92 ± 0.52^{k}
	20	4.91 ± 0.01^{d}	1.95 ± 0.02^{h}	75.45 ± 0.28^{d}
	40	4.25 ± 0.01^{g}	1.6 ± 0.08^{k}	45.74 ± 0.1^{l}

Mean with the same letter are not significantly different at 0.05 levels.

Table 4.8: A mean comparison effect of various doses gamma irradiation and callus harvesting time on total phenolic, flavonoid contents and antioxidant activity of *G. jasminoides* water extract cultured on solid WPM media supplemented with NAA (2 mg L^{-1})

Time (Days)	**Gamma ray** (Gy)	TPC	TFC	Antioxidant
35	0	5.32 ± 0.01^{b}	2.48 ± 0.01^{e}	76.94 ± 0.03^{c}
	5	5.04 ± 0.01^{e}	2.73 ± 0.004^{d}	73.96 ± 0.3^{d}
	10	4.86 ± 0.01^{f}	2.92 ± 0.003^{b}	68.34 ± 0.36^{f}
	20	$\mathbf{5.49 \pm 0.01^{a}}$	$\mathbf{3.00 \pm 0.01^{a}}$	$\mathbf{90.71 \pm 0.21^{a}}$
	40	4.82 ± 0.01^{fg}	2.7 ± 0.01^{cd}	65.13 ± 0.97^{h}
70	0	5.15 ± 0.01^{c}	2.01 ± 0.00^{j}	66.57 ± 0.69^{g}
	5	4.67 ± 0.01^{hi}	2.24 ± 0.01^{h}	63.64 ± 0.27^{i}
	10	4.77 ± 0.01^{g}	2.39 ± 0.01^{g}	59.08 ± 0.3^{j}
	20	5.31 ± 0.01^{b}	2.44 ± 0.01^{f}	83.5 ± 0.07^{b}
	40	4.65 ± 0.01^{i}	1.94 ± 0.00^{k}	56.76 ± 0.1^{k}
105	0	4.69 ± 0.01^{h}	1.83 ± 0.01^{lm}	58.59 ± 0.27^{j}
	5	4.41 ± 0.01^{j}	1.73 ± 0.01^{m}	56.40 ± 0.16^{k}
	10	4.28 ± 0.01^{k}	1.88 ± 0.01^{lm}	51.20 ± 0.52^{l}
	20	4.86 ± 0.02^{f}	2.05 ± 0.01^{i}	72.73 ± 0.28^{e}
	40	4.24 ± 0.03^{l}	1.64 ± 0.01^{n}	43.02 ± 0.1^{m}

Mean with the same letter are not significantly different at 0.05 levels.

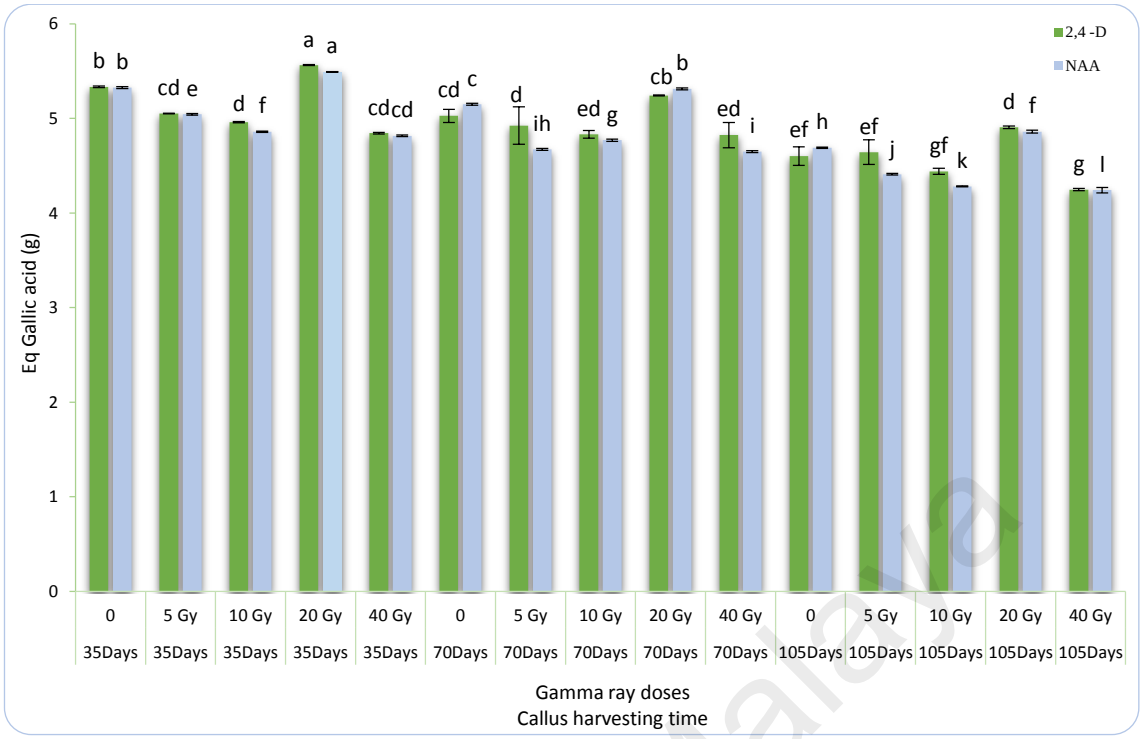


Figure 4.12: Effect of various doses of gamma irradiation and callus age on total phenolic content from *G. jasminoides* cultured on solid WPM media supplemented with 2,4-D (2 mg L^{-1}). Mean with the same letter are not significantly different at 0.05 levels.

4.3.2 Total flavonoid content (TFC)

In order to determine different effects of main factors (callus ages and at different doses of gamma ray) on total flavonoid contents, Duncan multiple range tests was applied. Based on the results, various callus harvesting times and gamma ray doses showed significant effects on TFC from collected callus on 2,4-D and NAA. Aside from individually influence of various gamma ray doses, higher effect was detected at 20 Gy (Appendix 12-a, b). Total flavonoid content was calculated to equivalent mg of Rutin in 1 g dry weight. The analysis of variance (Appendix 10-b, Appendix 11-b) and mean comparisons (Table 4.7, Table 4.8) revealed statistical differences between gamma irradiation, callus harvesting time and their interactions on TFC on 2,4-D and NAA. Flavonoid contents have been reported in *G. jasminoides* by Martins and Veronica (2015) and Yang et al. (2013).

The total flavonoid content in non-radiated callus was measured at 2.45 ± 0.01 and 2.48 ± 0.01 Eq Rutin g (57.7 and 58.1%) in 2,4-D and NAA. As shown in figure 4.13, the radiation effect has been specified by the trend line. All the irradiated treatments showed positive responses compared to control or non-radiated. This study was indicated that gamma irradiation at 20 Gy increased TFC to the highest amount (74, 72.9%) and showed a significant difference on 2,4-D (3.04 ± 0.01 Eq Rutin/g) and NAA (3.0 ± 0.01 Eq Rutin/g) when callus harvested at the early stage (35 days).

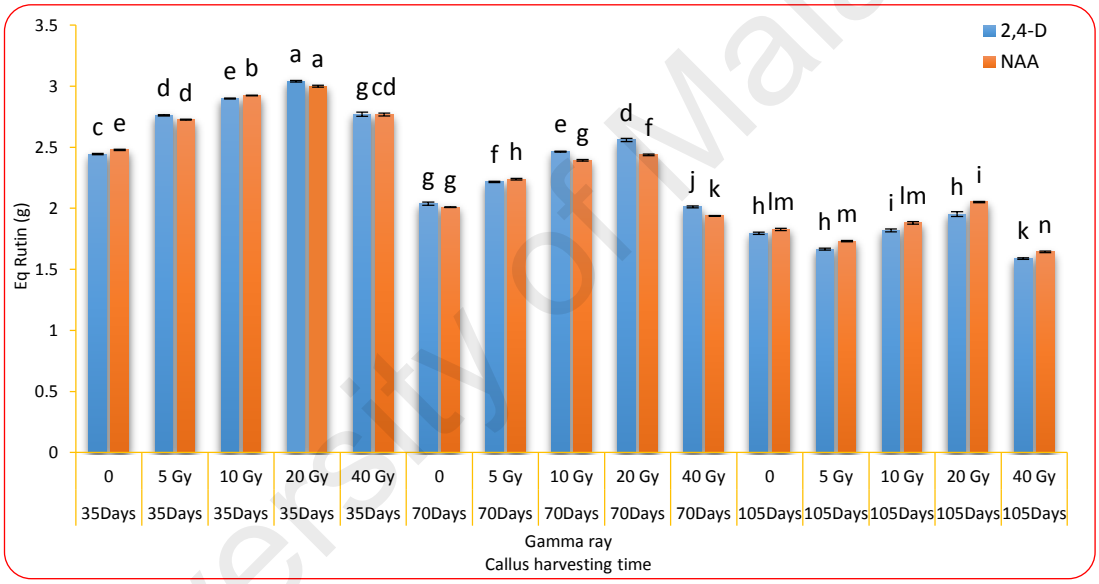


Figure 4.13: Effect of various doses of gamma ray and callus age TFC from *G. jasminoides* cultured on solid WPM media supplemented with 2,4-D (2 mg L^{-1}). Mean with the same letter are not significantly different at 0.05 levels

4.3.3 DPPH radical scavenging activity

Based on Duncan multiple range tests, various callus age affected separately on the percentage of DPPH inhibition on collected callus from 2,4-D and NAA. Gamma ray doses showed different effect on antioxidant activity from collected callus on WPM addition by NAA and 2,4-D.

Non-irradiation samples (control) at the fisrt collection time (35 days) showed DPPH inhabition 76.94 ± 0.03 on 2,4-D and 77.11 ± 0.26 on NAA. Callus age was decresed DPPH to 68.35 ± 0.26, 66.57 ± 0.69 and 45.74 ± 0.09, 43.02 ± 0.09 of initial culture on 2,4-D and NAA at 70 and 105 days, respectively. However, 20 Gy significantly resulted different compared to other doses (Appendix 12-a, b). As shown in figure 4.14, gamma ray at 20 Gy was increased antioxidant to 99.91 ± 0.08 (2,4-D) and 99.6 ± 0.31 (NAA) at an early stage of callus harvesting (35 days). Similar to TPC and TFC, 40 Gy gamma ray was suppressed antioxidant activity followed by harvesting callus. Leaf explants subjected to 40 Gy from 2,4-D and NAA showed 66.47 ± 0.93 and 65.13 ± 0.97 when harvested at 35 days initial culture. DPPH inhibition was dropped when callus collected at day of 70 (58.01 ± 0.96, 56.57 ± 0.01) and 105 days (43.02 ± 0.09, 45.74 ± 0.01) from 2,4-D and NAA, respectively. The analysis of variance was indicated significant differences in gamma irradiation on callus age and their interactions (Appendix 10-c, Appendix 11-c). The differences between means of antioxidant activity were analyzed and compared by Duncan multiple ranges. The report expressed as means ± standard deviations (Table 4.7, Table 4.8). To evaluate the association between secondary metabolite, Pearson correlation coefficient was applied (Table 4.9). The results showed positive correlated (r= 0.931 and r= 0.920) between total phenolic content and antioxidant activity from grown callus on 2,4-D and NAA, respectively.

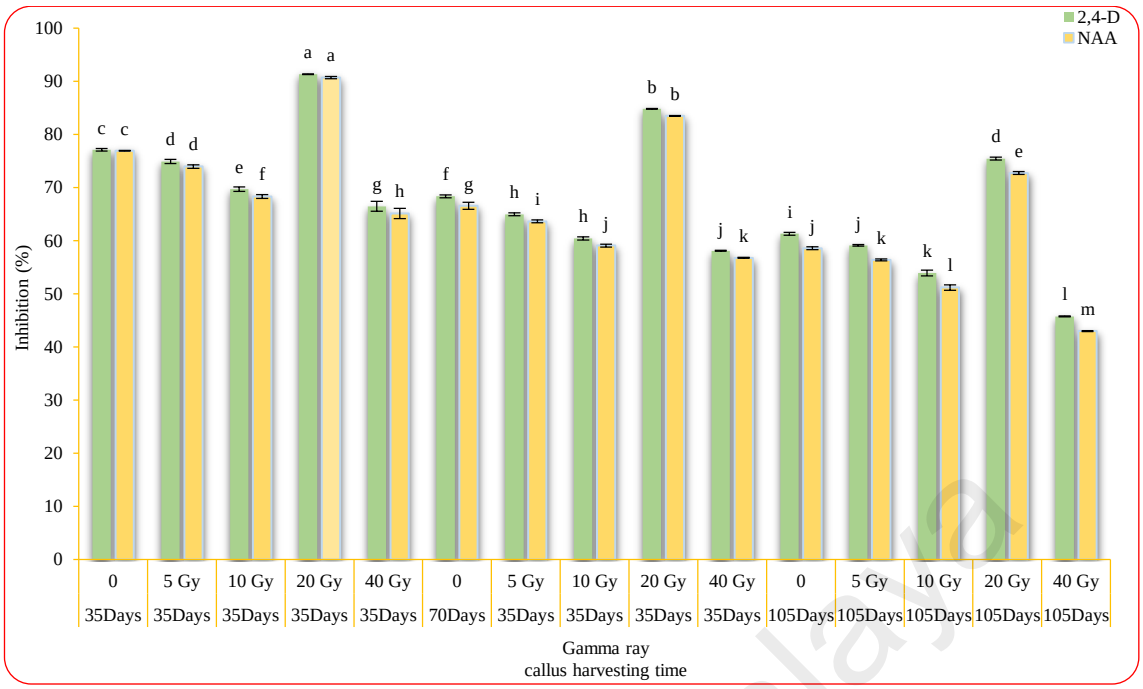


Figure 4.14: Effect of various doses of gamma ray and harvesting time on antioxidant activity of water extract of callus from *G. jasminoides* leaf explants cultured on solid WPM media supplemented with 2,4-D (2 mg L^{-1}). Mean with the same letter are not significantly different at 0.05 levels.

Table 4.9: Correlation coefficients among total flavonoid, total phenolic contents and antioxidant activity from grown callus on WPM media supplemented with 2,4-D and NAA collected of irradiation leaf explants of *G. jasminoides* Ellis

	TPC		TFC	
	2,4-D	NAA	2,4-D	NAA
TPC	0.78**	0.71**		
Antioxidant	0.70**	0.91**	**0.93****	**0.92****

** Correlation is significant at the 0.05 level (2-tailed).

4.3.4 Optimum auxin type in callus induction, total phenolic content, flavonoid content and antioxidant activity from gamma irradiation explants

The obtained data from grown callus on WPM supplemented with (2 mg L^{-1}) 2,4-D and NAA was analyzed and similar results was detected. In order to find the best PGR, independent sample test was applied (Appendix 13). The fresh weight of callus, antioxidant activity and total phenolic content from grown callus on 2,4-D and NAA revealed similar increment.

4.4 Effect of methyl jasmonate on callogenesis and callus growth in different auxin from leaf explants of *Gardenia jasminoides* Ellis

Callus explants included control were induced to WPM media supplemented with various concentrations of MeJA (0, 0.022, 0.220, 1.120, 2.240, 4.50 mg L^{-1}) and addition by NAA and 2,4-D (2 mg L^{-1}). However, MeJA in high concentration (4.5 mg L^{-1}) caused necrosis and explants died after the 14 days of initial callus induction (Fig 4.15). Based on the one-sample Kolmogorov-Smirnov test all the data were normally distributed. Primary weight of callus was considered as a covariate to prevent its possible effect and remove unequal callus weight at the induction time.

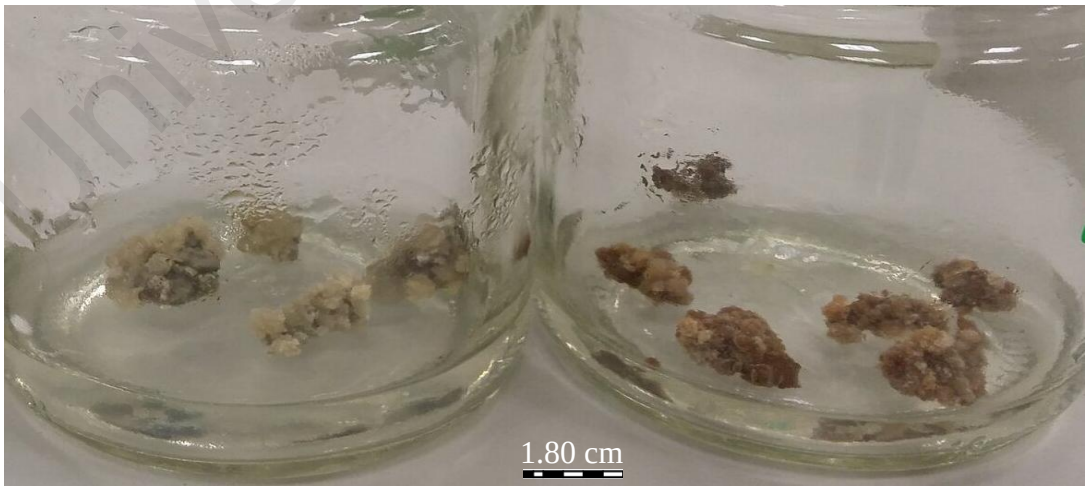


Figure 4.15: Necrosis callus from *G. jasminoides* after 7 days on WPM media supplemented with 4.5 mg L^{-1} MeJA (right), and normal callus on WPM media addition with 1.12 mg L^{-1} (left) of MeJA 14 days after initial callus induction.

Elicitation may have varied effects on different cell lines. Wu and Lin (2002) and Zhao et al. (2005) noted the positive effect of elicitation on callus growth and Cai et al. (2011) reported that cell cultures of *V. vinifera* were not affected by elicitors. Dhandhukia and Thakkar (2007), Wang et al. (2011) and Furmanowa et al. (1997) reported elicitation on solid media. Various callus harvesting times individually showed increasing at 105, 70 and 35 days on the fresh and dry weight of callus on 2,4-D and NAA (Fig 4.16).

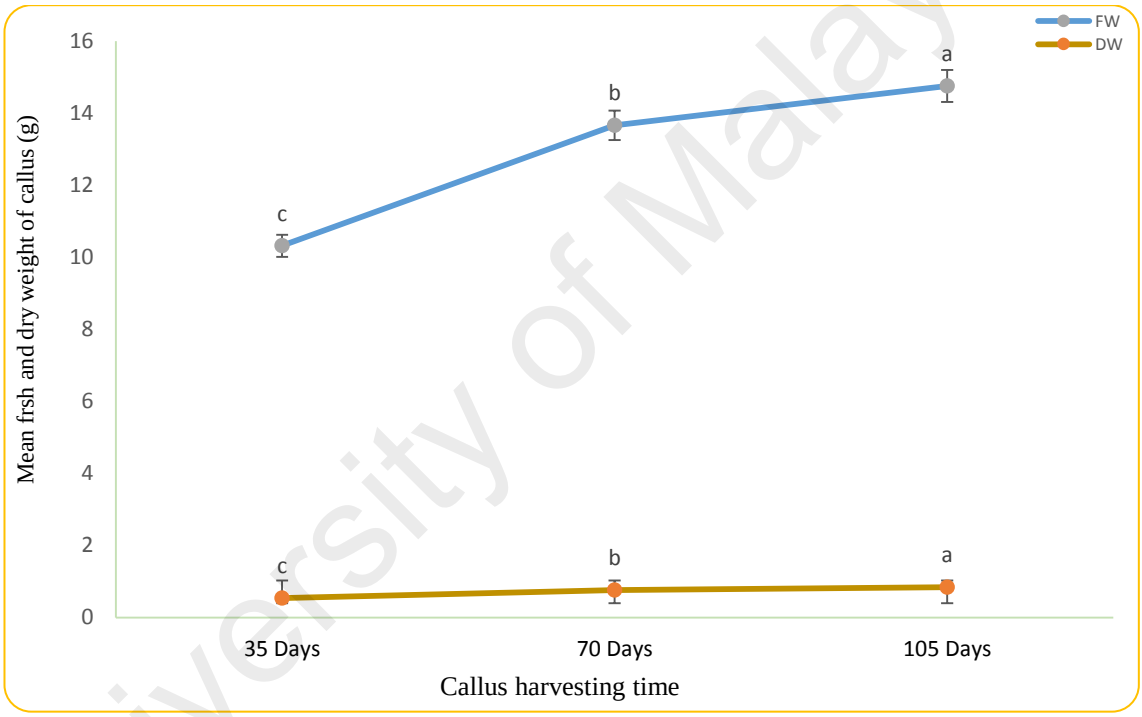


Figure 4.16: Effect of three different harvesting times on fresh and dry weight of callus from of *G. jasminoides* grown on WPM supplemented with 2,4-D or NAA. Mean with the same letter are not significantly different at 0.05 levels.

To evaluate the effects of all factors and their interaction on WPM medium, data were analyzed using GLM procedures. Due to the unequal weight of samples during inoculation, the initial weight of samples was considered as a covariate and its probable effect excluded from the results.

According to one-sample Kolmogorov–Smirnov test, all variables were normally distributed. The callus age significantly affected on CGR, the fresh and dry weight of *G. jasminoides* callus cultured on WPM media supplemented with 2,4-D or NAA at 0.05 and 0.01 levels. The amounts of adjusted R-squared showed a goodness of fit for the models (Table 4.10, Table 4.11).

Table 4.10: Summary of ANOVA for fresh weight, dry weight, and callus growth rate in callus induction of *G. jasminoides* growth in WPM medium supplemented with 2,4-D (2 mg L^{-1}) and various concentration of methyl jasmonate

Source	FW	DW	CGR
Time	185.25^{**}	0.42^{**}	0.1۲^{**}
MeJA	26.82^{**}	0.05^{*}	0.01^{**}
Time × MeJA	3^{ns}	0.0۱^{ns}	0.01^{**}
CV%	12.64	22	12.79
R^2	0.87	0.93	0.87

^{ns} Not significant * significant at 0.05 level ** significant at 0.01 level

Table 4.11: Summary of ANOVA for fresh weight, dry weight, and callus growth rate in Callus Induction of *G. jasminoides* growth in WPM medium supplemented with NAA (2 Mg L^{-1}) and various concentration of methyl jasmonate

Source	FW	DW	CGR
Time	187.03^{**}	0.4۲^{**}	0.131^{**}
MeJA	25.56^{**}	0.05^{*}	0.01^{**}
Time × MeJA	3.38^{*}	0.0۱^{ns}	0.01^{**}
CV%	12.79	22	12.64
R^2	0.82	0.93	0.87

^{ns} Not significant * significant at 0.05 level ** significant at 0.01 level

The interaction of MeJA and callus age showed significant influence on CGR at 0.01 levels on 2,4-D and NAA. No difference was observed on dry weight from grown callus on 2,4-D or NAA, however, significant differences of fresh weight was detected on NAA at 0.05 levels.

In order to determine variation between treatments, analysis of variance by SAS version 9.1 was applied. The differences between means were calculated and compared by Duncan's multiple range test. Data are reported as means ± standard deviations (Table 4.12, Table 4.13). The obtained results showed a sigmoid growth curve from given callus on 2,4-D and NAA. The exponential phase was detected between 35 and 70 days of culturing. Callus fresh weight on 2,4-D and NAA was increased from control (8.31 ± 0.4 g, 8.81 ± 0.29 g) to elicited media by 0.22 mg L^{-1} MeJA (12.٠٦ ± 0.٧ g, 12.49 ± 0.6 g) at the days of 35. Callus was developed slightly during the stationary phase at 105 days on 2,4-D and NAA (13.26 ± 0.8 g, 13.31 ± 0.32 g).

The results revealed that 0.22 mg L^{-1} of MeJA was most effective concentration on FW and DW compared to higher concentrations (1.12 and 2.24 mg L^{-1}). MeJA concentration (0.22 mg L^{-1}) was significantly increased dry weight at the 105 days of callus harvesting to 0.90 ± 0.01 g and 0.93 ± 0.01 g from 2,4-D and NAA versus control (0.82 ± 0.05, 0.85 ± 0.02 g). Similarly, same concentration was raised 1.19 and 1.17-fold biomass compared to control samples at 70 days. Callogenesis was achieved using leaf explants by addition 2,4-D and NAA.

Table 4.12: Mean comparison of callus fresh weight from *G. jasminoides* cultured on MeJA elicited WPM medium supplemented with 2,4-D or NAA (2 mg L^{-1}) harvested at different times

Time (Days)	MeJA (mg L^{-1})	2,4-D	NAA
35	0	08.31 ± 0.44^{f}	08.81 ± 0.29^{h}
	0.022	08.84 ± 0.19^{f}	09.43 ± 0.36^{h}
	0.220	12.06 ± 0.67^{de}	12.49 ± 0.58^{efg}
	1.120	11.14 ± 0.41^{e}	11.47 ± 0.62^{g}
	1.240	09.11 ± 0.35^{f}	09.4 ± 0.53^{h}
70	0	12.32 ± 0.6^{de}	12.97 ± 0.61^{def}
	0.022	13.01 ± 0.55^{cd}	13.66 ± 0.44^{cde}
	0.220	**15.39 ± 0.42^{a}**	**15.71 ± 0.45^{a}**
	1.120	13.42 ± 0.23^{cd}	13.10 ± 0.36^{dc}
	1.240	12.00 ± 0.63^{de}	12.01 ± 0.44^{fg}
105	0	13.26 ± 0.8^{bcd}	13.31 ± 0.32^{bcd}
	0.022	13.93± 0.49^{abc}	14.00 ± 0.27^{cd}
	0.220	14.39 ± 0.18^{ab}	14.62 ± 0.16^{ab}
	1.120	14.07 ± 0.29^{abc}	14.28 ± 0.43^{abc}
	1.240	13.27 ± 0.24^{bcd}	13.37 ± 0.14^{bcd}

Means with the same letter are not significantly different.

Table 4.13: Mean comparison of callus dry weight from *G. jasminoides* cultured on MeJA elicited WPM medium supplemented with 2,4-D or NAA (2 mg L^{-1}) harvested at different times

Time (Days)	**MeJA** (mg L^{-1})	**2,4-D**	**NAA**
35	0	0.48 ± 0.03^{f}	0.51 ± 0.02^{g}
	0.022	0.51 ± 0.01^{f}	0.55 ± 0.02^{g}
	0.220	0.70 ± 0.04^{de}	0.72 ± 0.03^{def}
	1.120	0.65 ± 0.02^{e}	0.67 ± 0.04^{f}
	1.240	0.53 ± 0.02^{f}	0.55 ± 0.03^{g}
70	0	0.72 ± 0.03^{de}	0.76 ± 0.04^{cde}
	0.022	0.76 ± 0.03^{cd}	0.80 ± 0.03^{bcd}
	0.220	0.86 ± 0.03^{ab}	0.88 ± 0.03^{ab}
	1.120	0.78 ± 0.01^{bcd}	0.82 ± 0.02^{bc}
	1.240	0.70 ± 0.04^{de}	0.70 ± 0.03^{ef}
105	0	0.82 ± 0.05^{abc}	0.85 ± 0.02^{ab}
	0.022	0.84 ± 0.03^{abc}	0.87 ± 0.02^{ab}
	0.220	$\mathbf{0.90 \pm 0.01^{a}}$	$\mathbf{0.93 \pm 0.01^{a}}$
	1.120	0.84 ± 0.02^{ab}	0.87 ± 0.03^{ab}
	1.240	0.83 ± 0.01^{abc}	0.86 ± 0.01^{ab}

Means with the same letter are not significantly different.

4.5 Effect of methyl jasmonate on secondary metabolite activites (total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity) from leaf explants of *Gardenia jasminoides* Ellis

To check normality of data, Kolmogorov – Smirnov test was applied and revealed all the variables were normally distributed. The amount of R-squared showed a high goodness of fit for the models (Table 4.14, Table 4.15). Callus induction on 2,4-D and NAA revealed significant differences when MeJA added to WPM and harvested at different times. However, only TPC on 2,4-D showed a non-significant difference at 0.05 levels.

Table 4.14: Summary of ANOVA for total phenolic, total flavonoid contents and Antioxidant activity in water extract callus of leaf explants from *G. jasminoides*, growth in WPM supplemented with 2 mg L^{-1} 2,4-D and various concentrations of MeJA harvested in three different harvesting times

Source	TPC	TFC	Antioxidant
Time	2.6^{**}	4.15^{**}	1430.59^{**}
MeJA	1.77^{**}	0.88^{**}	579.51^{**}
Time × MeJA	0.12^{ns}	0.03^{**}	14.03^{**}
CV%	5.05	2.09	0.77
R^2	0.86	0.99	0.99

^{ns} Not significant, **Significant at 0.01 level

Table 4.15: Summary of ANOVA for total phenolic, total flavonoid contents and Antioxidant activity in water extract callus of leaf explants from *G. jasminoides*, growth in WPM supplemented with 2 mg L^{-1} NAA and various concentrations of MeJA harvested in three different harvesting times

Source	TPC	TFC	Antioxidant
Time	2.01^{**}	4.29^{**}	3233.27^{**}
MeJA	1.26^{**}	0.91^{**}	1567.68^{**}
Time × MeJA	0.03^{**}	0.02^{**}	260.39^{**}
CV%	1.07	1.1	0.85
R^2	0.99	0.99	0.99

**Significant at 0.05 level

To evaluate the differences between main effects of three harvesting times and various concentrations of MeJA, Duncan multiple range test was applied. Based on the results, three harvesting time showed individual effects on secondary metabolite contents and antioxidant activities. Similarly, the pattern of accumulating secondary metabolite content and callus harvesting gamma detected same as gamma irradiation study. Harvesting time at the days 35 displayed significantly higher difference compared to others in both 2,4-D and NAA.

The callus induction treated with various concentrations of MeJA showed individual responses. According to Duncan multiple range tests, MeJA with 0.22 and 1.12 mg L^{-1} was increased TPC, TFC and antioxidant activity on develped callus on 2,4-D and NAA. In order to find the best effect of variation on producing secondary metabolite compounds, Duncan's multiple range test was applied. The differences between means of secondary metabolite activities from 2,4-D (Table 4.16) and NAA (Table 4.17) callus were analyzed and compared by DMRT. Data are reported as means ± standard deviations.

4.5.1 Total phenolic content (TPC)

Total phenolic content was calculated to equivalent mg of Gallic acid in 1 g dry weight. ANOVA table (Appendix 16-a, Appendix 17-a) detected the interaction of MeJA and callus harvesting time was not significantly affected on grown callus on 2,4-D. Total Phenolic content was detected on all treatments included controls. Based on Duncan's multiple range test, addition of 0.22 mg L^{-1} MeJA to 2,4-D and NAA was increased TPC in three harvesting times, but significantly was escalated at 70 days to (7.301 ± 0.027 Eq GA g) in 2,4-D. WPM media supplemented with NAA revealed significant differences when callus collected at 35 days (6.54 ± 0.13 Eq GA g) and 70 days (6.60 ± 0.01 Eq GA g).

Furthermore, callus age was affected on TPC; the early stage of harvesting (35 days) from WPM media addition with 0.22 mg L^{-1} of MeJA showed 1.9-fold in compared to 70 and 105 days (1.03 and 0.93 –fold) respectively.

4.5.2 Total flavonoid contents (TFC)

Using of methyl jasmonate (MeJA) elicitation for flavonoid enhancement in plant cell culture is an efficient method (Bota & Deliu, 2015; Danaee et al., 2015a; Moglia et al., 2008). Biosynthesis of flavonoids by MeJA elicitation was reported in *Panax ginseng* (Ali et al., 2007c) but Keinänen et al. (2001) noted that *Nicotiana attenuata* leaves do not responded to MeJA. It is crucial to screen potential elicitors for the hyper production of flavonoids in a shortened culture period by cell culture of *G. jasminoides*. In order to determine the different effects of main factors (callus ages and various concentrations of MeJA) on TFC, DMRT was applied. Based on the results, the callus age and MeJA diplayed statistical difference on TFC on 2,4-D and NAA. All concentrations of MeJA affected individually on flavonoid contents, however, higher effect was detected at 0.22 mg $L^{-1}$of MeJA (Appendix 18).

Total flavonoid content was calculated to equivalent mg of rutin in 1 g dry weight. The analysis of variance and mean comparisons revealed the statistical differences between MeJA, callus harvesting time and their interactions on TFC (Appendix 16-b, Appendix 17-b). Methyl jasmonate was increased TFC when callus collected at an early stage of 35 days from 2,4-D and NAA. To find the flavonoid accumulation in *G. jasminoides* and optimal cultivation time, cultures were analyzed throughout 3 collection periods. The TFC was significantly increased to 83 and 83.6% elicitation media with 0.22 mg L^{-1} of MeJA with 2,4-D (3.4 Eq Rutin g) and NAA (3.36 Eq Rutin g) at 35 days.

However, treated media with 1.12 mg L^{-1} MeJA was reduced TFC to 2.96 ± 0.01 and 2.98 ± 0.01 Eq Rutin g in 2,4-D and NAA, respectively. Flavonoid content was dropped to 36 % at a higher concentration of methyl jasmonate (2.24 mg L^{-1}) at the latest harvesting time (105 days).

4.5.3 DPPH Radical scavenging activity

Duncan's multiple range test detected individual effects of callus age on inhibition of DPPH on both calli (2,4-D and NAA). Addition of MeJA concentrations (0.22 and 1.12 mg L^{-1}) in 2,4-D and NAA was revealed significant difference on antioxidant activity (Appendix 18-a, b). Based on ANOVA table, callus age, MeJA concentrations and their interactions were indicated significant differences (Appendix 16-c, Appendix 17-c). The differences between means of antioxidant activity were analyzed, compared by Duncan's multiple range test and reported as means ± standard deviations illustrated in Table 4.16 (2,4-D) and Table 4.17 (NAA). Methyl jasmonate with 0.22 mg L^{-1} significantly was increased DPPH on 2,4-D (100 ± 0.37) and NAA (99.33 ± 0.37) at 35 days of callus collection versus to control (77.11 ± 0.26, 76.94 ± 0.03). The same concentration accelerated DPPH to 86.50 ± 0.1 and 85.60 ± 0.1 when harvested on 70 days. To finding the association between variables between total phenolic, flavonoid contents and antioxidant activity, Pearson correlation coefficient was applied (Table 4.18).

Table 4.16: A mean comparison of various concentrations of MeJA and harvesting times on total phenolic and flavonoid contents and antioxidant activity from *G. jasminoides* leaf explants (callus water extracts) cultured on solid WPM media supplemented with 2,4-D (2 mg L^{-1})

Time (Days)	MeJA (mg L^{-1})	TPC	TFC	Antioxidant
35	0	5.56 ± 0.01^{def}	2.479 ± 0.01^{e}	77.11 ± 0.26^{e}
	0.022	5.74 ± 0.01^{bcd}	2.58 ± 0.01^{d}	78.96 ± 0.09^{d}
	0.220	6.74 ± 0.63^{b}	**3.38 ± 0.00^{a}**	**100 ± 0.37^{a}**
	1.120	6.14 ± 0.02^{bc}	2.984 ± 0.01^{b}	85.05 ± 0.38^{c}
	1.240	5.40 ± 0.01^{cde}	2.758 ± 0.01^{c}	76.94 ± 0.24^{e}
70	0	5.24 ± 0.00^{def}	2.01 ± 0.00^{fi}	68.35 ± 0.27^{i}
	0.022	5.40 ± 0.03^{cde}	2.08 ± 0.00^{h}	69.4 ± 0.02^{h}
	0.220	**7.30 ± 0.03^{a}**	2.90 ± 0.09^{b}	86.50 ± 0.1^{b}
	1.120	5.72 ± 0.03^{bcd}	2.35 ± 0.02^{f}	77.57 ± 0.72^{e}
	1.240	5.27 ± 0.01^{def}	2.19 ± 0.08^{g}	71.08 ± 0.37^{g}
105	0	4.91 ± 0.01^{ef}	1.48 ± 0.01^{l}	61.31 ± 0.27^{j}
	0.022	5.09 ± 0.01^{f}	1.59 ± 0.01^{k}	59.05 ± 0.11^{k}
	0.220	5.58 ± 0.01^{cd}	2.07 ± 0.03^{h}	74.61 ± 0.03^{f}
	1.120	5.22 ± 0.03^{def}	1.97 ± 0.01^{i}	67.78 ± 0.29^{i}
	1.240	5.02 ± 0.08^{ef}	1.715 ± 0.01^{j}	57.94 ± 0.47^{1}

Mean with the same letter are not significantly different at 0.05 levels.

Table 4.17: A mean comparison of various concentrations of MeJA and harvesting times on total phenolic and flavonoid contents and antioxidant activity from *G. jasminoides* leaf explants (callus water extracts) cultured on solid WPM media supplemented with NAA (2 mg L^{-1})

Time (Days)	**MeJA** (mg L^{-1})	TPC	TFC	Antioxidant
35	0	5.49 ± 0.01^{ef}	2.445 ± 0.01^{e}	76.94± 0.03^{e}
	0.022	5.59 ± 0.00^{de}	2.559 ± 0.01^{d}	78.10 ± 0.09^{d}
	0.220	**6.54 ± 0.13^{a}**	**3.356 ± 0.002^{a}**	**99.33 ± 0.37^{a}**
	1.120	5.96 ± 0.01^{b}	2.955 ± 0.01^{b}	84.18 ± 0.38^{c}
	1.240	5.34 ± 0.00^{g}	2.709 ± 0.02^{c}	76.06 ± 0.24^{fe}
70	0	5.26 ± 0.01^{gh}	2.04 ± 0.01^{i}	66.57 ± 0.69^{i}
	0.022	5.45 ± 0.00^{f}	2.09 ± 0.00^{h}	68.50± 0.02^{h}
	0.220	**6.60 ± 0.010^{a}**	2.72± 0.01^{bc}	85.60 ± 0.10^{b}
	1.120	5.65 ± 0.00^{d}	2.40 ± 0.02^{f}	76.67 ± 0.72^{fe}
	1.240	5.22 ± 0.01^{h}	2.21 ± 0.03^{g}	70.18 ± 0.37^{g}
105	0	4.86 ± 0.02^{i}	1.46 ± 0.01^{m}	58.59 ± 0.27^{k}
	0.022	5.13 ± 0.01^{hi}	1.61 ± 0.01^{l}	60.22 ± 0.11^{j}
	0.220	5.82 ± 0.01^{b}	2.10 ± 0.01^{h}	75.79 ± 0.03^{f}
	1.120	5.22 ± 0.02^{h}	1.81 ± 0.01^{j}	68.96 ± 0.29^{h}
	1.240	4.98 ± 0.01^{j}	1.71 ± 0.03^{k}	59.12 ± 0.47^{k}

Mean with the same letter are not significantly different at 0.05 levels.

Table 4.18: Correlation coefficients among total phenolic, total flavonoid contents and antioxidant activity from grown of *G. jasminoides* Ellis callus on WPM media supplemented with 2,4-D, NAA with various concentrations of MeJA, collected at different time

	TFC		**TPC**	
	2,4-D	**NAA**	**2,4-D**	**NAA**
TPC	0.83**	0.90**		
Antioxidant	0.96**	0.96**	0.90**	0.96**

** Correlation is significant at the 0.05 level (2-tailed).

4.5.4 Optimum auxin type in callus induction, total phenolic content, flavonoid content and antioxidant activity from methyl jasmonate elicitation explants

The obtained data from grown callus on WPM supplemented with (2 mg L^{-1}) 2,4-D, NAA and various concentrations of MeJA were analyzed and similar results between NAA and 2, 4-D were detected. However, for finding the best PGR, independent sample test was applied (Appendix 19).

4.6 Effect of Salicylic acid on callogenesis and callus growth in different auxin from leaf explants of *Gardenia jasminoides* Ellis

Salicylic acid (SA) is an endogenous signal molecule and plays essential role in regulating stress response and plant developmental process (Klessig & Malamy, 1994). It is a key signal molecule for expression of multiple modes in plant stress resistance. As shown in figure 4.17, callus was induced on WPM media supplemented with NAA (2 mg L^{-1}) or 2,4-D (2 mg L^{-1}) and various concentrations of SA (0.5 mg L^{-1}, 2.5 mg L^{-1}, 5.0 mg L^{-1}, 7.5 mg L^{-1}, 12.5 mg L^{-1} and 20 mg L^{-1}). To evaluate the factors effect and their interaction, data were analyzed using GLM procedures. Due to the unequal weight of samples at a culturing time, the initial weight of callus samples considered as a covariate and its probable effect excluded from the results. According to one-sample Kolmogorov–Smirnov test, all variables were normally distributed. The amounts of adjusted R-squared showed a goodness of fit for the models (Table 4.18, Table 4.19). The various harvesting times and SA concentrations were affected significantly on CGR, fresh and dry weight of callus from *G. jasminoides* cultured on WPM media supplemented with 2,4-D or NAA at 0.01 levels. However, no statistical difference was observed on NAA. To determine significant variation between treatments, analysis of variance by SAS version 9.1 was applied. The differences between means were calculated and compared by DMRT. Data

are reported as means ± standard deviations (Table 4.21, Table 4.22). The obtained results showed a rapid growth on callus from control to 2.5 mg L^{-1} SA. Fresh callus was weighed out 8.31± 0.44 g, 8.81 ± 0.29 g at the first harvesting (35 days) from 2,4-D and NAA. Fresh weight was improved on 2.5 mg L^{-1} SA elicited media with 2,4-D (10.61 ± 0.18 g) and NAA (11.00 ± 0.19 g) at the first harvesting period (35 days); though, this difference was accelerated when callus collected at the 70 days on 2,4-D (14.65 ± 0.18 g) and at 105 days on media addition by both types of PGR's (14.92 ± 0.14 g, 15.39 ± 0.12 g). However, treated callus with 2.5 mg L^{-1} SA showed highest DW (0.62 ± 0.01, 0.64 ± 0.01 g) at days of 35 on 2,4-D and NAA, respectively. The increasing of DW at the second period (70 days) on the same concentration of SA showed 1.18 and 1.15-fold on 2,4-D and NAA, respectively. Aside from dry weight of callus harvested on 105 days which showed a remarkable difference, salicylic acid (2.5 mg L^{-1}) significantly increased DW on WPM media supplemented with 2,4-D when callus collected on 70 days (0.86 ± 0.01). The dry weight of callus grown on WPM media supplemented with 2,4-D (2 mg L^{-1}) significantly enhanced by 0.5, 2.5 and 5 mg L^{-1} SA after 105 days. However, higher and significant DW was measured in NAA supplemented with 2.5 and 5 mg L^{-1} SA.

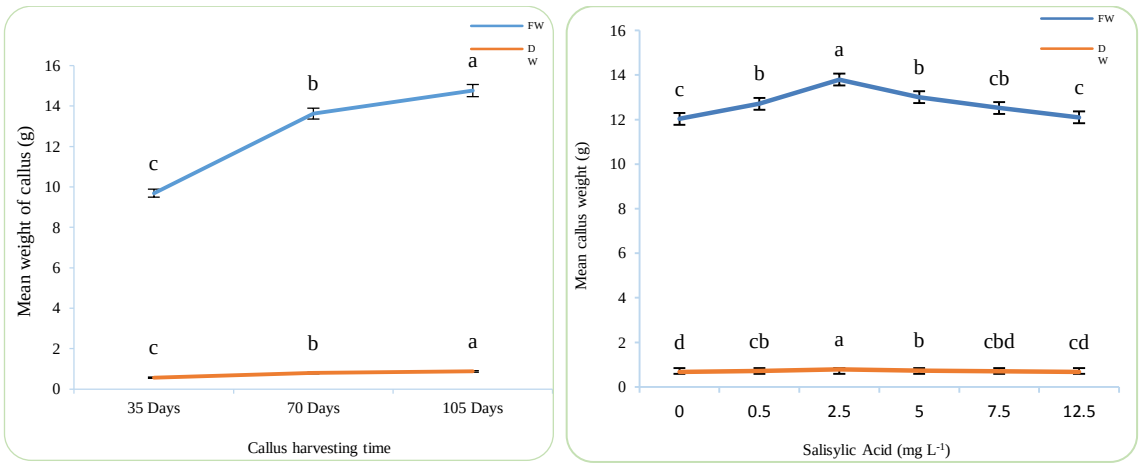


Figure 4.17: Effect of three different harvesting times (left) and various concentrations of SA (right) on the fresh and dry weight of callus from of *G. jasminoides* grown on WPM supplemented with 2,4-D or NAA.

Table 4.19: Summary of ANOVA for fresh weight, dry weight and callus growth rate in callus induction of *G. jasminoides* growth in WPM medium supplemented with 2,4-D (2 mg L^{-1}) and various concentration of salicylic acid

Source	FW	DW	CGR
Time	284.36**	1.06**	0.12**
SA	10.84**	0.04**	0.04**
Time × SA	0.93*	0.03*	0.01**
CV%	7.61	22	9.80
R^2	0.88	0.93	0.90

* Significant at 0.05 level ** significant at 0.01 level

Table 4.20: Summary of ANOVA for FW, DW, and CGR in callus induction of *G. jasminoides* growth in WPM medium supplemented with NAA (2 mg L^{-1}) and various concentration of salicylic acid

Source	FW	DW	CGR
Time	297.9**	1.11**	0.136**
SA	8.89**	0.03**	0.03**
Time × SA	0.67ns	0.00ns	0.00ns
CV%	7.69	22	9.80
R^2	0.87	0.93	0.90

ns Not significant * significant at 0.05 level ** significant at 0.01 level

Table 4.21: Mean comparison of callus fresh weight of *G. jasminoides* cultured on solid WPM medium supplemented with various concentrations of SA and 2,4-D or NAA (2 mg L^{-1}) harvested at different times

Time (Days)	**SA** (mg L^{-1})	2,4-D	NAA
35	0	8.31 ± 0.44^{g}	8.81 ± 0.29^{g}
	0.5	9.07 ± 0.13^{g}	9.41 ± 0.29^{fg}
	2.5	10.61 ± 0.18^{f}	11.04 ± 0.19^{e}
	5.0	10.15 ± 0.19^{f}	10.34 ± 0.22^{ef}
	7.5	9.01 ± 0.27^{g}	9.67 ± 0.23^{fg}
	12.5	8.34 ± 0.59^{ef}	8.9 ± 0.66^{g}
70	0	12.32 ± 0.57^{e}	12.97 ± 0.61^{d}
	0.5	13.21 ± 0.23^{bcde}	13.89 ± 0.22^{bcd}
	2.5	$\mathbf{14.65 \pm 0.18^{a}}$	14.98 ± 0.21^{ab}
	5.0	13.14 ± 0.40^{cde}	13.68 ± 0.44^{cd}
	7.5	12.84 ± 0.19^{de}	13.14 ± 0.17^{d}
	12.5	12.26 ± 0.08^{e}	13.1 ± 0.15^{d}
105	0	13.86 ± 0.8^{abcd}	14.31 ± 0.32^{abc}
	0.5	$\mathbf{14.33 \pm 0.32^{a}}$	14.83 ± 0.30^{abc}
	2.5	$\mathbf{14.92 \pm 0.14^{a}}$	$\mathbf{15.39 \pm 0.12^{a}}$
	5.0	14.3 ± 0.21^{ba}	14.96 ± 0.27^{ab}
	7.5	14.03 ± 0.22^{abc}	14.762 ± 0.49^{abc}
	12.5	13.89 ± 0.42^{abcd}	14.31 ± 0.70^{abc}

Means with the same letter are not significantly different.

Table 4.22: Mean comparison of callus dry weight of *G. jasminoides* cultured on solid WPM medium supplemented with various concentrations of SA and or NAA (2 mg L^{-1}) harvested at different times

Time (Days)	**SA** (mg L^{-1})	2,4-D	NAA
35	0	0.48 ± 0.03^{f}	0.51 ± 0.02^{g}
	0.5	0.53 ± 0.01^{bcd}	0.55 ± 0.02^{fg}
	2.5	0.62 ± 0.01^{e}	0.64 ± 0.01^{e}
	5.0	0.59 ± 0.01^{cd}	0.6 ± 0.01^{ef}
	7.5	0.52 ± 0.02^{f}	0.56 ± 0.01^{fg}
	12.5	0.48 ± 0.03^{f}	0.52 ± 0.04^{g}
70	0	0.72 ± 0.03^{d}	0.76 ± 0.04^{d}
	0.5	0.77 ± 0.01^{bcd}	0.81 ± 0.01^{bcd}
	2.5	**0.86 ± 0.01^{a}**	0.87 ± 0.01^{ab}
	5.0	0.77 ± 0.02^{cd}	0.8 ± 0.03^{cd}
	7.5	0.75 ± 0.01^{d}	0.77 ± 0.01^{d}
	12.5	0.72 ± 0.01^{d}	0.77 ± 0.01^{d}
105	0	0.82 ± 0.05^{abc}	0.85 ± 0.02^{abc}
	0.5	**0.85 ± 0.02^{a}**	0.88 ± 0.02^{ba}
	2.5	**0.88 ± 0.01^{a}**	**0.91 ± 0.01^{a}**
	5.0	**0.85 ± 0.01^{a}**	**0.89 ± 0.02^{a}**
	7.5	0.83 ± 0.01^{ab}	0.87 ± 0.03^{ab}
	12.5	0.82 ± 0.03^{abc}	0.85 ± 0.04^{abc}

Means with the same letter are not significantly different.

4.6.1 Effect of salicylic acid on secondary metabolite sctivities (total phenolic content (TFC), total flavonoid contents (TFC) and antioxidant activity) from leaf explants of *Gardenia jasminoides* Ellis

For checking normality of data, Kolmogorov – Smirnov test was applied and revealed all the variables were normally distributed. The amount of R- squared showed a very high goodness of fit for the models (Table 4.23, Table 4.24). Based on Duncan's multiple range test, the highest biological activities were detected from harvesting callus at 35, 70 and 105 days, respectively. Bioactive compounds from callus of *G. jasminoides* was gathered on SA elicited media (0.5 mg L^{-1}); however the maximum amount was detected on 2.5 mg L^{-1} of SA. As displayed in figure 4.18, synthesis of natural compound was declined with raising SA concentration.

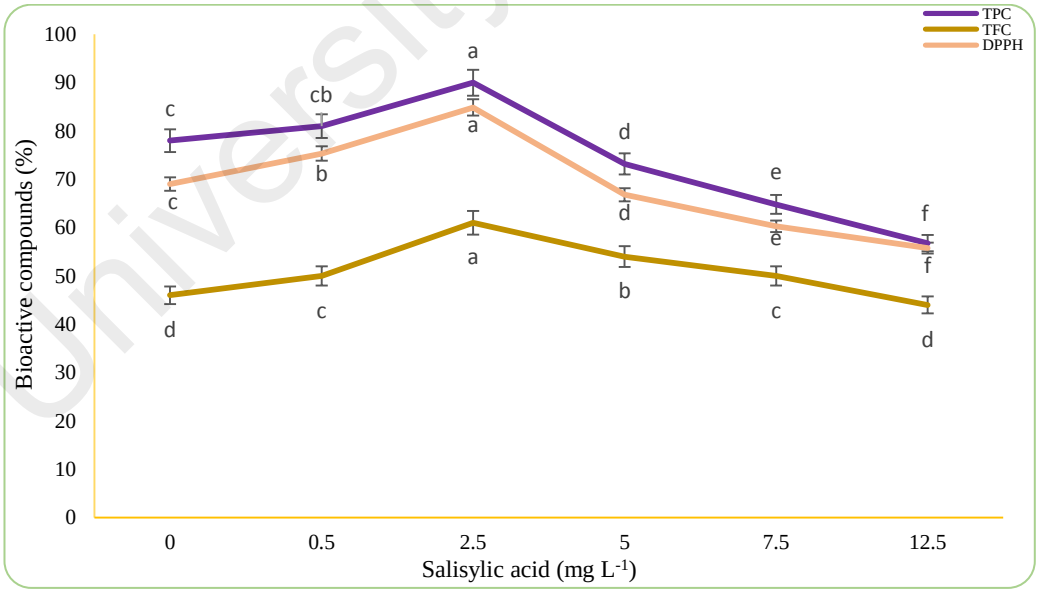


Figure 4.18: Effect of salicylic acid in various concentration on total phenolic, flavonoid contents and antioxidant activity

Table 4.23: Summary of ANOVA for total phenolic, total flavonoid contents and antioxidant activity in water extract callus of leaf explants from *G. jasminoides*, grown in WPM supplemented with 2 mg L^{-1} 2,4-D and various concentrations of SA harvested in three different times

Source	TPC	TFC	Antioxidant
Time	2.21**	7.028**	1304.38**
SA	2.63**	0.77**	986.51**
Time × SA	0.078**	0.38**	19.07**
CV%	2.69	1.24	0.81
R^2	0.97	1	1

**Significant at 0.01 level

Table 4.24: Summary of ANOVA for for total phenolic, total flavonoid contents and antioxidant activity in water extract callus of leaf explants from *G. jasminoides*, grown in WPM supplemented with 2 mg L^{-1} NAA and various concentrations of SA harvested in three different times

Source	TPC	TFC	Antioxidant
Time	1.42**	7.04**	3473.22**
SA	2.60**	0.73**	981.01**
Time × SA	0.04**	0.04**	18.58**
CV%	0.76	1.24	0.88
R^2	1	1	1

**Significant at 0.05 level

4.6.2 Total phenolic content (TPC)

Total phenolic content was calculated to equivalent mg of gallic acid in 1 g dry weight. Based on ANOVA table (Appendix 22-a, Appendix 23-a), all concentrations of salicylic, callus age, and their interactions were significantly affected TPC. Duncan's multiple range test detected that interaction influenced on TPC (Table 4.23, Table 4.24). The obtained TPC results at the first harvesting time was revealed 85.4 and 83.6 % (5.56 ± 0.01, 5.49 ± 0.01 Eq Ga g) from control treatments. Total phenolic content was significantly increased on 2.5 mg L^{-1} during first callus collection time to 101.33% (6.19 ± 0.04 Eq Ga g) and 100.84% (6.17 ± 0.06 Eq Ga g).

The lowest TPC with 47.74 and 48.31 % (3.90 ± 0.01, 4.06 ± 0.05) were recorded on harvested callus at 105 days treated by a high concentration of SA (12.5 mg L^{-1}) on 2,4-D and NAA, respectively.

4.6.3 Total Flavonoid contents (TFC)

Salicylic acid increases flavonoid contents in many plants such as *C. arietinum* (Shagufta et al., 2008), *A. absinthium* (Lee et al., 2013), *P. pulcher* (Danaee et al., 2015a), *Z. spina* (Galal, 2012) and *V. nuda* (Hao et al., 2006). To determine the different effect of main factors (callus ages and various concentrations of SA) on accumulation of TFC, Duncan multiple range tests was applied. Based on the results, callus age and SA concentrations were showed statistical differences on TFC from 2,4-D and NAA. All concentrations of SA were affected individually and higher amount of TFC was observed at 2.5 mg L^{-1} SA (Appendix 24-a, b). Total flavonoid content was calculated to equivalent mg of rutin in 1 g dry weight. The analysis of variance (Appendix 22-b, Appendix 23-b) and mean comparisons (Table 4.23, Table 4.24) were revealed statistical differences between SA, callus harvesting time and their interactions on TFC.

Total flavonoid enhancement was detected from collected cali at early stage of 35 days. SA enhanced the synthesis of flavonoid content from 57.20% (2,4-D) and 58.16% (NAA) on control treatments to 63.37% (2,4-D) and 64.61% (NAA) on elicited WPM by 0.50 mg L^{-1} of SA. However, TFC was recorded significantly higher 85.32 and 86.50 % on treated callus by SA (2.5 mg L^{-1}) from 2,4-D and NAA (3.44 ± 0.01, 3.49 ± 0.02 Eq Rutin g). This pattern was observed at 70 and 105 days of callus harvesting. The lowest amount was calculated on treated callus by 12.5 mg L^{-1} of SA when harvested on days the 105 and higher concentrations of SA (12.5 mg L^{-1}) was decreased TFC.

4.6.4 DPPH Radical scavenging activity

Duncan's multiple range test detected individual effects of callus age on inhibition of DPPH on callus from 2,4-D and NAA. However, SA concentrations in 2.5 and 5 mg L^{-1} was influenced on antioxidant activity on both callus and revealed significant variances on antioxidant activity (Appendix 24). Based on ANOVA table, callus age, SA concentrations and their interactions were indicated significant differences (Appendix 22-c, Appendix 23-c). The differences between means of antioxidant activity were analyzed, compared by duncan multiple ranges and reported as means ± standard deviations illustrated in Table 4.25 (2,4-D) and Table 4.26 (NAA). However, the various concentrations of SA were improved antioxidant activity. Elicited WPM media with 2.5 mg L^{-1} of SA was exhibited a highest radical scavenging activity among the different concentrations at day 35 on callus extract from 2,4-D (97.25 ± 0.16) and NAA (95.44 ± 0.11).

Table 4.25: A mean comparison of various concentrations of salicylic acid and callus age on total phenolic, total flavonoids contents and antioxidant activity from callus water extracts (leaf explants of *G. jasminoides* induced on WPM with 2,4-D) harvested at different times

Time (Days)	**SA** (mg L^{-1})	TPC	TFC	Antioxidant
35	0	5.56 ± 0.01^{cd}	2.45 ± 0.01^{g}	77.24 ± 0.28^{cd}
	0.5	5.70 ± 0.01^{bc}	2.66 ± 0.02^{e}	83.54 ± 0.09^{bc}
	2.5	$\mathbf{6.19 \pm 0.04^{a}}$	$\mathbf{3.44 \pm 0.01^{a}}$	$\mathbf{97.25 \pm 0.16^{a}}$
	5.0	5.38 ± 0.00^{d}	2.897 ± 0.01^{b}	72.65 ± 0.06^{ed}
	7.5	4.90 ± 0.01^{e}	2.719 ± 0.01^{c}	68.55 ± 0.08^{f}
	12.5	4.60 ± 0.02^{fg}	2.486 ± 0.02^{fg}	61.33 ± 0.29^{g}
70	0	5.24 ± 0.00^{cd}	1.98 ± 0.01^{k}	68.35 ± 0.27^{e}
	0.5	5.46 ± 0.01^{c}	2.23 ± 0.01^{i}	78.13 ± 0.48^{de}
	2.5	5.83 ± 0.01^{b}	2.81 ± 0.02^{c}	84.35 ± 0.28^{b}
	5.0	4.93 ± 0.03^{f}	2.52 ± 0.01^{f}	67.06 ± 0.34^{f}
	7.5	4.54 ± 0.02^{h}	2.31 ± 0.00^{h}	61.26 ± 0.61^{g}
	12.5	4.18 ± 0.02^{j}	2.036 ± 0.02^{j}	57.59 ± 0.22^{h}
105	0	4.91 ± 0.01^{o}	1.42 ± 0.02^{o}	61.31 ± 0.27^{f}
	0.5	4.61 ± 0.33^{n}	1.59 ± 0.01^{n}	64.34 ± 0.25^{g}
	2.5	5.35 ± 0.00^{bcd}	1.82 ± 0.00^{l}	73.04 ± 0.67^{de}
	5.0	4.84 ± 0.03^{m}	1.70 ± 0.03^{m}	60.67 ± 0.17^{f}
	7.5	4.52 ± 0.04^{o}	1.25 ± 0.02^{p}	51.05 ± 0.06^{g}
	12.5	3.90 ± 0.01^{p}	1.45 ± 0.02^{o}	48.32 ± 0.55^{i}

Mean with the same letter are not significantly different at 0.05 levels.

Table 4.26: A mean comparison of various concentrations of salicylic acid and callus age on total phenolic, total flavonoids contents and antioxidant activity from callus water extracts (leaf explants of *G. jasminoides* induced on WPM with NAA) harvested at different times

Time (Days)	**SA** (mg L^{-1})	TPC	TFC	Antioxidant
35	0	5.49± 0.01^{d}	2.48 ± 0.01^{g}	76.94 ± 0.03^{c}
	0.5	5.61 ± 0.01^{c}	2.71 ± 0.02^{e}	81.65 ± 0.09^{b}
	2.5	**6.17 ± 0.06^{a}**	**3.49 ± 0.02^{a}**	**95.44 ± 0.11^{a}**
	5.0	5.25 ± 0.01^{e}	2.94 ± 0.01^{b}	70.79 ± 0.02^{e}
	7.5	4.80 ± 0.01^{hi}	2.76 ± 0.01^{d}	66.32 ± 0.29^{f}
	12.5	4.43 ± 0.01^{k}	2.53 ± 0.02^{f}	60.11 ± 0.12^{i}
70	0	5.31 ± 0.01^{e}	2.01 ± 0.00^{k}	66.57 ± 0.69^{f}
	0.5	5.53 ± 0.01^{d}	2.27 ± 0.02^{i}	76.36 ± 0.48^{c}
	2.5	5.78 ± 0.02^{b}	2.85 ± 0.03^{c}	82.578 ± 0.3^{b}
	5.0	5.00 ± 0.02^{f}	2.55 ± 0.00^{f}	65.29 ± 0.34^{g}
	7.5	4.60 ± 0.02^{j}	2.35 ± 0.00^{h}	59.49 ± 0.61^{ij}
	12.5	4.25 ± 0.02^{l}	2.07 ± 0.02^{j}	55.82 ± 0.22^{k}
105	0	4.86 ± 0.02^{h}	1.48 ± 0.01^{o}	58.59 ± 0.27^{j}
	0.5	4.93 ± 0.01^{g}	1.64 ± 0.01^{n}	63.76 ±0.25^{h}
	2.5	5.31 ± 0.00^{e}	1.84 ± 0.01^{l}	72.46 ± 0.67^{d}
	5.0	4.77 ± 0.02^{i}	1.75 ± 0.03^{m}	60.11 ± 0.17^{i}
	7.5	4.47 ± 0.02^{k}	1.28 ± 0.01^{p}	50.46 ± 0.06^{l}
	12.5	4.06 ± 0.05^{m}	1.47 ± 0.03^{o}	47.74 ± 0.55^{m}

Mean with the same letter are not significantly different at 0.05 levels.

4.6.5 Optimum auxin type in callus induction, total phenolic content, flavonoid content and antioxidant activity from salicylic acid elicitation explants

The obtained data from grown callus on WPM supplemented with 2,4-D, NAA (2 mg L^{-1}) and various concentration of SA were analyzed and showed similar results between NAA and 2,4-D. However, to find the best PGR, independent sample test was applied (Appendix 25). To evaluate the association between total phenolic and flavonoid contents with DPPH, Pearson correlation coefficient was applied (Table 4.27). The results was detected positive correlation between total phenolic, flavonoid contents and antioxidant activity from grown callus on 2,4-D and NAA. The higher relationship between antioxidant and TPC was observed on WPM media treated with SA and 2,4-D (r= 0.967).

Table 4.27: Correlation coefficients among total phenolic, flavonoid content and antioxidant activity from grown callus on WPM media supplemented with 2,4-D and NAA collected from irradiation leaf explants of *G. jasminoides* Ellis treated with various concentration of SA

	TFC		TPC	
	2,4-D	NAA	2,4-D	NAA
TPC	0.68**	0.66**		
Antioxidant	0.79**	0.78**	0.97**	0.95**

** Correlation is significant at the 0.05 level (2-tailed).

CHAPTER 5 : DISCUSSION

5.15 Sterilization and auxin establishment study on callogenesis

The leaf explant was found to be susceptible to high concentration of Clorox. The same result was reported by Danaee et al. (2015b) on *P.pulcher* as a woody plant. A low concentration of Clorox and ethanol was not effective on the woody texture; however, for the leaf explants, high concentration of Clorox resulted in a low percentage survival of explants. Among the various types of plant growth regulators such as auxin (NAA, IBA and 2,4-D) and cytokinin (Kinetin, BAP) which have been applied to produce a high frequency of callus induction in crop species, 2,4-D has been proven as one of the most efficient PGR in callus growth and induction (Dodds & Lorin, 1995; Duangporn & Siripong, 2009). In addition, NAA plays an essential role in callus formation and biomass of growing woody plant (Bonyanpour & Khosh-Khui, 2013; Sen et al., 2014). The effect of 2,4-D is in agreement with Victório et al. (2010) who reported 2,4-D produced 100% friable calli in *Phyllanthus tenellus* Roxb as a woody plant and also supported Seran et al., (2006) who obtained embryo callus from *in vitro* propagation of *Camellia sinensis* supplemented with NAA. Al-Juboory et al. (1998), Mizukami et al. (1987) and Farzinebrahimi et al. (2014) used young leaves of *G. jasminoides* for callus induction. They reported that callus was appeared after one month of culture on MS medium supplemented with various PGR's. Michailidis (1995) was reported higher callus formation and somatic embryo cells formed on MS medium supplemented with 2,4-D and NAA. The same result on *in vitro* produced somatic embryos was published by Farzinebrahimi (2012).

5.16 Gamma ray assay on callogenesis and secondary metabolite activities

Adjusting *in vitro* condition of plant cell cultures such as the light, temperature, media nutrients, pH with addition of plant growth regulators or elicitors, may effectively increase the yield of the metabolite production. The boosted production of natural compounds from plant tissue culture through elicitation was revealed a new research area with important economic benefits for pharmaceutical industries (Namdeo et al., 2007). It was detected that low doses of gamma rays encouraged cell division, growth, and development in various plants. This phenomenon, named "hormesis", which has been discussed at length for various plant species (Jan et al., 2012). Osman et al. (2013), and Gupta et al. (2010) reported the relevance of plant growth regulators on callus weight. There are many reports on effect of low and high doses of gamma ray on callus induction and weigh. Agisimanto et al. (2016) reported that embryo callus from *Citrus reticulata* was appeared when irradiated at 10 and 20 Gy, whereas exposure to higher doses completely suppressed callus formation. Based on Chen et al. (2001), different varieties of japonica and indica rice demonstrated that the dose of 20 Gy gamma rays was improved the callus formation and this dose showed significant stimulation effect on regeneration of green plants from rice anther culture. Kulkarni et al. (2004) revealed that 18 Gy of gamma ray was improved callus formation, fresh and dry weight of banana cv. grand naine, whereas 40 Gy gamma showed lethal effect. Gamma irradiation at 25 Gy stimulated development of *P. americana* and inhibited the number of intermediate and mature stages of somatic embryos (Witja & Litz, 2004). The tissue responses were controlled by some parameters, including genotype, harvesting time, culture medium, PGR and the intact plant (Puchooa, 2005). Hasbullah et al. (2012) reported gamma ray may effect the cell wall and membrane and also can decrease callus growth at high doses.

Influence of gamma irradiation prohibit water intake and may be caused by the lesser amount of synthesis of endogenous hormones (auxin) to the cells. Decreasing of carbon gain with higher dose of gamma irradiation has also been reported by Kim et al. (2005) and Neelam et al. (2015). They suggested that gamma radiation blocks carbon translocation. The effects of gamma ray doses is varied; Ali et al. (2007a) reported that 10 and 20 Gy of gamma rays as optimum dose in callus fresh and dry weight in *S. officinarum*, but Puchooa (2005) observed the of lethal 15 Gy gamma ray on Anthurium and Moallem et al. (2013) reported no variation was detected after radiation on callus of *R. canina* after six weeks. According to Bajaj et al. (1970), development of callus tissues was dropped when exposed at 10-50 Gy gamma irradiation and extremely declined when subjected to higher irradiation doses (200-300 Gy). The growth was lethal at 400 Gy of irradiation dose. Similar results were also obtained on irradiated callus of *Gerbera jamesonii* (Hasbullah et al., 2012). Some physiological symptoms in a large range of exposed plants to gamma rays have been reported.

Effect of gamma ray with low doses was improved seed germination in *Capsicum annuum* (Kim et al., 2004). Wi et al. (2007) revealed that low dose irradiation slightly expanded mitochondria size. Farzinebrahimi et al. (2014b) reported that low dose of gamma irradiation was increased the carotenoid and growth in *Orthosiphon stamineus*. Using of gamma ray may cause a desire variation in ornamental or phytochemicals. Jamshidi et al. (2014) obtained higher yield in fresh and dry callus in *T. foenum* using NAA and 2, 4-D. The callogenesis result is in agreement with the previous report of optimally responding from NAA and 2,4-D supplemented in WPM on *G. jasminoides* (Farzinebrahimi et al., 2014).

Gamma irradiation is one of the most broadly utilized experimental approaches to enhance the synthesis and accumulation of secondary metabolite products. Interacting atoms and molecules with gamma radiation generate free radicals and modify essential components of plant cells. The protein synthesis, chlorophyll, lipid peroxidation, enzyme activity and accumulation of phenolic compounds are influenced by exposing of some plants to gamma rays (Hameed et al., 2008). Plant reactions to different stresses is varied (Ashraf et al., 2003) and it is confirmed that some range of gamma doses is strongly related to plant characteristics such as species, variety or cultivar, the developmental stage at the time of irradiation and even among the genotypes of the same species (Lee et al., 2009; Wi et al., 2007). Interaction of gamma rays with atoms or molecules resulted free radicals in cells, which depending on the irradiation level can damage or modify important components of plant cells and have been reported to affect the morphology, anatomy, biochemistry, and physiology of plants. These effects include changes in the plant cellular structure and metabolism such as enlargement of thylakoid membranes, variation in photosynthesis, inflection of the antioxidative activity, and accumulation of phenolic compounds (Jan et al., 2012). The results were revealed the probable mutations may have taken place in the *G. jasminoides* tissues due to the gamma radiation (Neelam et al., 2015; Puchooa, 2005). Therefore, most of mutagen has a more proficient dose that yields the high level of mutagenesis with lower somatic effects. Leaf explants irradiated with 5 and 10 Gy gave the higher response in terms of callus fresh weight. This *in vitro* condition result suggests that the type of chromosomal variations that took place eventually produced a change in the morphology. Hasbullah et al. (2012) and Esfandiari et al. (2008) reported high doses of gamma rays interrupts the biosynthesis of metabolites and activity.

Plant phenolic compounds can exploit as antioxidants, structural polymers (lignin), attractants (flavonoids and carotenoids), UV screens (flavonoids), signal compounds (salicylic acid, flavonoids) and defense response chemicals (tannins, phytoalexins). In many studies, phenolic compounds demonstrated higher antioxidant activity than antioxidant vitamins and carotenoids (Barros et al., 2008; Barros et al., 2007; Kortei et al., 2014; Yang et al., 2002). This results revealed that the accumulation of total phenolic content in the grown callus on 2,4-D and NAA was considerably influenced by various levels of gamma irradiation, and it had a significant ($p< 0.05$) effect on total phenolic of exposed treatments. In this study the irradiation treatments with 20 Gy gamma ray was significantly increased total phenolic content (6.75%) compared to non-irradiated factors. The previous studies have been proven that gamma ray influenced on the phenolic content. The soluble phenol in seed of *Eruca vesicaria* subjected to gamma ray was increased more than 60% (Moussa, 2006). Štajner et al. (2007) reported that 10k Gy gamma irradiation was raised 10% of total phenol in Soya been.

Taheri et al. (2014) stated that callus from leaves of *C. alismatifolia* gave highest total phenolic content when radiated with 20 Gy. Gamma irradiation at 10 Gy increased phenolic content in cinnamon (Cantos et al., 2000) and irradiation at 20 Gy boosted TPC in callus culture of *Rosmarinus officinalis* (El-Beltagi et al., 2011). Variyar and Bandyopadhyay (1998) reported that the phenolic content of soybean was increased when exposed to gamma irradiation from 15 to 20 Gy. Radiation can be affected by the direct or indirect mechanisms. Radiation hits the DNA and hits the molecular structure directly which leads to cell death or damage. In the indirect mechanism, the radiation hits the water molecules, the major constituent of the cell, and other organic molecules in the cell. The radiolysis of water yields free radicals such as hydroxyl (HO) and alkoxy (RO2).

Lee et al. (2009) revealed the increasing of total phenolic and flavonoids contents in irradiated plants is due to breaking the radicals to presence bonds such as the glycosidic of procyanidin trimer, tetramer and hexamer and leading to the formation of procyanidin monomers. Effect of gamma irradiation on enhancement of TFC was reported by some researchers (El-Beltagi et al., 2011; Wi et al., 2007). Moghaddam et al. (2011) revealed that flavonoid contents *in vitro* culture of *Centella asiatic* subjected to gamma irradiation at 20–30 Gy significantly increased after 8 weeks. Saidalahl et al. (2015) and Kortei et al. (2014) confirmed enhancement of TFC in plants by gamma radiation. TFC was extremely decreased along the callus age; it was recorded that 58% when harvested at 35 days, and remarkably was descent to 45 and 29 % at the days 70 and 105, respectively. Taheri et al. (2014) reported increasing of TFC in leaves of *C. alismatifolia* from 1.61 ± 0.48 Eq Rutin g to 2.87 ± 0.31 Eq Rutin g when exposed to 20 Gy gamma ray. The escalation in total flavonoids contents in irradiated plants at 20 Gy was reported by Ezzati et al. (2015) and El-Beltagi et al., (2011). Lee et al. (2009) mentioned increasing of TFC in radiation by gamma ray. The association between total phenolic and flavonoid contents with DPPH antioxidant has been published by many researchers (Agisimanto et al., 2016; Ahmad & Roshan, 2015; Ali et al., 2016; El-Beltagi et al., 2011; Jan et al., 2013; Kortei et al., 2014; Kukimura, 1986; Lee et al., 2009; Taheri et al., 2014; Wi et al., 2007).

The increment of total phenolic content under gamma ray could be degraded the larger compounds into smaller ones and assigned to the release of TPC from glycosidic forms (Harrison & Were, 2007; Taheri et al., 2014). Abo Hameed et al. (2013); Ahmad and Roshan (2015); Ali et al. (2016); El-Beltagi et al. (2011); Ezzati et al. (2015); Jaisi (2012); Kovács and Keresztes (2002); Moallem et al. (2013); Moghaddam et al. (2011); Pawar et al. (2010); Wi et al. (2007) reported similar results of the influence of gamma irradiation on DPPH antioxidant activity. *G. jasminoides* Ellis is a rich source of antioxidant. The high amount of antioxidant from *in vivo* leaf of *G. jasminoides* has been detected found

by Chen et al. (2010); Choi et al. (2007); Debnath et al. (2011); Hatzilazarou et al. (2006). However, this species showed high antioxidant content in *in vitro* leaf explant (Farzinebrahimi, 2012; Farzinebrahimi et al., 2014; Sayd et al., 2010). Furthermore, Uddin et al. (2014) suggested that this plant can be used as a control (vitamin C) in DPPH assay, due to high amount of TPC, TFC and DPPH inhibition. The recorded total flavonoid content from NAA detected higher than 2,4-D. Overall, the results demonstrated equal variances or homoscedasticity between grown callus on 2,4-D and NAA.

5.17 Methyl jasmonate assay on callogenesis and secondary metabolite activitis

In the present study, the synthetic auxin (2,4-D or NAA) were employed to induce the friable callus. However, no callus induction was observed in the PGR free medium. The stress signals are observed by several receptors through the transduction to multiple secondary messengers that finally lead the protective responses at the whole-plant level (Sasaki-Sekimoto et al., 2005; Shi et al., 2014; Shi & Chan, 2014). Plants have developed defensive systems under various environmental stresses that support plant tolerance even at high levels of potentially toxic compounds (Momoh et al., 2002; Pilon-Smits, 2005). Recently, extensive work has been carried out on the exogenous application of various plant growth regulators to improve plant stress tolerance to different abiotic stresses (Ali et al., 2015; Horváth et al., 2007; Jiang & Zhang, 2001). Similarly, exogenous application of MeJA either activates or inhibits plant growth under stress condition depending on its applied concentration and plant species. The friable callus formed in the PGR medium and the proliferation of cells was accelerated in the medium by a lower concentration of MeJA. The results of this research are in accordance with the previous studies reported by Biondi et al. (2001), Farooq et al. (2016), Gumerova et al. (2015) and Ram et al. (2013), who founded that MeJA in low concentration 0.22 mg L^{-1} exhibited positive

effects on biomass. Ncho et al. (2016) reported that TPC in *M. acuminata* was increased more than 2 times when explants maintained more than control. The same result on grapevine was also reported (Belhadj et al., 2006). Methyl jasmmonate with 4.5 mg L^{-1} caused explant to death, however, in 10 μM inhibited total phenolic content and dropping TPC was recorded at the lowest amounts when callus harvested at 105 days. Synthesize of secondary metabolite in plants is a common response to biotic and abiotic elicitors and these compound accumulation could be encouraged by exposure to elicitors (Zhao et al., 2005). The MeJA treatment of young red and black raspberry fruits was increased anthocyanins and phenolic compounds (Wang & Zheng, 2005).

Patel and Krishnamurthy (2003) reported that *C. roseus* with longer incubation period of elicitation showed higher yields of ajmalicine on elicitation. Similar observations were reported from various studies by Rijhwani and Shanks (1998) and Kulkarni et al. (2004). Methyl jasmonate (MeJA) is a plant hormone involved in regulating plant-herbivore interactions and using in the culture medium as an elicitor that improved the anthocyanin production in *V. pahalae* (Fang et al., 1999). Exogenous jasmonate has been found to trigger high concentrations of alkaloids, terpenoids and phenolics in many plants as well as have been shown to exhibit morphological and physiological effects (Mathew & Sankar, 2014; Tachinbana et al., 2007). MeJA has induced anthocyanin accumulation in *A. thaliana* (Peng et al., 2011), strawberry fruits (Fang et al., 1999), *V. vinifera* (Zhang et al., 2002) and in *Thevetia peruviana* (Zabala et al., 2010). The results on treated media by methyl jasmonate and association of callus age with TPC is consistent with previous reports by Danaee et al. (2015), Zabala et al. (2010) and Xiao et al. (2009). The cell growth exhibited a lag phase or slow growth period in the first 35 days. The flavonoid production is related to the cell growth and its content reached to maximum amount during stationary or declining phase at first stage (35 days).

In addition, TFC in cell suspension culture of *G. jasminoides* can be further improved by the supplementation of effective elicitors (Gadzovska et al., 2007; Zhao et al., 2010). Martins and Veronica (2015) and Yang et al. (2013) reported flavonoid content in *G. jasminoides.* The results of MeJA concentrations and callus age are in agreement with Wang et al. (2015) who reported higher flavonoid content in cell culture of *H. perforatum* on MeJA elicited (2 mg L^{-1}) media of and harvesting at an early stage of 20 days compared to 60 days. However, this finding also is in line with Danaee et al. (2015) which reported the similar effect of methyl jasmonate on callus age and TFC on *Phyllanthus pulcher*. *G. jasminoides* Ellis is rich of antioxidant and its value from leaf of the plant was studied by Farzinebrahimi et al. (2014), Sayd et al. (2010) and Debnath et al. (2011). There are some reports on low antioxidant activity in *in vitro* condition from *Brassica oleracea* (Farzinebrahimi et al., 2012) and *Asparagus officinalis* (Khorasani et al., 2010). The current finding proved the previous reports of Fan et al. (2011) and Sarmah and Baishya (2014) that about 80 % inhibition of *G. jasminoides* and Uddin et al. (2014) who revealed that this plant is capable to use as a control for DPPH assay because of higher phenolic content. These results showed positive correlation between total phenolic, flavonoid contents and antioxidant activity on 2,4-D and NAA. Furthermore, a higher correlation of antioxidant on TPC (r= 0.964) and TFC (r= 0.955) from elicited media with methyl jasmonate and NAA was detected. The association between total phenolic and total flavonoid content as two major components in antioxidant activities has been proven and is consistent with Danaee et al. (2015), Fang et al. (1999), Jalalpour et al. (2014). Antioxidant and total phenolic content from MeJA elicited media supplemented with 2,4-D was demonstrated higher amount compared to NAA. The results reveald that callus weight was in associated with TFC and total flavonoid contents, the fresh and dry weight of callus on WPM supplemented with NAA and MeJA, observed slightly higher than 2,4-D.

5.18 Salicylic acid assay on callogenesis and accumulation of secondary metabolite activities

Callus induction on the media free of SA was considered as control. Salicylic acid in high concentration of 20 mg L^{-1} showed a similar response with 4.5 mg L^{-1} of MeJA. Necrosis and died callus was observed after second weeks of initial callus culture (Appendix 20, Appendix 21). The cell growth revealed exponential phase, maximum fresh and higher dry weight at the final period of callus collection (105 days) and also showed individual effects on fresh and dry weight. However, callus significantly was increased on elicited media by 3 mg L^{-1} of SA when harvested on 105 days. However, the maximum callus growth was probably associated with SA concentration and the higher growth was observed on elicited media by 2.5 mg L^{-1} of SA. Effect of SA on callus growth was reported by Vlot et al. (2009), Klessig and Malamy (1994) and Salih and Al-oubaidi (2014). Rivas and Plasencia (2011) described the plant species, developmental stage, and concentrations are in associated with SA effects. Otherwise, salicylic acid was reported on embryogenesis, callus fresh and dry weight in *Ziziphus spina-christi* as a woody plant (Galal, 2012). Ibrahim et al. (2012) mentioned that the lower concentration of SA highly increased FW and DW of callus in *Phoenix dactylifera*. According to Hosseini et al. (2009), synthesis of growth retarding hormone ethylene is supported by a higher concentration of SA.

The results of this study are in agreement with earlier reports by Hao et al. (2006), Zhao et al. (2005) and Hosseini et al. (2011) who reported SA increased the dry weights of calluses and this increase may related to the acceleration processes connected to the cell division and its elongation. The elicitor type, concentrations and exposure time of elicitation are the achievement factors to gathering natural compounds (Rodrigues et al., 2014).

However, addition of salicylic acid as a member of phenolic group to media may increase the phenolic contents and antioxidant in plants. Shagufta et al. (2008) revealed that phenolic content improved both quantitatively and qualitatively with the increase in age of callus. Secondary metabolites, including phenolics, defend plants against a variety of herbivores and pathogenic microorganisms as well as various kinds of abiotic stresses (Dixon, 2001; Dixon & Paiva, 1995; Simic & Delaunay, 2012). Therefore, observing changes in phenolic contents in elicited plant material reported in common bean (Campos et al., 2003) and *H. perforatum* (Simic & Delaunay, 2012). The current results on effect of callus age on accumulation of total phenolic content is in agreement with Li et al. (2007), Vakil and Mendhulkar (2013), Rodrigues et al. (2014) and Danaee et al. (2015) who reported that increasing in TPC over time, reaching higher than control. The similar results of increasing fresh and dry weight of callus after addition SA were reported by some researchers and this finding are consistent with Delaney et al. (1994), Prakash and Srivastava (2008), Srivastava and Dwivedi (2000).

Horvath et al. (2007) mentioned that the salicylic acid is a messenger molecule in plants and encourages plant tolerance against biotic and abiotic stresses. However, it plays an imperative role on growth and development, ion uptake, transport and membrane permeability (Simaei et al., 2012). Matkowski (2008) reported that secondary metabolite production in a cell with addition of salicylic acid to the nutrient medium under *in vitro* condition was induced and increased. Accumulation of flavonoid content in alfalfa callus on SA elicitation media was studied (Cvikrova et al., 1991) and Dubravina et al. (2005) also reported the enhanced synthesis of flavonoids in cell dedifferentiation and growth *Taxus baccata in vitro* condition. Increasing of flavonoid compounds and dropping of TFC by higher concentration of SA in this study was similar with Rudell and Mattheis (2002) and Shabrangi and ArabbeigiJazi (2014) who found that the flavonoid content was decreased dramatically by higher concentration of salicylic acid. The results obtained in

the present work also are in agreement with the report of Babel et al. (2014) where the same observation was recorded for *C. borivilianum* cultures. It has been verified that salicylic acid is enhancing secondary metabolites and antioxidant activity in different plants species (Ghasemzadeh et al., 2012; Idrees et al., 2011; Tajik & Niknam, 2015). The positive correlation between the polyphenolic content of the extracts and its antioxidant activity is well documented (Cai et al., 2011; Elias et al., 2006; Huang & Mau, 2006; Yam et al., 2007). Therefore, total phenolic compounds in callus extracts might explain their high antioxidant activities. Inhibitory percentage of DPPH was dropped when elicited callus by a high concentration of SA harvested after 35 days.

The association between total phenolic and flavonoid content as two major components in antioxidant activities has been proven and is consistent with Danaee et al. (2015); Fang et al. (1999) and Jalalpour et al. (2014). The antioxidant value and total phenolic content from grown callus on elicited WPM media supplemented with 2,4-D and SA displayed higher amounts than NAA. The results of total flavonoid contents, the fresh and dry weight of callus on elicited media supplemented with NAA and SA, showed slightly higher than 2,4-D. Additionally, callus weight was associated with TFC and TPC.

CHAPTER 6 : CONCLUSION

The present study evaluated physical and chemical abiotic elicitors on induced callus of *Gardenia jasminoides* Ellis by using two different auxins (2,4-D and NAA) and collected at different harvesting time. Furthermore, callus extracts were analyzed for total phenolic and flavonoid contents and antioxidant activities. For investigation of explant sterilization the combination of 75% sodium hypochlorite (10 min) and 0.1% mercuric chloride (3 min) resulted in the lower contamination rate (8%) and the highest percentage of survival for the explants (73%). For effect the study of NAA and 2,4-D on callogenesis and total phenolic, flavonoid contents and antioxidant activity, it was found that despite the better callus induction compared to another auxin, no difference was detected on fresh and dry weight, callogenesis and secondary metabolite content from leaf explant of *Gardenia jasminoides* Ellis. This is the first research to study effect of gamma ray, methyl jasmonate, and salicylic acid on callus induction of *Gardenia jasminoides* Ellis on woody plant media (WPM). The investigation of auxin concentrations on callus culture revealed that among various concentrations, 2 mg L^{-1} of 2,4-D and NAA supplemented to WPM media showed 100% and 97.3% callus formation, respectively. For the effect of methyl jasmonate on callus growth total phenolic, flavonoid contents and antioxidant activity, callus culture on WPM supplemented with 2 mg L^{-1} 2,4-D and NAA elicited by 0.22 mg L^{-1} of MeJA showed the highest callus fresh (15.391, 15.71 g) and dry (0.901, 0.925 g) weight when harvested at 70 days. Grown callus on WPM addition with 2,4-D and 0.22 mg L^{-1} MeJA resulted in significant responses of TFC (3.38) and antioxidant activity (100) when harvested at days 35 days. However, formed callus on the same media after 70 days showed high TPC (7.301). The younger formed callus (35 old days) on WPM with NAA and the same concentration of MeJA resulted higher TPC (6.537), TFC (3.356) and antioxidant activity (99.332).

Effect of Salicylic scid on callus growth and secondary metabolite content revealed that addition of salicylic acid in a lower concentration of 2.5 mg L^{-1} and 2,4-D or NAA to WPM gave optimum response in callus fresh weight (14.9, 15.4 g) and dry weight (0.88 and 0.91 g) after days 105. WPM supplemented with 2.5 mg L^{-1} SA, 2,4-D or NAA revealed higher total phenolic contents (6.19, 6.2), total flavonoid contents (3.44, 3.49) and antioxidant activity (97.25, 95.44) when callus harvested at 35 days.

Recommendations for future work

Combination of abiotic and biotic elicitor is suggested. In addition, other aspect of studies such as precursor of crocin to study Crocin synthesis via leaf suspension culture, and evaluation of metabolomics of *Gardenia jasminoides* Ellis by liquid chromatography-mass spectrophotometry (LCMS) or nuclear magnetic resonance spectroscopy (NMR) are recommended.

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