Using Tissue Culture as an Alternative Source of Polyphenols Produced by *Ficus carica L.*

By

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Abstract

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Plants have long been used as sources of pharmaceuticals or other commercial products. Many of these products are difficult to synthesize at affordable prices. Furthermore, the extensive testing that is required of synthetic products in order to meet safety standards, compared to requirements for natural products, has stimulated interest in replacing many synthetic chemicals, especially food additives, by natural plant extracts. “Plantations” of medicinal plants, as with any cropped plants, are vulnerable to diminished yields due to outbreaks of disease or unfavorable changes in growing conditions, resulting in considerable economic loss to growers. Furthermore, some plants can not be grown as crops in the geographical areas where there is the most need for their products. In such cases, an alternative means of obtaining natural products from plants is through plant tissue culture. The fruit and leaves of *Ficus carica* (the edible fig) produce many polyphenolic antioxidants of potential therapeutic value. However, the plant is vulnerable to fig mosaic virus disease, and does not grow well out of semitropical and Mediterranean climates. In this work, *Ficus carica* tissue cultures were investigated as an alternate source of antioxidant polyphenols. It is well know that ntioxidants have anticarcinogenic, antibacterial, and antiviral properties and can be used as food

supplements. In an effort to determine culture conditions that resulted in the production of polyphenols comparable to those produced by intact plants, chemical and physical factors that affect yield, such as basal media composition, light intensity, temperature, growth hormones, and elicitors were tested.

As a result of this research, callus cultures were developed that contained an average of 4.26% polyphenols of their dry weight. Analysis based on TLC and HPLC, showed that the main antioxidants found in callus tissue are apigenin, isoquercitrin, astragalin (kaempferol glycoside), rutin, emodin, cyanidin, caffeic acid, tannic acid, chlorogenic acid, quercitin, kaempferol, taxifolin, catechin and epichatechin. These results show that tissue cultures of *Ficus carica* can be used successfully for production of antioxidant compounds used as food supplements. It is also shown that tissue cultures initiated from vegetative tissue can produce polyphenols usually found in fig fruit as well as in leaves and other parts of the plant.

Acknowledgments:

I take this opportunity to convey my heartfelt thanks to my advisor, Dr. Dominick V. Basile for suggesting the topic of my thesis, for sharing my enthusiasm for this dissertation and for his continuous advice, guidance and the patience for so many years. He has been very generous with his vast resources and creates a pleasant and unique environment of cooperation in his laboratory, constantly reinforcing the desire and excitement for scientific work and research.

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CONTENTS

List of Tables

List of Figurers

CHAPTER 1: INTRODUCTION

The medicinal properties of some plants were known to some people for thousands of years BC. As long ago as 5000 BC, Swiss and Central European Lake-dwellers cultivated over two hundred different plants, among which many such as (*Papaver somniferum L*., *Sambucus ebulus L., Fumaria officinalis L., Verbena officinalis Saponaria officinalis, and Menyanthes trifoliata L*) possess medicinal compounds (Sigerist, 1951). Of the 257 drugs mentioned in "The Medicine of Hippocrates," by Dierbach, "only 27 are no longer listed as medicinal plants today" (Riddle 1987). Theophrastus in his work, "Inquiry into Plants ", write of the many plants that were used in medicine and their uses (Hort, 1948). Today, about 7,000 of the drugs prescribed by physicians are derived from plants, and more than 3000 plants have been identified by the United States National Cancer Institute as having anti-cancer properties.

The demand for large quantities of pharmaceuticals has raised the interest of scientists to chemically synthesize them. Many of these plant products, however, have complex structure and the cost of their synthesis makes this approach uneconomical. Morphine, for example, is still extracted from *Papaver somniferum*, quinine from *Cinchona* trees, and important cardenolides such as digitoxin and digoxin from *Digitalis*. Food additives, like antioxidants, when prepared synthetically have to undergo expensive testing to meet the required safety standards. Even when compounds such as the antioxidants monoglyceride citrate, tert-butyhydroquinone, and n-propylgallate, extensively used in food industry, are synthetically prepared at an affordable cost, consumers are reluctant to accept them, and the desire for natural food additives has been constantly increased

(Aruoma, O.I., 1994). Furthermore, there is a large portion of plants not extensively surveyed for new biologically active compounds that can be used as pharmacological leads for new molecular designs. Plants, therefore, will continue to be a principal source of known and novel chemical compounds (Baladrin et al., 1985; Miller, J.S.et al., 2000).

At present phytochemical products are largely obtained from plants grown in commercial plantations. Some of these plants, for example *Taxus chinensis*, the bark of which is used for the production of the anticancer drug Taxol^{tm}, takes many years to grow to the stage at which the bark is worth harvesting. Monocultures of plants are more susceptible to disease and all cropped plants are vulnerable to unfavorable changes in environmental conditions such as drought that reduce the quantity and or quality of the products for which they are cultivated. For all these reasons, plant tissue cultures have been considered as alternative sources of useful plant products. Plant cells cultured in vitro, consequently, plant tissue cultures, have been found capable of producing many of the same compounds that the parent plants produce (Parr et al., 1988; Basile et al., 1993; Nair MSR et al., 1986; Jianyong and Jian-Jiang Zhong, 1999).

Plant tissues are usually cultured on media consisting of inorganic macronutrients and micronutrients, vitamins, growth regulators with or without solidifying agent (Murashige and Skoog,1962; Gamborg et al., 1968; Shenk and Hildebrandt, 1972). Determining the optimal combination of nutrients, growth hormones, pH, photoperiod, light intensity, temperature, and incubation period are crucial factors required to induce the excised tissues to synthesize the desired products (Parr, 1988). Elicitors (agents that trigger defense responses) also can induce the synthesis and/or increase the yield of secondary products in tissue cultures. Fungi, bacteria or other organisms that might be pathogens of

the parental plant often serve as sources of elicitors. Suitable elicitors can be autoclaved, and the defense-triggering components purified and added at known concentrations to the culture media (Parr, 1988; Brodellius et al., 1989; Spolansky et al., 2000). The production of anthraquinone, for example, was increased by 50% when suspension cultures of *Cinchona ledgeriana* were treated with heat-killed mycelia of *Phytophthora cinnamoni* (Parr, 1988). Similarly, Taxol^{tm} yields were doubled when suspension cultures of *Taxus chinensis* were treated with 40 mg/l of fungal elicitor (*Aspergillus niger*) isolated from the inner bark of Taxus (Chuangui, Wang et al., 2001).

In this work, tissue cultures from excised petioles of *Ficus carica* incubated under a variety of carefully controlled conditions are used to produce measurable quantities of antioxidant polyphenols. *Ficus carica L.* (Moraceae), common fig plant, is mostly grown in tropical, semitropical, and temperate climates like Mediterranean countries where the best fruits are produced (Condit 1941, 1947, 1955). Figs are mostly preserved dry because fresh fruits are very perishable (Kim et al., 1992; Budavari 1996). They are very rich in nutrients and especially in polyphenols (Edenharder et al., 1998; Vinson 1999), but because of their vulnerability to spoiling, they are not readily available to consumers and possibly not known to many people living in regions where the plant does not grow. The objective here is to determine whether tissues isolated from *Ficus carica* can be cultured under conditions that result in yields of polyphenols comparable to those presently be obtained from field-grown figs sufficient to be used as food supplements or other beneficial uses. To this end, the main polyphenols in these extracts are identified .

Polyphenols are among the secondary plant metabolites with manifold functions in plants. They may inhibit insect feeding, be toxic to pathogenic bacteria, fungi, and

viruses as well as being attractants to pollinators and seed dispersants. They also may help prevent development of cancer in humans by their antioxidant, free radical scavenging, antiprolifiration, and antiprogression activities (Kellogg et al., 1994; Rijnkels et al., 1997; Sachiko et al, 1998).

CHAPTER 2: LITERATURE REVIEW

Polyphenolic compounds

Natural phenolic compounds are secondary plant metabolites that are wide spread in the plant kingdom. They are derived primarily from phenylalanine in plants via the shikimate pathway and range from simple phenols and phenolic acids to polymerized polyphenols. “Phenols” or “polyphenols” can be defined as substances possessing an aromatic ring in which one or more hydrogens are replaced by hydroxyl groups, including esters, methyl ethers, and glycosides (Harborne, 1980). Most phenolics have two or more hydroxyl groups. In phenols, however, the hydroxy group is attached to an unsaturated carbon nucleus and they are mostly weak acids due to dissociability of the _OH group. They are bioactive substances found extensively in fruits and other plant parts eaten by people. They form hydrogen bonds if no steric inhibition due to side chains occurs and they have the ability to form chelate complexes with metals and are easily oxidized to form polymers. The darkening of cuts of plant parts is a result of this reaction, which actually may have an inhibiting effect on plant growth. Most phenolics are formed from more than one ring, and this affects basic properties. Some scents like the coumarins, cinnamic acid, sinapinic acid, the coniferyl alcohols and others (phenylpropanol derivatives) can also act as intermediates of the biosynthesis of lignin, the primary substance of wood (Haslam, 1996,1989; Haslam et al., 1992; Harborne and Baxter, 1999). Table 2.1 shows the most important classes of phenolic compounds.

Table 2.1. The most important classes of phenolic compounds in plants (according to J.B. Harborne, 1980)

Number of C-atoms	Basic skeleton	Class	Examples
6	C6	simple phenols benzoquinones	Catechol, hydroquinone 2,6- Dimethoxybenzoquinone
7	C6 - C1	phenolic acids	Gallic, salicylic
8	C6 - C2	acetophenones, tyrosine derivatives phenylacetic acids	3-Acetyl-6-methoxybenzaldehyde Tyrosol p-Hydroxyphenylacetic
9	C6 - C3	hydroxycinnamic acid polypropene, coumarin, isocoumarin, chromones	Caffeic, ferulic Myristicin, eugenol Umbelliferone, aesculetin Bergenon Eugenin
10	C6 - C4	napthoquinone	Juglone, plumbagin
13	C6 - C1 - C6	xanthone	Mangiferin
14	C6 - C2 - C6	stilbene, anthrachinone	Resveratrol Emodin
15	C6 - C3 - C6	flavonoids, isoflavonoids	Quercetin, cyanidin Genistein
18	(C6 - C3)2	lignans, neolignans	Pinoresinol Eusiderin
30	(C6 - C3 - C6)2	biflavonoids	Amentoflavone
n	(C6 - C3)n (C6)n (C6- C3- C6)n	lignins catecholmelanine condensed tannins	

Hydroquinone (Figure 2.1 A) is a simple aromatic phenolic compounds in which two atoms of hydrogen are replaced by two hydroxyl groups. Its derivative sesamol (Figure 2.1 B), probably the most widespread diphenol, found in sesame oil, "exhibits antimutagenic activity against oxygen species mediated mutagenicity" (Kaur I.P. and A. Saini, 2000), and its derivative sesaminol (not shown) is a powerful antioxidant (Van Sumere, C. F., 1989).

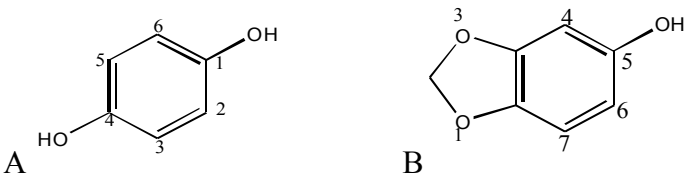
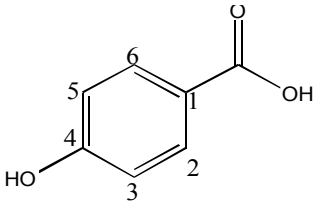
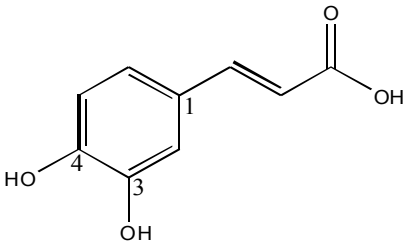


Figure 2.1. A; Hydroquinone, C6 H6 O2. B; Sesamol

Phenolic acids comprises a diverse group including hydroxybenzoic and hydroxycinnamic acids (Figure 2.2), found in most plants and have been the subject of many medical, biological and chemical studies. Phenolic acids in plants are not found in free form. They may be found in food plants as esters or glycosides conjugated with compounds such as flavonoids, hydroxyfatty acids, alcohols and glucosides. The carboxyl groups are very reactive. Most convert into esters or amides if combined with aliphatic alcohols and phenols or amino compounds, respectively. The most important derivatives of hydroxycinnamic acid are p-coumaric, caffeic and ferulic acids, and most frequently they occur in conjugated forms as esters rather than glycosides. A member of this group, chlorogenic acid, is mainly responsible for enzymatic browning, especially in apples and peas (Eskin, 1990). A degradation of it (1,3,4,5-Tetrahydroxycyclohexanecarboxylic acid), has been implicated in the astringency of coffee. The concentration of quinic acid increases with the thermal degradation of chlorogenic acids. Of the hydroxybenzoic derivatives, vanillin is best know for its flavor. Gallic acid is in an esterified form in tea catechins . It may occur in soluble form in plants either as quinic acid esters or hydrolysable tannins (Nishimura, H. et al., 1984).



p-hydroxybenzoic acid



Caffeic acid

Substituent	Hydroxybenzoi acid derivatives	Hydroxycinnamic acid derivatives
2-OH	salicylic acid	o-coumaric acid
4-OH	p-hydroxybenzoic acid	p-coumaric acid
3,4-di-OH	procatechuic acid	caffeic acid
3-OCH_3 4-OH	vanilic acid	ferulic acid
3-OH 4-OCH3	iso-vanilic acid	iso-ferulic acid
3,4,5-tri-OH	gallic acid	

Figure 2.2. Some of the primary hydroxybenzoic and hydroxycinnamic acids derivatives. If the hydroxy groups in p-hydroxybenzoic acid and caffeic acids rings are displaced and the right hydrogens are replaced by the respective substituents, different phenolic acids are formed as is shown in this figure.

A good example of naphthoquinone is plumbagin (Figure 2.3 A), which may have chemotherapeutic properties by inducing cell death in human cervical cancer cell line, ME-180 (Priya Srinivas et al., 2004). Mangiferin (Figure 2.3 B) is a xanthone polyphenolic compound (C19 H18 O11) which has been found to protect cardiac and renal tissues from oxidative damage in rats induced by streptozotocin (Muruganandan, S. et al., 2002).

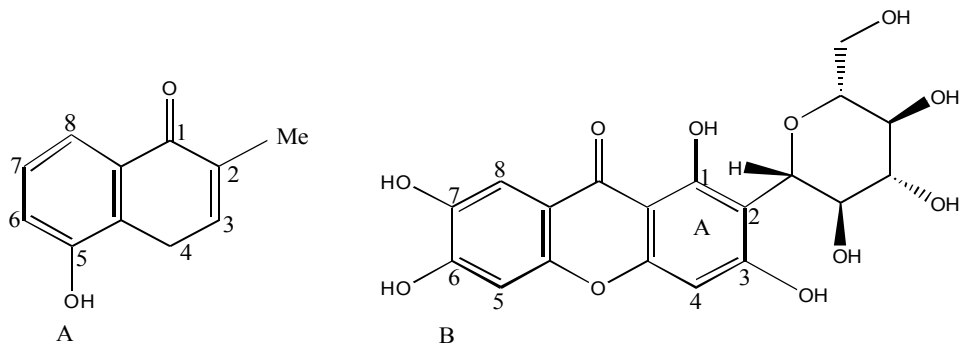


Figure 2.3. A, Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone). B, Mangiferin

Resveratrol (Figure 2.4), found primarily in red wine which contains about 160 μg/ounce, is a stilbene antioxidant with anti-inflammatory properties and potential for the prevention of many types of cancer (Sanders and McMichael., 1998).

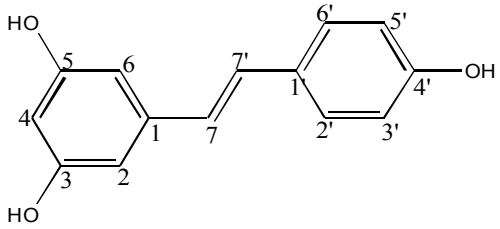


Figure 2.4. Resveratrol, a potent polyphenolic antioxidant

Flavonoids have a common phenolic carbon skeleton, C6-C3-C6 (Figure 2.5, page. 10). More than 4000 flavonoids have been identified (Hollman et al., 1997a), and many of them appear to be important components of many fruits and vegetables. They differ in biological properties with the other phenolics mainly due to oxidation pattern of the central pyran ring. In plants they are coupled to other molecules, often to glucosyl residues. They are responsible most of the water soluble pigments of fruits and vegetables, and together with the rest of phenolic compounds and other secondary metabolites protect plants from diseases, ultraviolet light, and oxidative damage and probably perform the same function for humans. There is a large variety of flavonoids

and are considered as the most important group of phenolics in food (Harborne and Baxter, 1999. The variability is mainly based on the hydroxylation or methylation pattern of the three ring systems. A correlation between two flavonoids points usually to a relationship between the producing plant species, proven to be useful traits for the study of plant phylogeny. They divide into four main subgroups, flavones, flavanones, catehcins and anthocyanidins (Table 2.2, Figure 2.5).

Table 2.2. Main groups of flavonoids and their constituents found in plant food (Nijveldt, RJ et al., 2001)

Flavones	**Flavanones**	**Catechins**	**Anthocyanidins**
Apigenin	Fisetin	Catechin	Cyanidin
Chrysin	Hesperetin	Epicatechin	Delphinidin
Kaempferol	Narigin	Epigallocatechin gallate	Malvidin
Luteolin	Naringenin		Pelargonidin
Myricetin	Taxifolin		Peonidin
Rutin			Petunidin
Sibelin			
Quercetin			

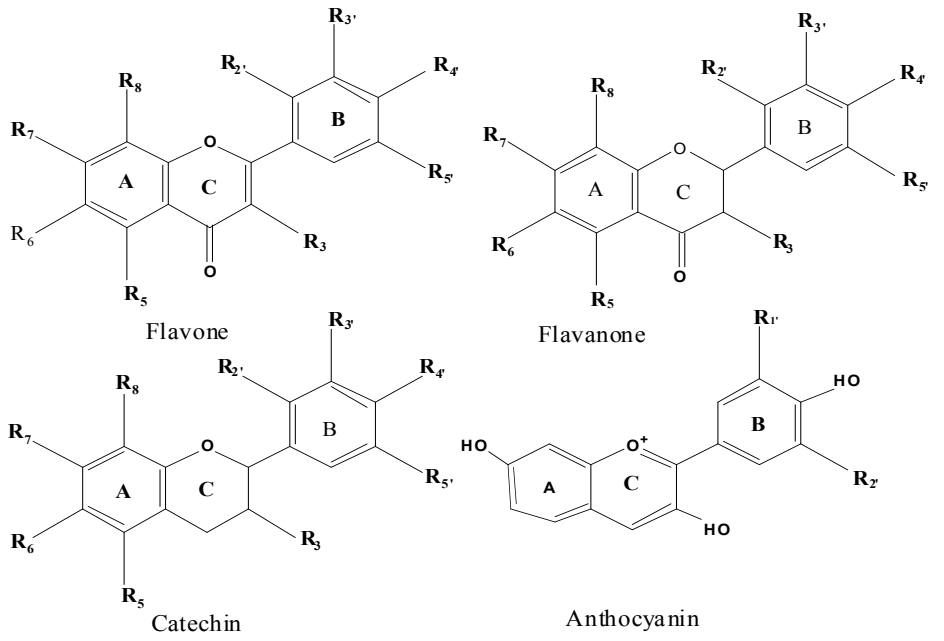
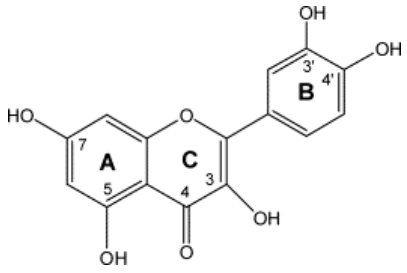
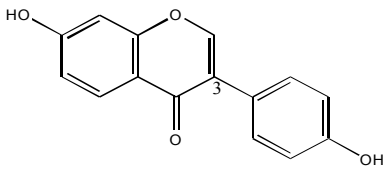


Figure 2.5. Skeletan structure of common flavonoids

Flavones and their glycosides have a slight yellow color and typically found in plants. They are characterized by a planar structure due a double bond in the central aromatic ring. In the isoflavonoids (isoflavones) the B ring is connected to carbon 3 of the central ring instead of carbon 2. One of the most common and best-described flavonoids, quercetin (Figure 2.6), belongs to flavones. Quercetin is found in abundance in onions, apples, broccoli, and berries (Nijveldt RJ et al**.,** 2001**)**





A B

Figure 2.6. A: Quercetin is a typical flavonoid found in many plants. B; Daidzein, an isoflavone.

Flavanones and their glycosides are mostly found in citrus fruits. They do not have a double bond at 2,3 position in the C ring (Figure 2.5, flavanone). A good example of this group is naringenin (Figure 2.7). Other main flavonones found in citrus are hesperetin and eriodictyol.

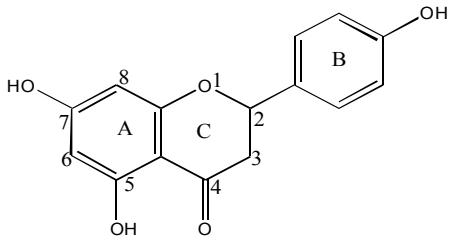


Figure 2.7. Naringenin. Its glycoside, naringin, is the most frequent flavanone in citrus

Catechins are found in most plants but are most abundant in tea leaves. All teas contain catechins, but white and green teas have the highest concentrations of catechins, constituting 30% of dry weight (Chi-Tang Ho, 1992). They are very reactive in atmospheric oxygen and can condense into oligomers and polymers known as theaflavins and thearubigins. Black tea, because is oxidized during processing, has the highest concentrations of theaflavins and thearubigins. The most common catechins are (+)-catechin (Figure 2.8), (-)-epicatechin, (+)-epicatechin, (-)-epigallocatechin, (-)-epicatechin gallate and (-)-epigallocatechin gallate (EGCG).

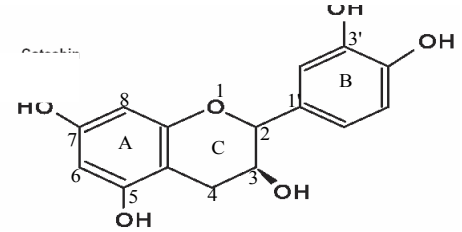


Figure 2.8. Structure of (+)-catechin, one of the most common catechin

Anthocyanidins and their glycosides (anthocyanins) are the colorants found in some part of almost every flowering plant. They are responsible for bright orange, pink, scarlet, red, mauve, blue and violet colors of flower petals as well as similar colors of fruits in higher plants (Nishimura et al., 1984). A well known function of the anthocyanidin pigments is the attraction of pollinators and seed dispersers. They, as well as other flavonoids, play a key role in signaling between plants and microbes, in defense as antimicrobial agents and in UV protection (Stafford, 1991). Figure 2.9 shows cyanidin , one of the most frequently occuring anthocyaninidin in flowering plants. Other anthocyanidins are pelargoniden, delphinidin , malvidin, petunidin and paonidin.

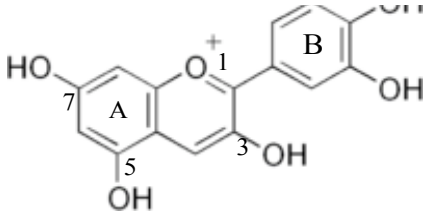


Figure 2.9. Cyanidin, a typical anthocyanidin

Lignans and antilignans are dimeric compounds made basically of two C6C3 units (Figure 2.10). If two C6C3 units (A) are linked by a b,b'-bond, a lignan base (B) is formed. If two C6C3 units (A) are linked by a bond other than a b,b'-bond, the basic structure for a neolignan is formed (IUPAC, 1999).

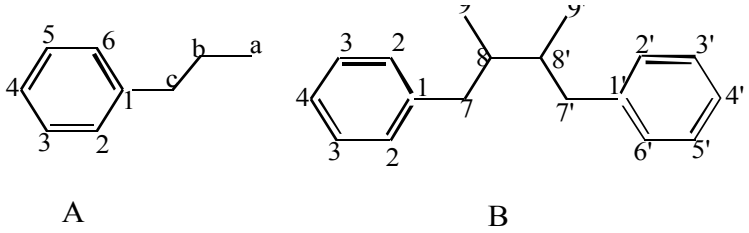


Figure 2.10 .A, one of the two C6C3 parts of lignan or neolignan. B, basic lignan compound

Amentoflavone (Figure 2.11) is a good example of biflavonoids found in many plants. It is a potent antioxidant and mild antidepressant with antiviral and anticancer activity (Banefjee, T. et al., 2002).

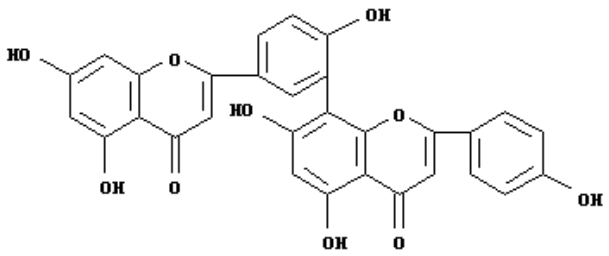


Figure 2.11 . Amentoflavone, a biflavonoid formed by the oxidative coupling of two chalcones.

Another group of phenolic compounds is proanthocyanidins or condensed tannins (Figure 2.12). They are polyflavonoids, consisting of chains of flavan-3-ol units and they have the tendency to complex with carbohydrates and proteins. If the chains are short, they are colorless but become yellowish to brown as the polymerization increases. If, however, they are heated in acidic solution, they reverse into corresponding polymerizing units in red to violet color(Haslan, E., 1989).

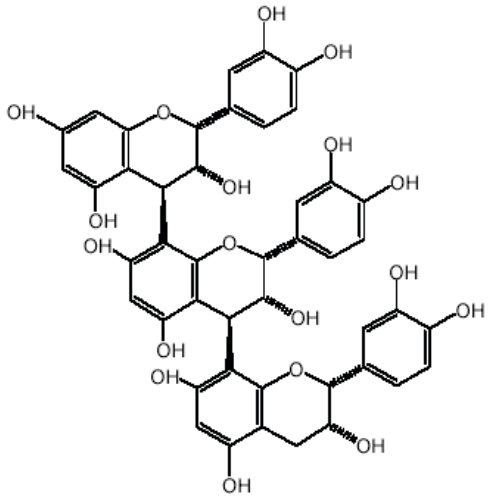


Figure 2.12. Procyanidin C-1, a condensed tannin

Much effort has been made lately at elucidating the flavonoid biosynthetic pathway. Work on maize, snapdragon, and petunia led to the isolation of regulatory and structural genes of many flavonoid (Holton and Cornish, 1995). Work on arabidopsis facilitated analysis of the organization and regulation of the flavonoid pathway. The advantage of using arabidopsis for studying flavonoid biosynthesis is that all of the enzymes of central flavonoid metabolism are encoded by single-copy genes, except flavonol synthase which seems to be encoded by six genes. Loci for regulatory and structural genes in the

arabidopsis genome have been identified mainly on the basis of mutations that eliminate or reduce pigmentation in the seed coat. (Holton TA and Cornish EC, 1995, Winkel-shirley, 2001)

Health Effects of Polyphenols

The antioxidant properties that almost all polyphenols have is very beneficial to human health due their ability to neutralize free radicals, compounds with one or more atoms with unpaired electrons in one of their orbitals (generally the outer orbital). A free radical always tries to replace the missing electron by taking it from another molecule. An oxygen free radical, for example, can take an electron from DNA base, oxidizing it (causing loss of an electron in oxygen of it). It can result to endless proliferation of the cell and possibly lead to diseases, like cancer, respiratory and cardiovascular problems, arthritis and many more (Raloff, 1996; Kellogg et al., 1994). In a similar way, oxygen free radicals can cause oxidation to low density lipoproteins (LDL) resulting to atherosclerosis, or cause oxidation to proteins and cellular lipid and damage cellular membranes, mitochondria, heart and other tissues. Free radicals in humans are formed normally during metabolism, or through pollutants, vigorous exercise, emotional stress, radiation, cigarette smoke, drugs and other factors. Our body produces endogenous enzymes like superoxide dismutase and glutathione peroxidase that can prevent oxidative damages, exogenous antioxidants (some vitamins, minerals, and other phytonutrients), however, can also prevent oxidative damage or scavenge free radicals by donating an electron to the free radical when the organism is short of endogenous antioxidants

(Kahkonem et al., 1999). Vitamin C (ascorbic acid) and vitamin E (a-tochoferol) have long been known as “free radical scavengers”. In vitro, vitamin C (humans cannot synthesize vitamin C) for example, scavenges peroxyl, ethyl, sulfenyl, and urate free radicals produced by pollutants, cigarette smoke and other causes. In addition vitamin C helps the inactive form of vitamin E to return in its active form. Vitamin E is effective antioxidant for cardiovascular protection and biological membranes**,** and found to be beneficial for athletes exercising at high altitudes (Subudhi et al. 2001)

Tea polyphenols (mostly catechins) have been extensively investigated and are found to have a preventive function as antioxidative “radioprotective; anti-mutagenic; anti-tumor; anti-hyper-cholesterolemic; anti-hyperglycemic; fat-reducing; anti-hypertensive; anti-ulcer; anti-bacterial; and anti-viral”, and “inhibit a-amylase and control obesity and diabetes by diminishing the hydrolysis of starch to glucose” (Hara, Y., 1995). Y. Hara., whose PhD thesis title is “Physiological Activities of Tea Polyphenols” has also found that “polyphenols are bactericidal against pathogenic bacteria, yet ineffective against acid forming beneficial bacteria, and prevent the formation of dental plaque and caries at far lower concentrations than present in an everyday cup of tea” (Hara, Y., 1994) which contains about 80-100 mg polyphenols. Green or black tea or total extracts of tea polyphenols as well as purified compound, such as EGCG of green tea were also tested on animals (rodents) for cancer (tumorigenesis) inhibition on skin and internal organs. It was found that tea polyphenols inhibited “tumor multiplicity,” and in the majority of the studies, tumorigenesis was blocked (Fung-Lung Chung et al., 2003). Prevention of cancer, coronary heart disease, arthritis, stroke, osteoporosis, liver disease, bacterial and viral infectivity as well as protection against human prostate cancer cells by tea

polyphenols have been reported also by other authors (Gupta S, et al., 2001; Ahmad, N. et al, 1994). Green tea polyphenols, in addition to a variety of anticarcinogenic and antiinflammatory activities, enhance the intercellular communication of gap junctions (Stoner and Mukhtar, 1995).

Epidemiology suggests that extra virgin olive oil is associated with reduced risk of cardiovascular disease and colon cancer, owing mainly to abundance of phenolic antioxidants that olive oil contains, like simple phenols (hydroxytyrosol, tyrosol), flavonoids and lignans (acetoxypinoresinol, pinoresinol). Recent studies have shown that olive oil antioxidant polyphenols play a protective role against human atherogenesis and colorectal carcinogenesis (Owen RW et al., 2000; Carluccio MA et al, 2003)

Pycnogenol (polyphenolic compound from the bark of pine**),** and resveratrol are also powerful antioxidants. Resveratrol, which together with other polyphenols (anthocyanins and catechins) is found in much higher quantities in red wine than in white, is a polyphenolic antioxidant that has been shown to have cardioprotective effect to those consuming moderate amounts of wine, and in experiments had been shown to protect the heart from arrhythmia and damage of cardiac cells (Takimoto, 1982; and Katalinic, V. et al., 2004).

Polyphenols like quercentin (in many fruits and vegetables), anthocyanins , proathocyanins, and narigenin (mostly in citrus) are also among the most talked about nutritional compounds (Chi-Tang Ho, 1992). They are claimed as antioxidants, cancer preventing, protecting from heart disease and hypertension, having anti-inflammatory activity, and strengthening blood vessels (Bravo, 1998; Stoner and Mukhtar, 1995). Studies in vitro showed that quercetin decreased primary tumor growth, prevented

metastasis in a model of pancreatic cancer, inhibited proliferation of colon cancer cells and blocked the formation of lipid peroxides, while luteolin (mostly citrus bioflavonoid), in vitro, caused apoptosis to pancreatic cells and inhibited proliferation of thyroid cancer cells (Lee, LT et al., 2002; Yamashita, N. et al., 2000; Yin, F. et al., 1999).

Apigenin, a flavone, was shown to prevent over-proliferation of cells by binding to estrogen receptor sites on membranes of these cells, and inhibited the growth of human leukemia cells. In an other study apigenin and luteolin caused leukemia cells to mature into healthy lymphocytes (Hirano T,et al. 1989; Kuiper, GG. et al., 1998; Wang, IK et al., 1999). Orientin (flavonoid) is free radical scavenger found in many plants. In experiments, it significantly reduced the adverse effect of radiation on human blood cells, mice gastrointestinal tract and bone marrow, and it also protected mice exposed to radiation from lipid peroxidation in the liver (Uma Devi, P. et al., 2000; Vrinda B. and Uma Devi, P., 2001). Rutin, a bioflavonoid found in many fruits and vegetables,is used to treat hemorrhoids. It is also used to treat insufficient blood circulation in legs that may cause varicose and leg edema (MacLennan, WJ, 1994; Titapant, V. et al., 2001), and the flavonoid myricetin was found to have protective properties against prostate cancer (Knekt, P. et al., 2002), while kaempferol (flavonoid, strong antioxidant in many fruits and edible plants) prevents atherosclerosis by inhibiting LDL oxidation. (Middleton, E. and Kandaswami, C., 1992).

Polyphenols seem to function through a variety of mechanisms to prevent diseases. Ellagic acid, for example, anticarcinogen found in many fruits and vegetables, binds

directly to DNA receptor sites protecting these sites from binding carcinogens. It is also suggested that polyphenols act synergistically. Kaempferol, for example, is more effective in to fighting cancer cells when it is used together with quercetin (Ackland, ML et al., 2005; Kowalski, Jan et al., 2005). Dr. Albert Szent-Gyorgyi, the Nobel Prize winning researcher who discovered flavonoids, also discovered that pure vitamin C was less effective for treating patients with blood vessel problems than vitamin C combined with polyphenols (Gil, MI et al., 1999). It seems that polyphenols are in a unique combination in each plant species, difficult to be synthesized in a lab, since many polyphenols are unknown, and although may be in very little quantities in each plant their activity may be highly significant. Cells of the immune system also –like T cells, B cells, NK cells, mast cells, and neutrophils – have been shown to be more effective in the presence of different flavonoids (Middleton, E. and Kandaswami, C,, 1992). Therefore, using in our diet compound polyphenols of a plant may be more beneficial than using single ones.

Ficus carica L.

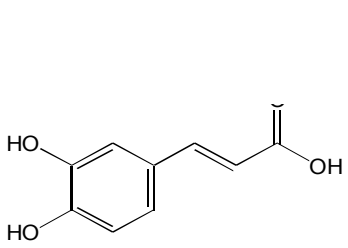
History: *Ficus carica L.* (Moraceae) has its origin in Iraq or Syria (De Candolle, 1886) and has been cultivated in Near Far East and Eastern Mediterranean for thousands of years (Zohary, 1988). In United States it was introduced by Spaniards in 1575 (Fisherman, 1986). Fig trees are now grown in tropical, semitropical, and temperate climates and some varieties such as the King fig , are well adapted to survive temperatures as low as 20^{o}F (Condit, 1941, 1947, 1955). Nevertheless, localities with characteristically wet winter and dry hot summers, as in Mediterranean countries, are where the best fruits are produced. There are over 700 varieties of *Ficus carica* (Condit, 1955) and they are divided to varieties that need pollination to produce mature fruit, like

Smyrna, and to ones that do not need pollination, like Brown Turkey and Black Mission, which are the varieties most cultivated in United States (Storey et al., 1977, 1976).

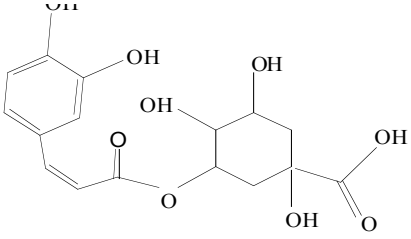
Fig trees can be raised from seed but the most common way is propagation by cuttings about 2cm thick and 20-30 cm long taken from 2-3 years old trees (Krislev et al., 1975). They grow up to 25 m, depending on the cultivar, with numerous branches and a trunk up to 40cm thick in some cultivars. The roots spread out to about 20 m and up to 4 m dip if the soil permits. The leaves, green in color, are palmate and deeply divided into 3-7 lobes with irregular margins, fairly thick blade of about 25 mm with rough the upper and soft-hairy the lower surface. The fruit, usually a pear shaped syconium 3-10 cm long, is soft when ripe and varies in color from yellow to brown or black. Numerous minute flowers without petals are on the inside wall of the syconium. The somatic chromosome number in fig is 2n=36 and the chromosome that bears the sex alleles is indistinguishable from the rest of chromosomes (Storey, 1975). In cultivars that have separate male and female plants, like "Smyrna", the pollination of the female flowers is curried out by the tiny wasp, *Blastophaga grossorum.* The male plants (caprifigs) produce pollen, and unpalatable fruits (Tous, 1996). The seeds of pollinated varieties, about 20-2000 per fruit, are tiny and yellow-brown in color, while the figs of non-pollinated varieties, like the black mission and brown turkey, do not have real seeds but hallow drupelets with an endocarp layer inside (Galil, 1977). Most varieties yield two crops of fruit each year: The breba, early in the summer on the old shoots, and the main crop later in the summer on the shoots of the current season (Givan, 1997). Fig trees are vulnerable to nematodes, stem-borer *Batocera Rufomaculata,* lepidopsterous *Azochis gripusalis,* coleopterous *Epitrix* and *Colaspis, Asterolecanium* sp., *Saissetia haemispherica, Cerotelium fici* (causes leaf rust, common problem), and a fig mosaic virus which is incurable so that the tree must be destroyed (U.S department of Agriculture). To obtain pathogen free plantlets and large-scale production whenever needed, which is basic requirements for a

commercial orchard, and for supporting the research on transgenic plants obtained through genetic transformation, successful efforts are being made to generate fig plants through tissue cultures (micropropagation) initiated from shoot tips or leaf explants (Muriithi et al., 1983; Pontikis and Melas, 1986; Noble and Romano, 1998; Yakushij et al., 2003).

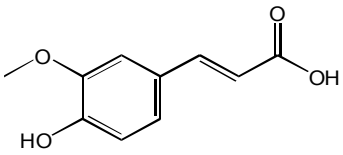
Natural products of *Ficus carica*: *Ficus carica* contains more than 700 different chemical compound (USDA, 2004). Figs are mostly preserved dry because fresh fruits are very perishable. Fresh or dry they are rich in potassium, calcium, ascorbic acid, vitamin A, carbohydrate, major amino acids serine, aspartic acid, proline, alanine (Kim et al, 1992; Budavari, 1996) and a lot of polyphenolic antioxidants. (Edenharder et al., 1998; Vinson, 1999). The main polyphenols found in fig fruit are flavones (apigenin, astragalin, kaempferol and rutin), catechins (catechin and epicatechin), flavonones anthcyanidin (cyaniding), chlorogenic acid, gallic acid and syringic acid. Many polyphenolic acids are found in plant (caffeic acid, chlorogenic acid, ferulic acid and tannic acid) as well as the flavones kaempferol and quercitin. The fig leaves contain luteolin (flavone), quercitin, luteolin, caffeic acid, chlorogenic acid and tannic acid (Figure 2.13), (Robinson and Robinson, 1932; Puech *et al*., 1975; Solomon *et al*., 2006; Duke J., 1992; Vaya and Mahmood, 2006; Del Caro and Piga, 2008; Veberic et al., 2008).



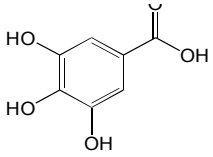
Caffeic acid



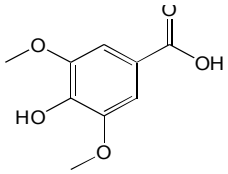
Chlorogenic acid



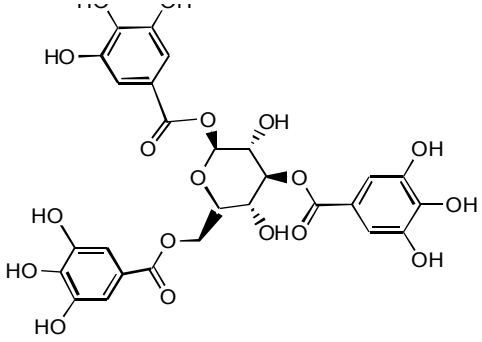
Ferulic acid



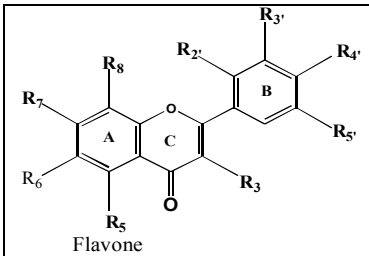
Gallic acid



Syringic acid

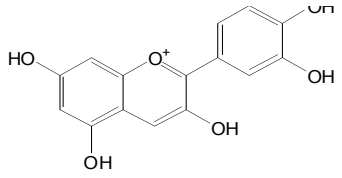


Tannic acid ($C_{27}H_{24}O_{18}$)

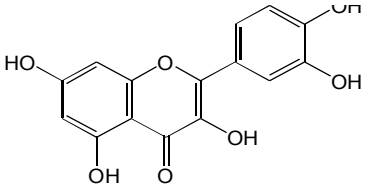


Flavone

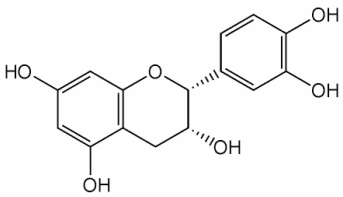
Compound	R_3	R_5	R_6	R_7	R_8	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$
Apigenin	H	OH	H	HO	H	H	H	OH	H
Astragalin	GL	OH	H	HO	H	H	H	OH	H
Isoquercetrin	GL	OH	H	HO	H	H	OH	OH	H
Luteolin	H	OH	H	HO	H	H	OH	OH	H
Kaempferol	OH	OH	H	HO	H	H	H	OH	H
Quercetin	OH	OH	H	HO	H	H	H	OH	OH
Rutin	RU	OH	H	HO	H	H	H	OH	OH



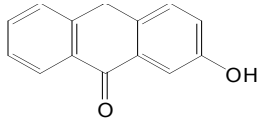
Cyanidin



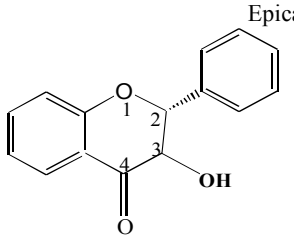
Cathechin



Epicatechin



Emodin



Taxifolin

Figure 2.13. Polyphenols found in *Ficus carica L.* GL: glucose, RU: rutinose

The unripe fruit and the rest parts of the tree, mainly the leaves, contain latex. Latex contains caoutchouc (not a source for commercial caoutchouc), resin, albumin, cerin, sugar and malic acid, warfarin (anticoagulant), terpenoids, ficin (proteolytic enzyme used for milk clotting and meat tenderizing), coumarins and other biochemical compounds (Ashy, 1981; Innocenti, 1982) .

Leaves also contain pentosans, carotene, stigmasterol, sitosterol, tyrosine, quazulene and benzaldehyde. Quazulene and benzaldehyde as well as coumarins have shown anticarcenogenic properties (Vinson, 1999; Vinson et al., 1998; Jeong and Lachance, 2001). Of the coumarins in fig, the most prevalent, especially in the leaf extracts, are the linear furocoumarins, psoralen and bergabten with a concentration of 0.41% psoralen and 0.39% bergapten in high season when hottest and dry (Abu-mustafa et al, 1977; Zaynoun et al., 1984).

Furocoumarins are isolated mostly from four plant families, Moraceae, Leguminosae, Umbelliferae and Rutaceae, and some are chemically synthesized in laboratories**.** The two forms of furocoumarins that exist in nature are the linear and angular (e.g angelicin) structure of furocoumarins. The linear forms are more phototoxic than the angular ones, and bergapten, which is used for the same purpose that psoralen is used in clinics, is less powerful than psoralen (Caporale et al., 1970, 1972)

It was noticed very early that fig latex was a skin irritant and could cause irritant dermatitis and pigmentation if not removed promptly (Dioscorides, 50AD). It had, however, therapeutic effect when applied on warts, skin ulcers, eczema, and vitiligo (Behl et al., 1966). Kitchevatz (1934) noticed that the latex of leaves and other part of the plant was photosensitizer (makes the skin more sensitive to light), and the role of sunlight in the reaction of fig sap was noted (Arthur and Shelley, 1955; Grant, 1962**).** Ullman (1945, 1952) investigating the effect of latex on rabbits and rats, found that some

fractions of it were very toxic and lethal , depending on doses, while some were found to have therapeutic effect on malignant tumors, lung and breast, spontaneous and transplanted cancer. Later, psoralens were isolated from the fig sap and characterized, and their photoreactivity and pigment-stimulating properties were investigated for mutagenesis and carcinogenesis in vitro and vivo. In 1970s and 80s, the photochemotherapeutic effectiveness of psoralen in combination with UVA radiation system was recognized and used for treatment of psoriasis, mycosis fungoides and many other skin diseases (Stern et al., 1998). For the last 15 years, psoralens have been used to treat skin diseases, but also for skin tanning. Especially noteworthy is their use for treating non-melanoma skin cancer cutaneous CTCL (Stern et al., 1998). UVA (320-400 nm range) and UVB (about 1,000 times more powerful than UVA) sources are used with psoralen in order to make the skin more responsive to the rays. UVA bombards the DNA inside the skin cells and injures it, and impairs the immune function in the skin. It excites psoralen to a state that causes covalent binding of the psoralen with nuclear DNA. (Kavli,1984; Wamer et al.,1995). With the right dose of UVA, the effects of psoralen on cells can be beneficial in case of psoriasis or some cancerous cells, since cross-links and "mono-adducts" prevent cell proliferation. It can also be effective against restenosis following agioplasty (Jacques et al., 1994). Therapeutic furocoumarins can be administered locally, or orally in higher doses, and the dose of light is strictly monitored to avoid carcinogenesis and burning (Stern et al., 1998). Patients receiving PUVA are treated usually three time a week until remission of the cancer occurs, and since the skin is sensitized, patients are not exposed to sunlight for certain time after treatment. Psoralen and bergapten found also to have anticarcenogenic activity in vitro against gastric carcinoma BGC-823, and colon HTC (Yin et al., 1997)

Plant Tissue Culture for natural product production

Plant tissue culture technology was inspired by the belief in the potential of plant cells to start to divide and differentiate under the influence of a suitable culture conditions. Haberlandt (1902) first tried unsuccessfully to prove the "totipotency" (the ability of a single cell to regenerate a whole plant) of the plant cells by using monocotyledonous plants. Many other unsuccessful attempts followed until Robbins (1922) was able to culture and maintain cultures of pea and maize roots in vitro.

White with the culture tomato roots (1934), and in tobacco callus (1939) followed. White also noticed that root meristems were free of virus in his cultures. In the 1950s callus cultures of Dicots, Monocots and Gymnosperms were produced successfully and the important role of phytohormones as growth regulators in tissue cultures was demonstrated. During this period, plantlets from callus and from cell suspension cultures were regenerated (Skoog and Miller, 1957; Steward et al., 1958). Eventually, whole plants from single cells of tobacco, and somatic embryos from single cells of carrot were formed (Vasil and Hildebrandt, 1965; Backs -Hussemann and Reinert, 1970).

Many factors influence the initiation and maintance of tissue cultures of different plant species. Age, size, condition, source of the explant as well as when the explant is collected constitute one set of factors. The composition of the culture medium, the quantity and quality of light, pH and temperature play also significant roles (Basile and Basile, 1988; Endress , 1994). Introducing foreign DNA into plant cells has made it possible to alter the genetic properties of the cells and produce transgenic plants. Horsch et al (1984) used *Agrobacterium tumefaciens* as mediator to transfer foreign genes to *Nicotiana tabacum* and form transgenic *Nicotiana tabacum* plants, and Huang et al.

(1993) obtained the first transgenic conifer, *Larix deciduas*, through *Agrobacterium rhizogenes* "mediated transformation". Flores and Filner (1985) also used *Agrobacterium rhizogenes* mediated transformation to produce hairy roots of *Hyoscyamus muticus* that resulted in the production of higher levels of secondary metabolites than in the parent plant.

It is now well established that plant cells cultured in vitro are capable of producing many of the same compounds that are produced by the parent plants, and in many cases at higher levels, than the parent plants, and in some cases cultured tissues have produced compounds not known to be present in the parent plant (Parr et al, 1988; Endress R., 1994; Zafar et al., 1992).

As mentioned earlier, because of the disadvantage of producing useful drugs from medicinal plants, for which the growth and production of desired compounds are highly dependent on the constraints imposed by restricted environmental factors, geographical location, and danger of assault from pests and disease, much effort has been given to develop tissue culture procedures for the production of secondary metabolites that have commercial and or therapeutic value. Mitsui Petrochemical Industry Co. Ltd. in Japan for example was able to produce shikonin on a commercial scale from *Lithosperrmum ethythrorhizon* tissue cultures and Nitto Denko Co. Ltd. in Japan demonstrated mass production of ginsenoside from *Panax ginseng*. Shikonin was one of the first secondary metabolites to be produced commercially by tissue cultures. It is used as an anti-inflammatory, antibiotic, and to treat burns and hemorrhoids. Ginsenoside is behind the claim of ginseng's effect which is used to prevent infections, lower cholesterol and increase energy and endurance. (Misawa, 1994; Curtin, 1983; Hara et al., 1987 ; Nair et

al. (1986) and Basile et al. (1993) also reported a significant amount of artemisinin produced from tissue cultures of *Artemisia annua* L. Artemisinin is an antimalarial drug effective against the drug-resistant malarial parasite, *Plasmodium*. Scopolin, a polyphenolic compound with anti-inflammatory properties and used for treating capillary fragility and other physiological disorders was produced at a level of industrial use from tissue cultures of *Nicotiana tabacum* (Giller et al., 2000). Santos-Gomes et al.(2003) were able to isolate 12 measurable polyphenolic antioxidant compounds from calli of tissue cultures of *Salvia officinalis* L (sage).Many secondary metabolites such as the anthraquinones, ajmalicine, rosmarinic acid,ubiquinone-10 and benzylisoquinoline were also produced from tissue cultures in quantities of many times higher than that obtained from comparable amounts of intact plant tissue (Misawa, 1994).

CHAPTER 3: THE TISSUE CULTURES OF *FICUS CARICA*

Summary

Petiole discs of *Ficus carica* inoculated on Gamborg's B5 basal media containing 0.5mg/l BAP and 0.5 mg/l NAA, 3% sucrose, solidified with 4,5 g/L Gellan and incubated at 29ºC±1 under high light intensity and a 16 hours photoperiod resulted in the relatively rapid callus initiation and growth. Once these conditions were established, no further changes in the basal media formulation were required during the course of this research. This medium produced healthy yellowish friable callus, suitable for maintaining cultures for many years if subcultured every 50 days and for successfully inoculating suspension cultures. Suspension cultures, however, although exhibiting a satisfactory mass increase, could not be maintained in a healthy condition for more than two months. This protocol proved useful for repeatedly initiating fig cultures used for production of polyphenols and other secondary metabolites as well.

Introduction

There are no reports of tissue cultures initiated from fig leaf petioles used for any purpose**.** Tissue cultures of fig, however, have been used to produce fig plants through micro propagation starting with tissues excised from differed parts of the plant. Successful micro propagation was achieved from excised shoot tips (Muriithi et al., 1982; Pontikis and Melas, 1986; Noble and Romano, 1998). The shoot tips, in contrast to leaf petioles, already have differentiated apical meristems and leaf primordia. In another report, (Yakushij et al., 2003), leaf explants produced in shoot cultures were used. This resulted in the generation of adventitious buds that developed into to shoots that were

subsequently transferred to medium supplemented with IBA in order to produce roots. An attempt to initiate callus tissue was not mentioned by these authors. Recently, Ferreira and Pasqual (2007) used leave explants of the cultivar 'Roxo de Valinhos' inoculated on MS medium with 2, 4-Dichlorophenoxy acetic acid (2, 4-D) and kinetin each at a range of concentrations. These investigators were able to obtain callus initiation and subsequently plantlet regeneration was obtained with cultures supplement with 4.52 µM of 2,4-D and 2.32 µM kinetin.

No attempts known to me have been made to produce antioxidant phenolics from cultures of fig tissue. Suspension cultured fig tissues, however, have been successfully used to produce ficin (Nilsson and Townsley, 1982; Nassar and Newbury, 1989; Miguel and Lima-Costa, 1998; Cormier et al., 1989).

Similarly, there are no reports of fig leaf petioles being used to initiate tissue cultures for any purpose. In this research, excised petioles of *Ficus carica* leaves were used to initiate tissue cultures in order to investigate the potential of vegetative tissue for producing antioxidant phenols similar or the same as produced in fig fruits. Tissue isolated from fig leaf petioles were inoculated onto media of various nutrient-hormone concentrations and incubated under a variety of carefully controlled conditions designed first to initiate to callus, and subsequently to induce the tissue to produce measurable quantities polyphenolic antioxidants that could be used as food supplements or other medicinal use. Culture conditions were developed that resulted in obtaining same polyphenolic compounds produced by field-grown fig plants. Extracts of the cultures were analyzed and the antioxidant compounds identified by using chromatographic methods.

Materials and methods

Reagents. Methanol, ethanol, hexane, ethyl acetate, acetone, hydrochloric acid, formic acid, acetic acid, sulfuric acid, sodium carbonate, and phloroglucinol were purchased from Fisher Scientific (Suwannee, GA). Evans blue, Diphenyl-2-picryl-hydrazyl (DPPH), Folin-Ciocalteau's phenol reagent, pectinase, L-phenylalanine, (+)-catechin, were purchased from Sigma (St. Louis, MO); 1-phenyl-3-(1,2,3- Thiadiazol-5-yl (Thidiazuron), phytohormone solutions [Indole -3-butyric acid (IBA), alpha-naphthaleleneacetic acid (NAA), 2,4-D, 6-benzylaminopurine (BAP) and kinetin were purchased from Research Organic Inc, (Cleveland, Ohio); plant cultulre media, Gamborg's B-5 basal medium with minimal organics and Murashige and Skoog medium with macro-and micronutrients and vitamins were purchased from Sigma (St. Louis, MO) and Caisson Laboratories, Inc. (Rexburg, ID) respectively.

Plant Material. Petioles of *Ficus carica* (Black mission) were collected from the trees grown in the research green house of Lehman College, CUNY. After washing them with hand soap, 11-12 mm long sections of petioles were placed in 6 x 13 cm screw-capped jars, immersed in 95% ethanol for 1-2 min, followed by immersion in 20% v/v Clorox™ containing 0.1% w/v detergent for 20 min with occasional shaking and rinsing them in three 1-2 min changes of sterile, distilled water. The petioles were transferred to plastic Petri dishes and cut into discs about 2 mm thick. The petiole discs were used to inoculate heat-sterilized Gamborg's B5 basal media, preadjusted to pH 5.6±0.1 containing vitamins, 20 g/L sucrose, 4.5 g/L Gellan, auxin (NAA) and cytokinin (BAP) as shown in Fig. 3.1.

Figure 3.1. Petri dish of B-5 medium inoculated with 2 mm thick petiole discs

Experiments

Based on results obtained in pilot studies and preliminary work (Table A.1, page 72), a number of experiment were conducted to optimize tissue cultures for good callus growth and high yield of polyphenolic antioxidants (Figure 3. 2).

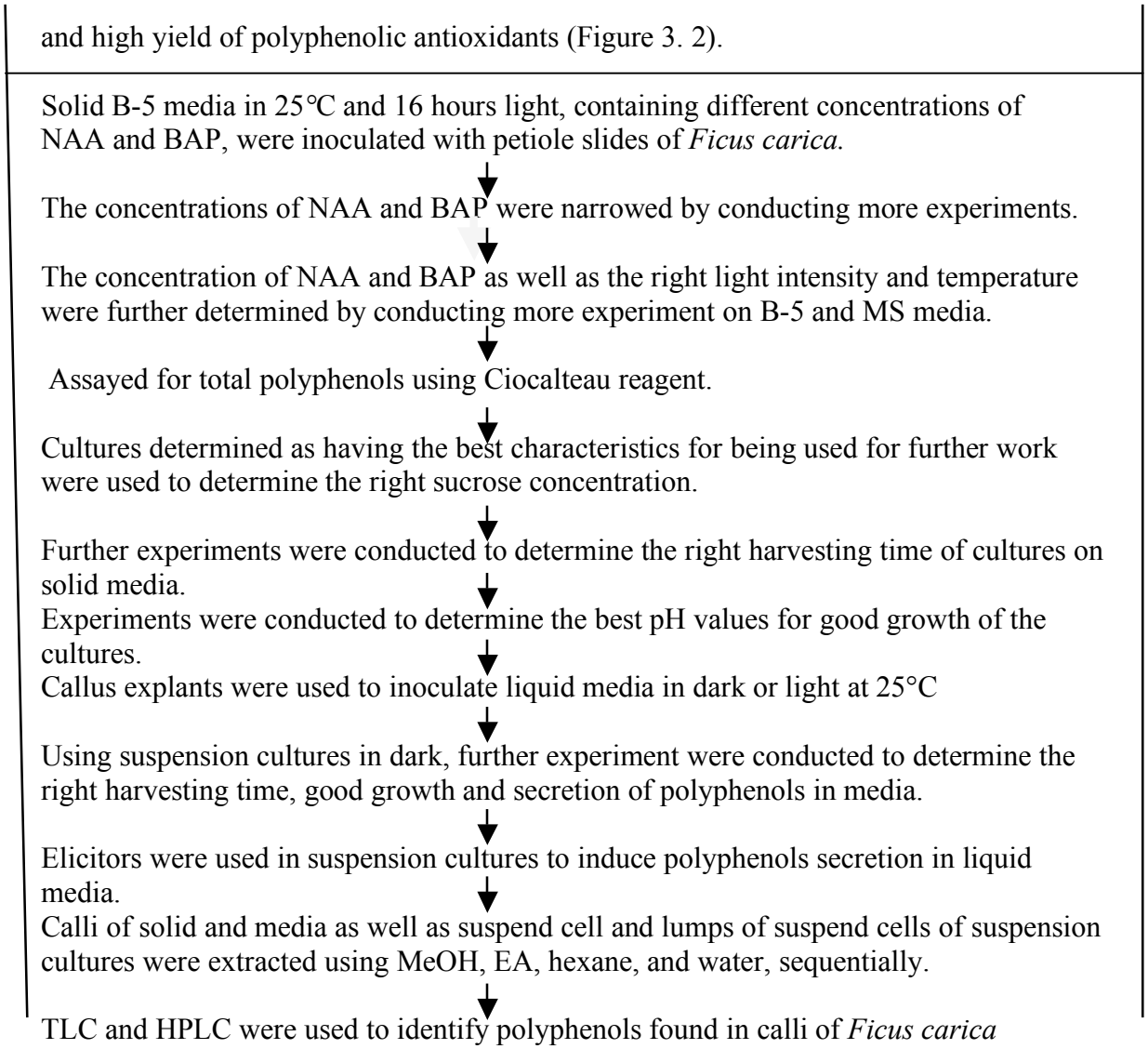


Figure 3.2. Flow chart of tissue culture experiments of *Ficus carica.*

Experiment 1. for the first two designed experiments Gamborg's B5 medium with minimal organics was used as the sole nutrient source. To determine an effective nutrient – hormone combination, disposable Petri dishes of media (8 replicas for each treatment), supplemented with phytohormones in different combinations, in one series or 4 different concentrations of NAA (1, 5, 10, and 15 mg/L) by 4 different concentration of BAP (1, 5, 10, and 15 mg/L), in a Latin Square design were employed. The cultures were incubated at 25^{o}C±1 and 16 hour photoperiod provided by cool white fluorescent lights (85±5% µmol $m^{-2}s^{-1}$). They were visually examined in four weeks and an estimate made of the best growth, friability and texture were made. Cultures showing favorable characteristics (A, E, I, F,H and O, Table 3.1) were maintained under the same environmental and nutritional conditions that produced them and subcultured in plastic Petri dishes or 100 ml glass baby jars for further investigations.

Table 3.1. Auxin cytokinin combination of cultures on B5 Media in the 1st experiment with *Ficus carica* tissue cultures. Cultures A, E, F, H, I, and O, under visual examination, displayed satisfactory growth and texture composition

NAA / BAP	1 mg/L	5 mg/L	10 mg/L	15 mg/L
1 mg/L	A	B	C	D
5 mg/L	E	F	G	H
10 mg/L	I	K	N	O
15 mg/L	P	S	V	Z

Experiment 2. To narrow the auxin-cytokinin concentrations, based on the results of visual examination of these cultures, a second set of experiments using 25 more NAA-BAP combinations, or 5 different concentrations of NAA (0.1, 0.5, 1, 2, and 4 mg/L) with 5 different concentrations of BAP (0.1, 0.5, 1, 2, and 4 mg/L employing the same multifactorial design and the same conditions of incubation were conducted, this time

using 8 replicas for each treatment. They were again examined visually in four weeks for best growth, friability and color. Cultures showing favorable characteristics (1, 2, 6 and 7, Table 3.2.) were taken as a base for the experiment 3.

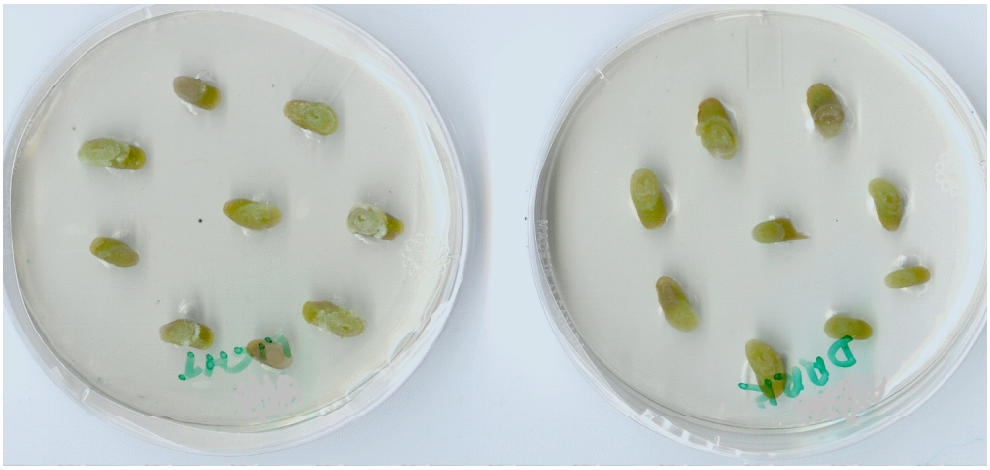
Table 3.2.Showing the auxin (NAA) cytokinin (BAP) combinations in B5 media in the second experiment of *Ficus carica* tissue cultures. Visual examination showed no difference in the characteristics of interest. Cultures 1, 2, 6 and 7 were selected for further experiments

NAA / BAP	0.1 mg/L	0.5 mg/L	1.0 mg/L	2.0 mg/L	4.0 mg/L
0.1 mg/L	**1**	**2**	3	4	5
0.5 mg/L	**6**	**7**	8	9	10
1.0 mg/L	11	12	13	14	15
2.0 mg/L	16	17	18	19	20
4.0 mg/L	21	22	23	24	25

Experiment 3. To further narrow the conditions for the best cultures to be investigated, a third set of experiments was undertaken. In these experiments the influence of a different nutrient formulation, the presence or absence of light, as well as different incubation temperatures were evaluated. In these experiments B-5 as well as MS media were used, both under the same conditions except the carbon source. In general, 30 g/L sucrose for MS media and 20 g/L for B-5 media are recommended, for cultures maintained on these media (Skoog and Miller, 1957; Gamborg et al., 1968). These cultures were supplemented with NAA concentrations of 0.05, 0.1, and 0.5 mg/L and BAP concentrations of 0.05, 0.1, and 0.5 as shown in Table 3.3. Separate sets of 5 replicas were prepared and each set incubated either in dark at 25ºC ±1 and 29ºC ±1 or in the light at 25ºC ±1 and 29ºC ±1. These cultures were subcultured in 2 weeks on the same media after their weight was taken, and examined in the following four weeks for texture, friability, color, weight, and total polyphenolic antioxidants (Table 3.3, Table A.3, page 76).

Table 3.3. Auxin/cytokinin ((NAA/BAP) combinations in B5 and MS media in the third experiment of *Ficus carica* tissue cultures under different light intensity and temperature. The friability, the size and color of the cultures were examined visually and their weight and total polyphenolic compounds were measured FF: friable, F: semifriable, EE: excellent growth, high percent weight increase, E: low percentage weight increase, YY: healthy, yellowish color, Y: yellow green or yellow brown, PP: high percent polyphenols, P: moderate percent polyphenols. Not labeled cultures do not have any favorable characteristics. Cultures in bold letters have the most favorable characteristics.

	MS														
	25°C									29°C					
	Dark			Constant light			16 hrs light			Dark			16 hrs light		
NAA / BAP	0.05	0.1	0.5	0.05	0.1	0.5	0.05	0.1	0.5	0.05	0.1	0.5	0.05	0.1	0.5
0.05	FY E	FB E		FY EP	FY E		FY	FY		FB E	FB E		F Y E	FY P EE	
0.1	FB E	FB E PP		FY E	FY E		FY	F Y P		FB E	FB E		FY EE	FY EE P	
0.5		FB E	FB E		FY E P	FY EE		F Y E	F Y EE		FB E	FB E PP		FY EE P	FY EE P
B-5															
0.05	FB E	FB E		FF YY E	FF YY EE		**FF YY P**	**FF YY**		FB E	FB E		**FF YY EE**	FF YY E	
0.1	FB E	FB E		FF YY EE	FF YY E		**FF YY**	**FF YY P**			FB E		FF YY E	FF YY EE P	
0.5		FB E PP	FB		FF YY EE	FF YY EE P		FF YY EE P	**FF YY EE P**	F B EE	FB E PP	FB E		FF YY EE	**FF YY EE P***



Figrure 3.3. Fig petiole discs inoculated on B-5 medium and incubated in the light (right) and dark (left) for two weeks.

Experiment 4. As a result of the third set of experiments, callus cultured on B5 in light and 29ºC±1 supplemented with 0.5 mg/L NAA and 0.5 mg/L BAP) were selected for further work. Inoculum derived from the foregoing experiments was used to evaluate the influence of the concentration of sucrose on the general appearance and mass growth. Sets of replicates were inoculated on B-5 medium containing 20 and 30 g/L sucrose after the weights of the inocula were taken. The cultures otherwise were treated as the parent culture and were evaluated in four weeks (Table 3.4).

Table 3.4. Sucrose effect on callus growth in a period of 50 days. The dry weight of the inoculum was estimated by freeze drying callus, from the same cultures, equal to amount of inocula). Std dev: standard deviation. DF: degree of freedom; P: probability

		Inoculum in20 g/L	20 g/L (50days)	Inoculum in30 g/L	30g/L (50 days)	
mean		10 mg (dry, Estimated)	250 mg (dry)	10 mg (dry, Estimated)	344mg, (dry)	
Std dev		2	62	2	76	
Std error		0.01	16	0.01	20	
Paired t-test			DF 13		DF13	P: .0059

Experiment 5. To test the possible effect of pH on the nutrient-hormone combination that produced the most favorable results thus far, weighed pieces of callus (about 5 grams for every 5 replicas) were inoculated into 100 ml baby food jars containing 20 ml of B-5 basal media with vitamins, 30 g/L sucrose, 0.5 mg/L NAA and 0.5 mg/L BAP. The pH of the media was adjusted to pH4.5, pH5, pH5.5, pH6 and pH 6.5 in each of the 5 replicate cultures. The appearance was evaluated and the percentage mass increase and the antioxidant polyphenolic content of the cultures were measured 4 weeks after inoculation.

Experiment 6. To investigate the rate of increase of callus growth and polyphenolic content in solid tissue cultures, an experiment with cultures incubated in 16 hours photoperiod on B5 supplemented with 0.5 mg/L NAA and 0.5 mg/L BAP) was conducted. Ten cultures were examined visually and harvested every 10 days for a total of 55 days, starting at culture initiation. Callus tissue of five groups of cultures (10-15 cultures in baby jars, combined) maintained on B5 containing 30 g/L sucrose, 0.5 mg/L NAA and 0.5 mg/L BAP, was freeze dried, ground and extracted in 100% methanol. Fifteen samples were taken out of the supernatant (3 of each group) and assayed for total polyphenolic compounds using Ciocalteau reagent. Part of the extracts were hydrolyzed by using HCl acid and adjusting the pH at 1.8. Of the supernatants, 15 samples were taken out and examined for total polyphenols.

Cultures exhibiting the best properties thus far were those on B-5, treated with 0.5 mg/l NAA, 0.5 mg/l BAP at 29ºC±1, 16 hours light at pH 5.5. These were friable to semi friable, had a relatively high biomass and an average of 2.8 % of dry weight as total polyphenols. As shown in Table 3.5, many cultures incubated in the dark contained more polyphenolic antioxidants, but their biomass was very low, they were less friable and less healthy looking compared to those incubated in the light. Cultures on basal media other than MS and B5 produced inconsistent results and therefore were eliminated from further investigation. As shown in Table 3. 5, tissue cultures incubated at 29ºC±1 have higher percent growth increase and better phenol context and composition than those incubated at 25ºC±1. Those incubated in light (85±5% µmol 3 $m^{-2}s^{-1}$ cool white fluorescent lights) have higher percent increase in growth than those incubated in dark, but the highest production of polyphenols was obtained from cultures incubated in dark.

Effect of sucrose concentration on tissue cultured on solid media. Sucrose is the most widely used carbon source for plant tissue cultures. Plant tissue cultured on MS and Gomborg's B-5 media usually contain 30 g/L and 20 g/L sucrose in the media, respectively. Some times, however, the production of certain secondary metabolites by plant tissues is improved by using sucrose concentrations other than those given in the original publications. (Zenk, M.H. et al., 1977). To test the effect of different sucrose concentrations on biomass and polyphenol production, cultures were initiated under the same conditions specified above, except that each of 10 replicates will contain either 20 or 30 grams per liter of sucrose. As may be seen by referring to Figure 3.4, the biomass of the cultures containing 30 g/L increased significantly compared to 20 g/L control.

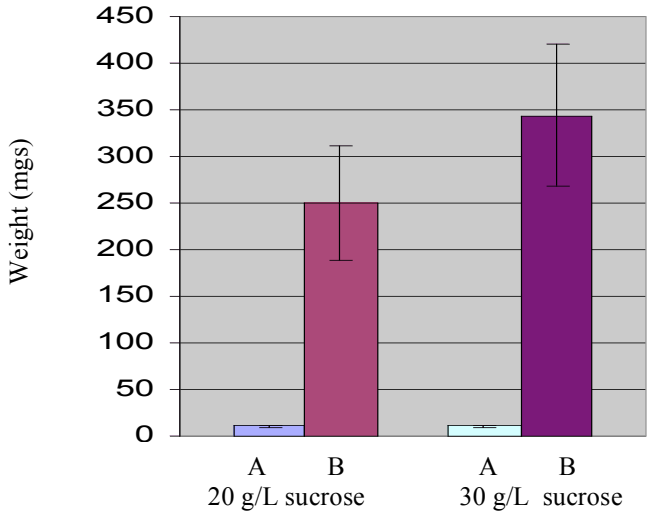


Figure 3.4. Effect of sucrose on callus growth (dry weight increase in mgs) in 7 weeks of culture on media containing 20 or 30 g/L sucrose. A: average weight of inocula. B: Average weight of callus in 7 weeks. The bars show standard deviation. The dry weight of inocula was estimated by freeze drying equal amount of callus with that of inocula.

pH effect on tissue cultured on solid media. As has been mentioned above, in most of reports, the best results with reference to favorable callus growth and adequate secondary

metabolite production were obtained on culture media adjusted to pH5.5±0.2. In this work, no significant improvement was obtained by using the pH treatments that differed from pH5.5 (Table 3.5 and 3.6). The percentage mass growth of cultures of pH 5.5 is significantly higher that that of pH 6.5 (P: 0.01) but it is not significantly higher than the remaining treatments.

Table 3.5. Effect of pH on callus growth cultured on solid B5 media supplement with 0.5 mg/L NAA and 0.5 mg/L BAP. . DW: Dry weight.

pH	4.5	5.0	5.5	6.0	6.5
DW Mean % increase	1594.993	1481.688	1706.434	1348.424	1411.299
Std Deviation	280.51	294.21	439.98	557.63	271.78
Std Error	125.45	131.26	196.77	249.38	121.54
P value (t-paired test)	(paired with pH 5.5) 0.34	(paired with pH 5.5) 0.13		(paired with pH 5.5) 0.21	(paired with pH 5.5) 0.01
N	5	5	5	5	5

Table 3.6. The effect of pH on phenolic content. pH4.5-6.5 has no significant effect on phenolic content of callus cultured on solid B5 media supplement with 0.5 mg/L NAA and 0.5 mg/L BAP. The mean absorbance is proportional to phenols in callus.

pH	4.5	5.0	5.5	6.0	6.5
Mean absorbance at 750nm	0.103	0.106	0.121	0.106	0.124
Std Deviation	0.044	0.044	0.048	0.022	0.023
Std Error	0.016	0.016	0.017	0.007	0.008
P value (t-paired test)	(paired with pH 5.5) 0.11	(paired with pH 5.5) 0.15		(paired with pH 5.5) 0.16	(paired with pH 5.5) 0.54
N	8	8	8	8	8

It became apparent during the course of this research that callus cultures reach a plateau at about 50 days after their initiation. The color of the callus then changes gradually from yellowish to yellow brown and the solidified media begins to liquefy in cultures on B-5 solid media supplemented with 0.5 mg/L NAA and 0.5 mg/L.

To investigate the changes in phenol content in callus that occur as the cultures reach a plateau, an experiment was conducted in which 50 cultures on B-5 solid media supplemented with 0.5 mg/L NAA and 0.5 mg/L. were incubated with a 16 hours photoperiod at 29ºC±1. Every 10 days, 10 of these cultures were harvested and assayed for polyphenolic content. As shown in Table 3.7 and Figure 3.5, the polyphenolic content decreases in the beginning but then increases reaching a plateau at about 45-50 days. These results suggest that 45 to 50 days after initiation is the best time to harvest tissue to obtain the highest yield of polyphenols. Presumably, the plateau in growth occurs because of nutrient depletion, while phenolic synthesis increases because nutrients that are first directed to the growing cells become incorporated into metabolites required for phenolic production.

Table 3.7. Absorbance at 750 nm of callus cultures for a period of 55 days. The higher the absorbance the higher the polyphenol content.

Days	0	15	25	35	45	55
Mean	0.1521	0.0891	0.0598	0.1282	0.1314	0.1424
Std Dev.	0.0287	0.0319	0.0134	0.0140	0.0317	0.0417
Std Error	0.0091	0.0101	0.0042	0.0044	0.0100	0.0132
Count	10	10	10	10	10	10

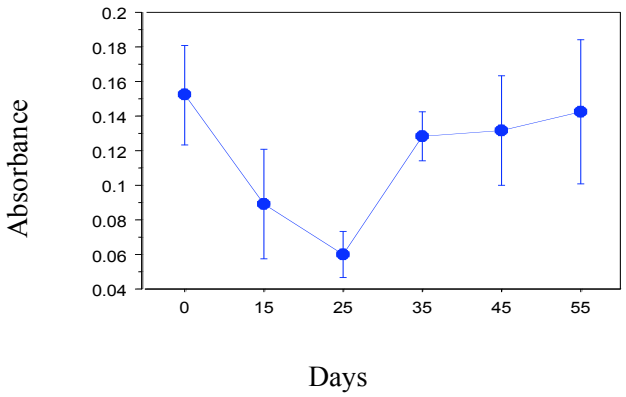
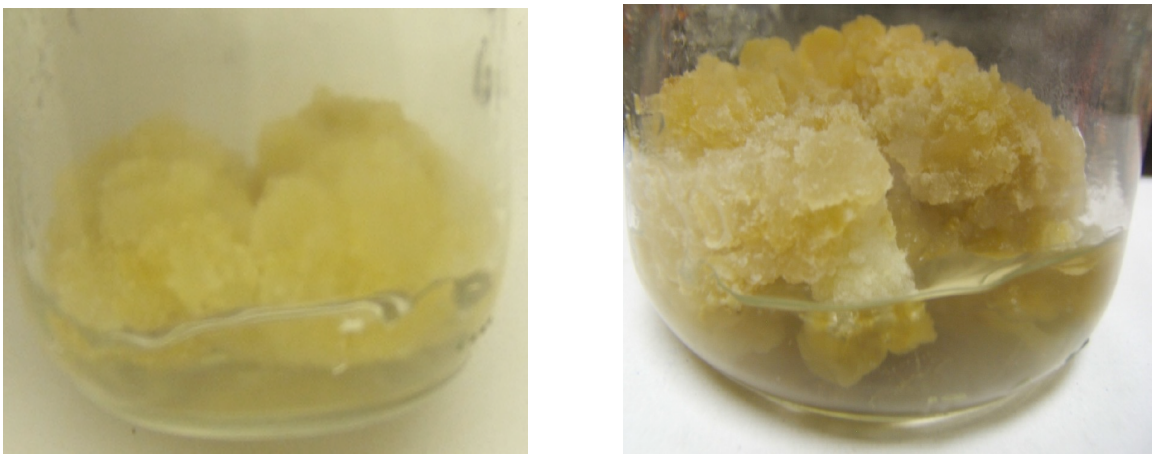


Figure 3.5. Callus absorbance changes at 750 nm in a period of 55 days. The higher the absorbance the higher the polyphenol content.The bars represent standard deviation (SD).

Figure 3.6 illustrates calli of cultures optimized for the production of phenolic compounds. They display good growth, friability and the yellow color indicative of good health. The culture at the left (A) is 50 days old and has reached a plateau of increased mass and phenol production. In contrast, the culture at the right (B) is 90 days old, is yellow brown and liquefaction of the media is evident.



A B

Figure 3.6. Fifty and ninety days old callus, A and B, respectively, on B-5 basal media in100ml jar containing 30 g/L sucrose, 0.5mg/L NAA, 0.5 mg/L NAA and incubated in light (16 hours photoperiod)

Determination of total polyphenols in solid media cultures. Antioxidant polyphenols were determined using Folin-Ciocalteau assay (Appendix , page 78) in catechin equivalent (Curve Sample, Figure A.3, page 79). Cultures in dark have higher content of polyphenols (up to 5.2% of freeze dried callus in 4 weeks old cultures) but they are slower growing, have low friability, and tend to discolor early, need frequent subculture and most of them die early. Cultures exhibiting satisfactory combined results were those cultured on B-5, supplemented 3% sucrose, 0.5 mg/l NAA, 0.5 mg/l BAP and incubated at 29ºC±1 and 16 hours light. Their polyphenolic content was originally determined at 2.8 % of freeze dried callus of 4 weeks cultures (Table 3.3 and Table A.3, page 76).

Under these conditions, cultures attained higher biomass and satisfactory polyphenolic production (Figure 3.6, Table A.3, page 76). As has been mentioned in Chapter 2, polyphenolic compounds in plants are often found as glycosides. Vinson (1999), after hydrolyzing fresh fig tissue with hydrochloric acid, found that total polyphenolic content increased by about 25%. In order to more accurately determine the total polyphenolic content in *F. carica* callus, samples (15 in number) of calli cultured for different periods of time over 50 days were extracted in 100% methanol for 24 hours under argon after being ground and then they were assayed for total polyphenols. Equal number of samples collected from the same cultures were further hydrolyzed for 30 minutes after adjusted the pH at 1.8 using hydrochloric acid and then assayed for total polyphenols. The results show that the polyphenolic content in hydrolyzed samples increased significantly

compared to those not hydrolyzed, 4.26%±0.96 of dry callus while those not hydrolyzed is 2.98%±0.8 (Table 3.8).

Table 3.8. Samples of solid cultures on B5 media supplemented with 0.5 mg/L NAA and 0.5 mg/L BAP were extracted in 100% MeOH and tested for phenols using Ciocalteau assay. In hydrolyzed samples the MeOH was adjusted to pH1.8.

	Number of samples	Mean (Percent phenols)	Std Deviation	Std Error
Not hydrolyzed samples (dry)	15	2.98	0.80	0.21
Hydrolyzed	15	4.26	0.96	0.25

Experiment 7. Establishing suspension cultures. Pieces of callus tissue was removed from cultures maintained on solidified B5 containing 30 g/L sucrose, 0.5 mg/L NAA and 0.5 mg/L BAP and used to inoculate liquid media of the same composition. The inoculum, approximately one gram of fresh weight, was introduced into 250 ml conical flasks containing 50ml of liquid media and incubated at room temperature (24ºC±2) on gyrotory shakers at 110 rpm) either in the dark or under constant cool white fluorescent lights. Ten suspension cultures (5 in dark and 5 in light) were renewed every two weeks after visual examination for apparent tissue increase and testing for total polyphenolic antioxidants secreted in the media by withdrawing aliquots of culture medium. Sixteen suspension cultures (8 in dark and 8 in light) of about the same weight were examined visually for apparent tissue increase every 10 days, harvested in 40 days and testing for total polyphenolic antioxidants secreted in the media by withdrawing aliquots of culture medium. The cultures were combined (light and dark cultures separately) filtered by using Watman filter paper (No1), freeze dried, their weight was taken, ground and assayed (five replica) for total polyphenols. This experiment resulted in no significant difference in the polyphenols secreted into the culture media of suspension cultures in

the light compared to those in the dark (Table 3.9) The relative phenolic content of the suspended mass of the cultures in the dark, however, was slightly higher comparing to those in light ($P < 0.08$, Table 3.9) by t-test. Consequently, another experiment, using suspension cultures incubated in the dark (experiment 8) was conducted, to further investigate the tissue growth and polyphenolic content in suspension cultures.

Table 3.9. Effect of light and dark on phenol production in suspension cultures. The higher the absorbance the higher the polyphenolic content

	Total dry weight of inocula (grams)	Total dry mass in 40 days (grams)	% increase of mass in 40 days	Absorbance (750 nm) of polyphenols in suspended callus	Absorbance (750nm) of secreted polyphenols
Treatments in light	0.201	0.637		0.0290±0.007	0.0425±0.013
Treatments in dark	0.196	0.606		0.0415±0.013	0.0412±0.007
samples				5	8
Paired t-test, Prob <t				0.08	0.41

Experiment 8. To evaluate the relative rate of growth and polyphenolic increase in suspension cultures, based on the results of the above mentioned cultures (Table 3.6, page 40), a separate experiment with suspension cultures in the dark on B5 was conducted. The possibility of using suspension tissue cultures for obtaining antioxidant polyphenols could be based on the results of this expersiment. Five cultures were examined every week for a total of 9 weeks, starting 2 weeks after inoculation. The combined five cultures were filtered and the aggregate cell mass was freeze dried, weighed and assayed for total phenolics.

Suspension cultures in B-5 media (1 g callus/50ml) containing 0.5mg/l NAA and 0.5 mg/l BAP on shaker (110 rpm) in dark were observed. The mean values of dry weight was found (2nd row

Table 3.10) and the percentage antioxidant polyphenols was estimated (3rd row Table 3.10) in catechin equivalent by using Ciocalteau assay. Antioxidant polyphenol content in the media of these cultures was also estimated (4th row Table 3.10) by using the same method.

Table 3.10. Suspension cultures grown in dark Abbreviations. Inoc.: inoculant, wt: weight, mg: milligrams, polyph.: polyphenols. The calculations and statistical evaluation were perfomed using JMP and Statview for Windows 2000.

week	1 (inoc)	2	3	4	5	6	7	8	9
Mean wt (mg)	38.6±1.3	89.2±12	102.2±16	165.8±58	240.8±84	416.2±47	442.2±11	452.2±70	467.0±57
% polyph.	2.992	1.43	1.49	1.34	1.42	1.24	0.57	0.84	1.62
Polyph. µg/ml		1.14	6.03	18.54	18.94	26.89	30.86	40.65	53.29

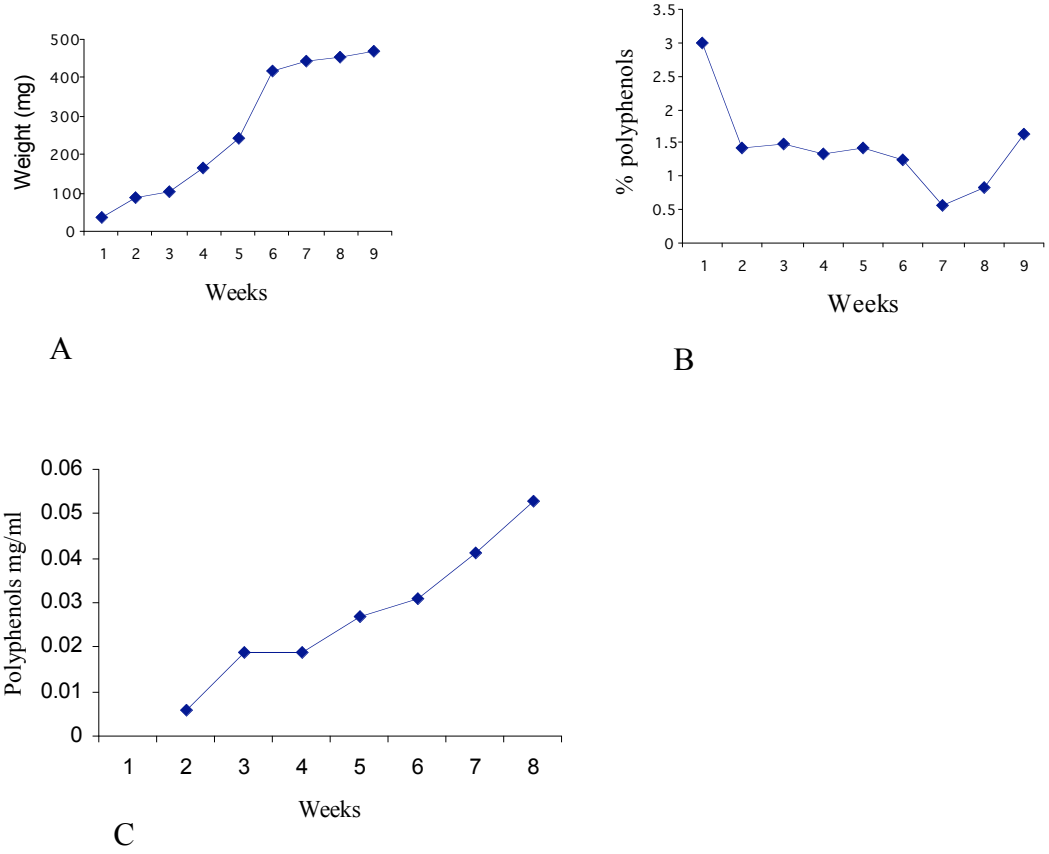


Figure 3.7. Analysis of callus growth and polyphenol concentration in suspension cultures over a period of 9 weeks. A; increase of biomass in mg. B; the % content of polyphenols in biomass of suspension cultures, and C; the increase of polyphenols content in the media of suspension cultures. The graphs were created using Statview for Windows 2000.

Experiment 9**.** To find out if any of a variety of elicitors would increase production of phenolic antioxidants by suspension-cultured tissue, sets of five cultures were used for each treatment. For each elicitor and for each concentration tested, five cultures were incubated in the dark. The elictors tested were jasmonic acid, chitosan, pectinase, and DMSO. At 7 and 10 and 30 days after inoculation elicitors were introduced into the cultures. Aliquots of these cultures were visually examined 0, 1, 2, 3 and 6 days after elicitation and assayed for total phenolic antioxidants secreted in the media and cell viability using the Folin-Ciocalteau assay (Singleton and Rossi, 1965), and Evan's blue assay **(**Gaff et al., 1971**),** respectively. To measure the total polyphenols remained in the cells after elicitation, suspended cells and cell aggregates were collected by filtering the culture medium through a Buchner funnel fitted with Whatman No.1 filter paper. The tissue was freeze- dried, homogenized and extracted in 100% MeOH under argon for 2 hours and examined for total polyphenolic content using the Folin-Ciocalteau assay. For most of cultures treated with different elicitors there was not any indication of antioxidant polyphenols increase. However, increase of secreted polyphenols, without lowering the cell viability in 6 days was observed in media of suspension cultures treated with 10 µL of pectinase (*Asperigus niger*) in 50 mL suspension culture containing 1g callus. Cultures treated with 20, 30, 40, and 80 µl had good polyphenolic secretion in media the first day and good cell viability, but the cell viability dropped significantly thereafter. (Figure 3.8,Table 3.11).

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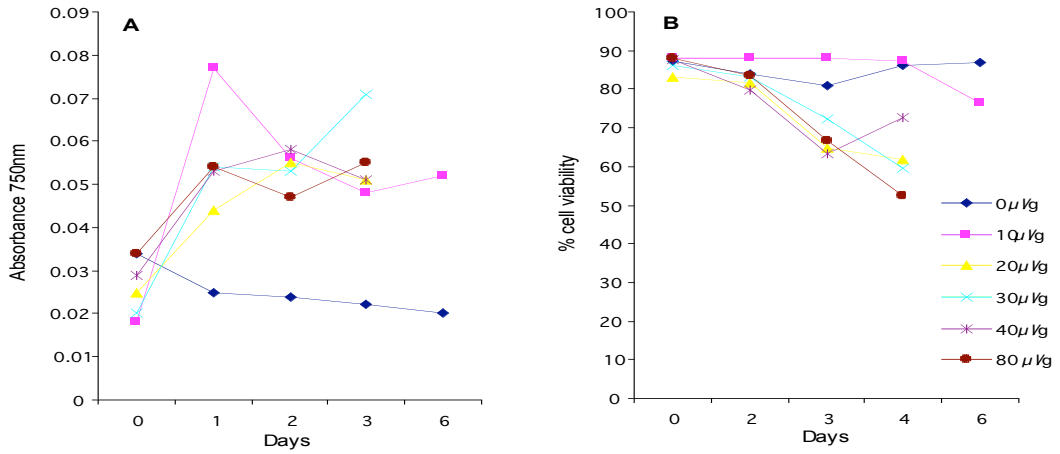

Figure 3.8. The effects of pectinase (*Aspergillus. niger*) on 50 days old suspension *Ficus carica* tissue cultures. Pectinase is applied in different concentrations (20, 30, 40 and 80 µL) for three days and 6 days (10 µL) to 50 mL cultures inoculated with 1g of callus. A: Shows the absorbance in 750 nm. The higher the absorbance the higher the polyphenolic content secreted in the medium. B: shows the cell viability during the elicitation period.

Table 3.11. Suspension cultured elicited with pectinase of *Aspergillus niger.* increase of absorbance (abs) and therefore increase of sectreted polyphenols in media, without lowering the cell viability in 3 days was observed in media of suspension cultures treated with 10 µL of pectinase. Cell viability remains high at least for six days.

Pectinase µl/50ml	0 days		1 day		2 days		3 days		6 days	
	Mean Abs 750 nm	Mean Cell viability (%)	Mean Abs 750 nm	Mean Cell viability (%)	Mean Abs 750 nm	Mean Cell viability (%)	Mean Abs 750 nm	Mean Cell viability (%)	Mean Abs 750 nm	Mean Cell viability (%)
0	0.034 ±0.009	87.3 ±3.3	0.025 ±0.007	83.8 ±8.3	0.025 ±0.008	80.8 ±4.4	0.022 ±0.07	86.2 ±3.8	0.02±0.005	87 ±3.4
10	0.018 ±0.004	88.13 ±1.2	0.077±0.01	87.86 ±2.9	0.056 ±0.02	88 ±3.6	0.048 ±.01	87.45 ±2.9	0.05 ±01	76.4 ± 8.7
20	0.025 ±0.001	83.3 ±5.3	0.044 ±0.001	81.8 ±5.9	0.044 ±0.001	64.7 ±17.5	0.051 ±0.001	61.7 ±11.7		
30	0.020 ±0.008	86.2 ±5.6	0.054±0.001	83.0±5.9	0.054 ±0.001	72.2 ±5.0	0.071 ±0.002	59.7 ±19.3		
40	0.029 ±0.002	87.7 ±4.4	0.053±0.002	79.8±14.9	0.053 ±0.002	63.2 ±23.2	0.051 ±0.002	72.5±4.7		
80	0.034 ±0.002	88.0 ±5.0	0.054±0.002	83.5±8.2	0.054 ±0.001	66.8 ±16.9	0.055 ±0.001	52.5 ±32.7		
	1.211	2.858	3.559	2.898	8.71	3.347				

Results and Discussion

Pilot studies. It was noticed that callus inocula of different size showed different growth rates. For this reason, care was taken that the inocula of similar in size or weight was used in each experiment. In pilot experiments using solidified B-5 medium, callus was initiated almost in all cultures in about 10 days (Figure 3.3, page 36). Cultures containing no hormones started initiating callus but within two weeks became necrotic. Cultures containing only cytokinins became necrotic in about 6-8 weeks, while cultures containing only auxin could initiate callus and survive for more than a year if subcultured periodically. Cultures containing auxin and cytokinin could grow well and live for at least 5 years, if subcultured regularly.

Designed experiments. Based on results of pilot studies, five additional sets of experiments were conducted and designed to further optimize culture conditions for the best possible production of antioxidant polyphenols. It was assumed that the healthier the cultures, the better the chances of their achieving greater biomass in turn resulting in a higher yields of desired products. Therefore the initial three sets of experiments in this series were focused on establishing tissue cultures that were consistently light in color, (non necrotic) friable, with a relatively high biomass. Many cultures maintained on either B-5 or MS at pH 5.5 supplemented with 0.5 mg/l NAA and 0.5 mg/l BAP and incubated at 29ºC±1 subjected to a 16 hour photoperiod did in fact support callus development with all the desired characteristics (Table3. 3, page 35). Nevertheless, it was deemed desirable that additional experiments were conducted in which pH range and sucrose concentrations were varied. None of these experiments resulted in inducing significant

increases in detectable phenolic compounds. Cultures, however, at pH 5.5 and containing 30 g/L sucrose showed significantly higher biomass compared to cultures at pH 4.5, 5.00, 6.00 and 6.5, and the biomass in cultures on B-5 media containing 30 g/L sucrose was higher than in those containing 20 g/L. Finally, another experiment was conducted to investigate the biomass changes of cultures before they reach a plateau in increased growth.

In the first experiment mentioned above on B5 media containing NAA (1, 5, 10, and 15 mg/L) and BAP (1, 5, 10, and 15 mg/L), 24 days after inoculation,of cultures A, E, I, F, H and O displayed comparable growth, yellowish to green color and semi friable callus on the upper surface of the explants, and in general, better growth and callus texture than the rest of the cultures. Since H and O contained relatively high concentrations of hormones and did not appear to be better than the rest of the 6 best, they were not included for further investigation. Cultures with NAA concentrations of 0.1-0.5 mg/L and BAP concentrations of 0.1-0.5 mg/L, looked comparable to cultures containing higher hormonal concentrations, and therefore these lower auxin cytokinin combinations were selected for further experiments

Elicitation experiment. The purpose of this experiment was to use elicitors to induce secretion of phenols of cells to media before the cells are broken down, and the cells be reused for phenol secretion after been transferred to new media and re-elicited. Of these treatments, as mentioned in Materials and Methods, only cultures treated with pectinase (*Aspergillus niger)* produced increase of antioxidant polyphenols. Suspension cultures treated with 10 µL of pectinase (*Aspergillus niger*) in 50 mL media containing 1g callus produced increase of secreted polyphenols, without lowering the cell viability in 6 days.

Cultures treated with 20, 30, 40, and 80 µL had good polyphenolic secretion in media the first day and good cell viability, but the cell viability dropped significantly thereafter. Elicitation for longer than 6 days does not increase further secretion of polyphenols. It is suggested here that pectinase of *Aspergillus niger* at a concentration 10 µL in 50 mL suspension cultures can successfully be used for eliciting polyphenols of cells in suspension cultures.

CHAPTER 4: ISOLATION AND IDENTIFICATION OF PHENOLIC ANTIOXIDANTS PRODUCED BY *FICUS CARICA L.* CALLUS CULTURED

Summary

This research was initiated in an effort to optimize *Ficus carica* tissue cultures for relatively rapid callus growth combined with a relatively high yield of useful polyphenolic antioxidants. It was shown that petiole discs of *Ficus carica* inoculated on Gellan-solidified Gomborg's B-5 basal medium with minimal organics, 3% sucrose and supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA and incubated at 29ºC±1 under high light intensity at a 16 hours photoperiod resulted in relatively rapid callus initiation and growth. These culture conditions produce healthy yellowish friable callus development, suitable for maintaining cultures for many years if subcultured every 50 days as well as for successfully initiating suspension cultures. Using the Folin-Ciocalteau assay, the total polyphenolic antioxidant content in cultures incubated under the conditions given above was determined to be 4.26% of the freeze dried weight of the callus tissue. Ethyl acetate and water extracts had high antioxidant activity while in hexane extracts the antioxidant activity was not significant. Isolation and identification of the polyphenolic compounds were established by chromatographic methods (TLC, HPLC). It was found that the main known polyphenolic compounds found in both fig fruit and vegetative tissues were produced by callus tissue when cultured under the conditions described herein.

Suspension cultures exhibited a satisfactory increase in biomass and moderate polyphenolic antioxidant production. Suspension cultures could not be maintained in a healthy condition for more than two months under the conditions employed. Further

efforts are needed to establish suitable conditions for producing polyphenolic antioxidants in suspension cultures in quantities and extended incubation periods comparable to that obtained from cultures incubated on solidified media. Pectinase of *Aspergillus niger* is a good elicitor of cell polyphenolic content secretion in suspension cultures without lowering the cell viability.

Introduction

There are no reports regarding the use *Ficus carica* tissue cultures for the production of polyphenolic antioxidants. Furthermore the information on polyphenols of *Ficus carica* fruit, or vegetative tissues currently available is limited. It has been reported however, that the polyphenolic antioxidants in fig fruit is anywhere between 4 to 50 times higher than in any other fruit (Edenharder et al., 1998; Vinson, 1999). Robinson and Robinson, (1932), in a survey of anthocyanins, reported that cyanidin glucocides were present in *Ficus carica.* Puech et al. (1975) used paper chromatography, densitometry and gas chromatography to characterize the anthocyanins found in the skin of ripened *Ficus carica* "Mission". Cyanidin glycosides (cyanidin-3,5-diglycoside, cyanidin 3-rhamnoglucoside, cyanidin 3-monoglucoside, and cyanidin 3-rhamnoglucoside) and pelargonidin 3-rhamnoglucoside were identified. They found anthocyanins were higher in *Ficus carica* "Mission" than other cultivars such as "Kadota" and "Calimyrna.". Solomon et al. (2006) used hydrolyzed extract of *Ficus carica L*. fruit and analyzed it using reverse-phase liquid chromatography (RP-LC), HPLC and NMR. They found that the content of anthocyanins were higher in Mission (11 mg/100g fresh weight) among six cultivars examined. The main anthocyanin was cyanidin-3-O-rhamnoglucoside. The total

polyphenols were 281 mg/100 g FW (fresh weight) and the total flavonoids 21 mg/100g FW. Vaya and Mahmood (2006) used reversed phase high-performance liquid chromatography (HPLC) and ultraviolet and visible range diode array detector (UV/VIS) and electrospray ionization (ESI)-mass spectrometry (MS) detectors and determined the major flavonoid content of leaf extracts (70% ethanol). They found the major compounds were quercetin and luteolin, with a total of 631 and 681 mg/kg extract, respectively. Del Caro and Piga (2008), working on green and black fresh fig fruit, used high performance liquid chromatography/ diode array detector (HPLC-DAD) analysis for the extracts of the peels and the pulp of the fruit. The results showed that the concentration of phenols were higher in the peels than in the pulp and higher in the black fruit than in the green. The rutin content in peels was up to 527 mg/kg for green fruit and up to 1,071 mg/kg for black. The pulp contained only cyanidin 3-O-rutinoside. Additionally, cyanidin 3-O-glucoside, chlorogenic acid and another cinnamic acid were detected in the peel of the green and black fruit. Veberic et al (2008), working on *Ficus carica L.* cultivars (Skofjotka Crna petrovka Miljska figa) and using HPLC-PDA system, found that the highest content of phenolic compounds in the fruit of these cultivars were rutin (28.7 mg per 100 g FW), (+)-catechin (4.03 mg per 100 g FW), chlorogenic acid (1.71 mg per 100 g FW), epicatechin (0.97 mg per 100 g FW), gallic acid (0.38 mg per 100 g FW) and syringic acid (0.10 mg per 100 g FW). According to Duke J. (1992), caffeic acid, kaempferol, quercetin, and ferulic acid are found also in plant (concentration was not determined), and rutin in leaves is estimated to be 500-1000 ppm. Total flavonoids in leaves were found to be about 5300 ppm.

Based on the above reports, anthocyanins (mainly cyanidin-3-O-rutinoside and cyanidin—O-rhamnoglucoside, (−)-Apigenin, (apigenin-4,6-arabinosyl-8-c-glucoside, apigenin-4,6-arabinosyl-8-c-arobinose), astragalin, catechin chlorogenic acid, emodin, epicatechin, isoquercitrin, (kaempferol glycoside), ferulic acid gallic acid, kaempferol-3-beta-d-rutinoside, rutin and syringic acid are found in fig fruit. Caffeic acid, tannic acid, chlorogenic acid, quercitin, kaempferol and ferulic acid are found in plant. Quercitin, rutin, luteolin, caffeic acid, tannic acid and chlorogenic acid are also found in fig leaves

An analysis of the polyphenolics extracted from *Ficus carica* tissue cultured using the conditions determined during the course of the research reported herein has shown that the main known polyphenolic compounds found in the fruit, as well as the vegetative tissues of this plant, can be also be produced by callus tissue cultures of this plant (Figure 4.2, page 62 and Figure 4.3, page 65).

Materials and Methods

Chemicals: (+)-catechin, (+)-epicatechin, gallocatechin, epigallocatechin gallate, kaemferol, caffeic acid, L-ascorbic acid, chlorogenic acid, p-coumaric acid, tannic acid, cyanidin, emodin, myricetin, rutin hydrate and (±)-taxifolin were purchased from Sigma (St. Louis, MO); apigenin, and Diaion Hp20 resin were purchased from Aldrich (Milwaukee, Wisconsin); 1,1-myricetrin, quercetin and quercetin-3-β-D-glucoside were purchased from Fluca.

Analysis of the crude extracts. Calli of solid cultures, and cells or cell aggregates harvested from suspension cultures were freeze dried, ground using a mortar and pestle, extracted in 100% MeOH and separated as shown in the flow chart (Figure 4.1).

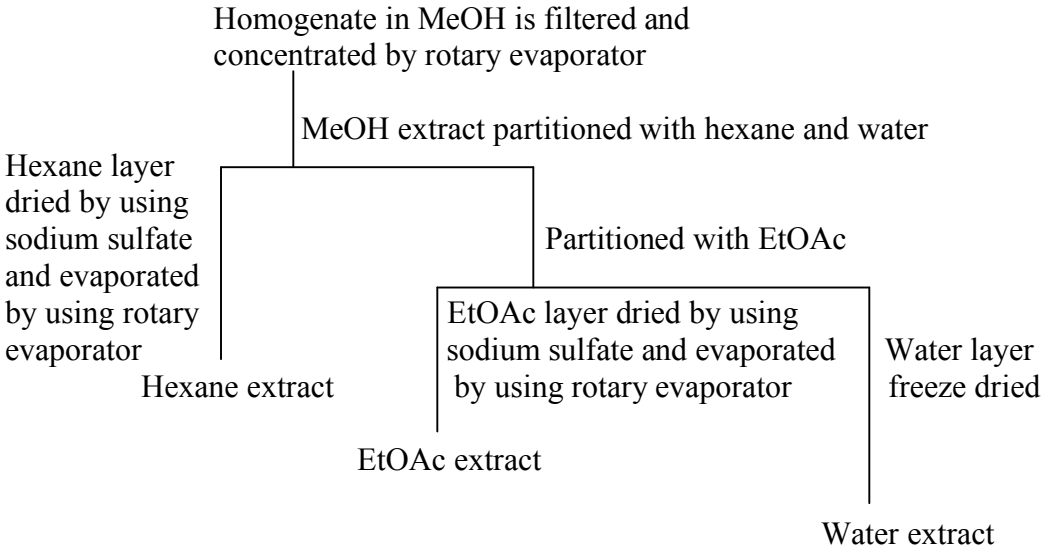

Figure 4.1. Flow chart showing separation method used for tissue cultures material after it was homogenized and extracted in MeOH under argon on shaker.

The total polyphenolic antioxidants of the methanol extracts were measured using the Folin-Ciocalteau assay. The antioxidant activity of the hexane, EtOAc, and water extracts were measured using a DPPH assay.

Identification of antioxidant polyphenols. The identity of polyphenols present in hexane, ethyl acetate and water extracts of callus tissues were made using Thin Layer Chromatography (TLC) and HPLC.

Isolation of phenolic extracts. Water extracts were loaded on columns of Diaion HP20 resin and eluted with water, followed by 50% water-methanol and then 100% methanol, successively. The ethyl acetate extracts were loaded on a Sephadex LH-20 column and eluted with methanol, ethyl acetate, and acetone, successively. The pooled fractions collected were dried *in vacuuo*, under argon, or lyophilized and analyzed by using HPLC.

TLC analysis. TLC analysis was performed as a preliminary attempt to identify the main polyphenolic antioxidants (Sherma, and Fried, 1996). Extracts as well as the standards were diluted in 90% MeOH (1 mg/mL) and spotted on G/UV254 plates (Altech Associates Inc, Deerfield, Il) using 10 µL pipettes (5 µL for the extract spots, 2 µL for the standard spots). The plates were developed with benzene (BEN):ethyl acetate (EA):acetic acid (AA):formic acid(FA) in a proportion of 5:5:1:1 or 8:2:1:1 or 10:2:1:1 or 7:3:1:1 or10:5:1:1 or 7:3:1:1, EA:W (water):AA: FA in a proportion of 10: 2.6: 1:1 or 10:2:1:1, or 12:7:1:1, and H (Hexane):EA: AA: FA in a proportion of 8.5:4.5:1:1. The compounds were visualized under the UV light and/or by spraying the plates with 5% sulfuric acid in methanol or 50% Folin-Ciocalteau reagent in ethanol or 400 µm DPPH, in ethanol, and heated at about 80^{o}C to visualize the spots . The colors of the spots were enhanced by Photo Suite Image. The intensity was examined visually and compared to that of the standards. The extracts that only started being visible were represented by (+), the highest bright were represented by (+++) and the moderate bright were represented by (++).

HPLC Analysis. Samples of the extracts, as well as the standards, dissolved in methanol containing 0.5% phosphoric acid, were filtered using 0.45 µm pore size. A Waters 2695 separations Module equipped with a 996 Photodiode Array (PDA) detector controlled by Empower software was used. A Synergi Fusion, 5 µm, 4.6 X 250 mm I.D, C18 reversed-phase column was used for the separation. The solvents used for gradient elution were: A, water with 0.05% Trifluoroacetic acid (TFA) and B, Acetonitrile with 0.05% TFA. The samples and the standards were eluted as follows: at 0 min 88% A and 12% B, at 25 min 79% A and 21% B , at 30 min 75% A and 25 B, and at 35 0% A and 100% B. The total run time was 35 min and the flow rate was 1 mL/min The sample

injection volume was 20 µL and the sample temperature 4ºC while the column temperature was 30ºC.

Compounds were identified by comparing their retention time and their UV spectra by those of the standards. Pure standards of : apigenin, caffeic acid, (+)-catechin, chlorogenic acid, cyaniding, emodin, (+)-epicatechin, epigallocatechin gallate, gallic acid,gallocatechin, kaemferol, p-coumaric acid, myricetin, myricetrin, quercetin-3-β-D, rutin hydrate, tannic acid, and (±)-taxifolin were used to determine retention time, separately for each standard, comparing with their spectra. Then the standards were mixed, and analyzed using the same method mentioned above and the order of their retention time were determined. The spectra of the standards and that of the samples were recorded between 210 and 350 nm and detected at 268 nm. The retention time of the mixed samples, ethyl acetate extracts, water extracts, hexane extracts, fractions of ethyl acetate extracts and fractions of water extracts were determined by using the same solvents and methods used for analyzing the standards. The unknown compounds in the samples were identified by comparing their retention time and spectra with the retention time and spectra of the known standards.

Results and discussion

Antioxidant . The antioxidant activities of ethyl acetate extract, water extract and 50:50 H2O:MeOH were IC50=28.35±6.1, IC50=53.32±5.8 and 17.58±2.02, respectively. Table 4.1). Hexane extracts showed inconsistent activity, mostly above 300 µg, presumably due to degrading of the compounds during extraction. Emodin, however, was present in TLC analysis in hexane extract and in ethyl acetate extract (not hydrolysed).

Table 4.1. Free radical scavenging activity of ethyl acetate and aqueous extracts of *Ficus carica* callus by DPPH method. Ascorbic acid was used as positive control

Sample	Scavenging activity IC50	R^2
Ethyl acetate	28.35±6.1	0.9041±0.031
Water extract	53.32±5.8	0.9816±0.014
Water extract eluted with H2O:MeOH, 50:50	17.58±2.02	0.9840±0.013
Ascorbic acid as a control	14.5±1.6	0.9963 ± 0.002

TLC analysis.The results obtained by TLC analyses of ethyl acetate and water extracts of tissue cultured on solid media are given in Table 4.2 and Figure 4.2. Chlorogenic acid is found in high intensity in ethyl acetate extract and water extract. Apigenin, (+)-atechin, Gallic acid and Quercetin-3-β-D glycoside are in moderate intensity and lesser amounts of the rest polyphenolics. The main polyphenolic compounds found in the fruit, the leaves and the stem, therefore, are found in calli as well. In TLC plates, many unidentified compounds reacted with applied DPPH, turning to orange spots, an indication of presence of polyphenolic antioxidants additional to those already identified. As mentioned in Chapter 3, finding new or different compounds than those found in the parent plant is quite common, presumably because of the environmental differences under which the tissue cultures grow compared to those experienced by the parent plants. Additional work is proposed to identify these as yet unidentified phenolic compounds by HPLC using additional known standards, and by NMR.

Table 4.2. Polyphenolic compounds found in callus of *Ficus carica* tissue cultures determined by TLC. The solvents used for the TLC plates were benzene-ethyl acetate- acetic acid- formic acid, , or ethyl acetate- water- acetic acid- formic acid, or hexane-ethyl acetate - acetic acid- formic acid. Intensity; +++: High, ++: Moderate, and +: low.

Compounds	Intensity	Extract	TLC solvent	Detection reagents
Apigenin	++	EA	BEN : EA: AA: FA, 10:2:1:1	5% sulfuric acid in MeOH
Caffeic acid	+	EA	BEN : EA: AA: FA 10:5:1:1	5% sulfuric acid in MeOH
Catechin	++	EA	BEN : EA: AA: FA 7:3:1:1	50% Folin Ciocalteau reagent (2N)
Chlorogenic acid	+++	EA, W	EA:W:AA: FA, 10: 2.6: 1:1	50% Folin Ciocalteau reagent (2N)
Emodin	+	H	H:EA: AA: FA 8.5: 4.5:1:1	UV
Epicatechin	+	EA	BEN : EA: AA: FA 7:3:1:1	50% Folin Ciocalteau reagent (2N)
Gallic acid	++	EA	BEN : EA: AA: FA 7:3:1:1	50% Folin Ciocalteau reagent (2N)
Isoquercetrin	+	EA	BEN : EA: AA: FA, 10:2:1:1	5% sulfuric acid
Kaemferol	+	EA	EA:W:AA: FA, 10:2:1:1	50% Folin Ciocalteau reagent (2N)
Myricetrin	+	EA	BEN : EA: AA: FA, 10:5:1:1	5% sulfuric acid in MeOH
Quercetin-3-β-D	++	EA	BEN : EA: AA: FA 5:5:1:1	50% Folin Ciocalteau reagent (2N)
Rutin	+	EA	EA:W:AA: FA, 10: 2.6: 1:1	50% Folin Ciocalteau reagent (2N)
Taxifolin	+	EA	BEN : EA: AA: FA 5:5:1:1	50% Folin Ciocalteau reagent (2N)

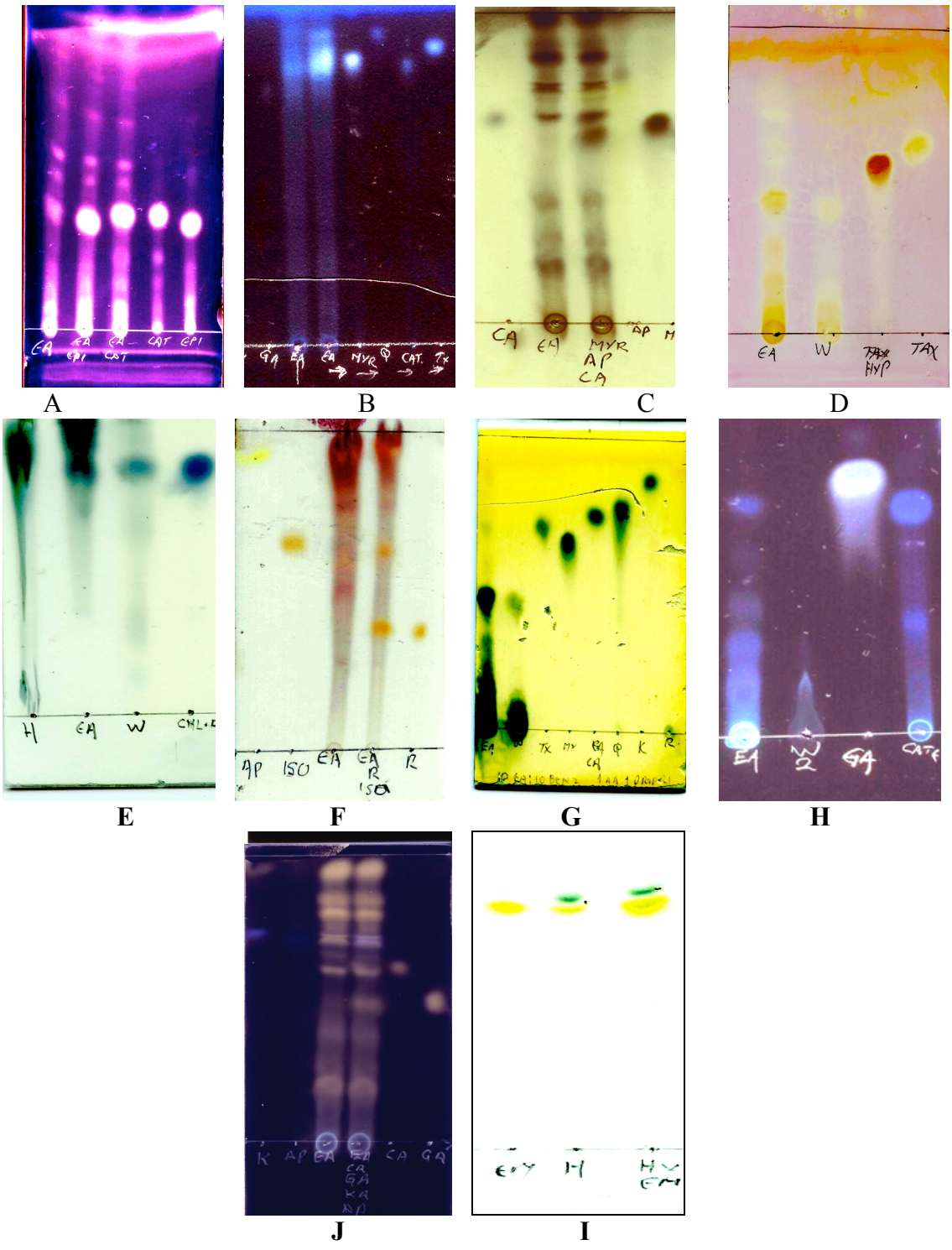

Figure 4.2. TLC analysis (Description on the next page)

A: Ethyl acetate (EA) extract eluted with benzene (BEN): EA: acetic acid(AA): formic acid (FA), 7:3:1:1. B: EA extract eluted with BEN : EA: AA: FA, 5:5:1:1. C: EA extract eluted with BEN : EA: AA: FA, 10:5:1:1, and detected with 5% sulfuric in methanol. D: EA and water (W) extracts, eluted with EA:W:AA: FA, 10: 2.6: 1:1, and detected with DPPH. E. Hexane, EA and water extracts eluted with EA:W:AA: FA, 10: 2.6: 1:1, and sprayed with 50% Folin Ciocalteau reagent. 5% sulfuric acid. F: EA extract eluted with BEN : EA: AA: FA, 10:2:1:1 and detected with DPPH. G: EA and W extracts eluted with EA:W:AA: FA, 10:2:1:1 and visualized with 50% Folin Ciocalteau reagent. H: EA and W2 (fraction of water in Diaion HP20 column chromatography) extracts, eluted with BEN : EA: AA: FA 7:3:1:1 and visualized with 50% Folin Ciocalteau reagent J: Hexane extract, eluted with EA: W: AA: FA, 12:7:1:1 and visualized under UV 254. I: H:EA: AA: FA and visualized under UV 254. Abriviations; AP: apigenin, CA; caffeic acid. CAT or CATE catechin, CHLOR; chlorogenic acid, EM: Emodin. EPI, epecatechin, GA: gallic acid, HYP; hypercentin, ISO: isoquercetrin, K; kaemferol, MYR: myricetrin Q: quercetin R: rutin, TX or TAX: taxifolin . The color of the plates were enhanced using Photo Suite Image to improve contrast.

HPLC analysis. The retention time of the mixed standards, as mentioned in " material and methods", were compared with the respective retention time and spectra of the mixed ethyl acetate, water, and hexane extracts in consideration of the retention time of the separated standards and their spectra. In mixture of standards, Figure 4. 2, the analyzed standard are Given; 1: Gallic acid. 2: Tannic acid. 3: (+)-chatechin; 4: Chlorogenic acid. 5: Epicatechin. 6: Caffeic acid. 7: EGCG. 8: Coumaric acid. 9: Ferulic acid. 10: Taxifolin. 11:Cyandin. 12: Myricetin. 13: Rutin. 14: apigenin. 15: Kaemferol. 16: Quercetin. 17: Emodin. The standards are compared with the retention time and spectra of of the compounds in ethyl acetate (not hydrolyzed) and water extracts (not hydrolyzed). UV spectra for polyphenolic identified in the ethyl acetate and water extracgts are shown in Figure B.5 (page 91). According to this analysis, most of the standard polyphenols are found in the extracts (Figure 4.3). It has been noticed that identified polyphenols in water extract were also present in ethyl extract where most of polyphenols were found. In hexane extracts identifiable compound compared with the known standards were not present. In the ethyl acetate fractions the identified compounds were the same of those in the crude ethyl acetate extract (data is not shown). The quantification of the identified compounds as well as the isolation and identification of possible novel polyphenols in the mentioned extracts is considered as future work.

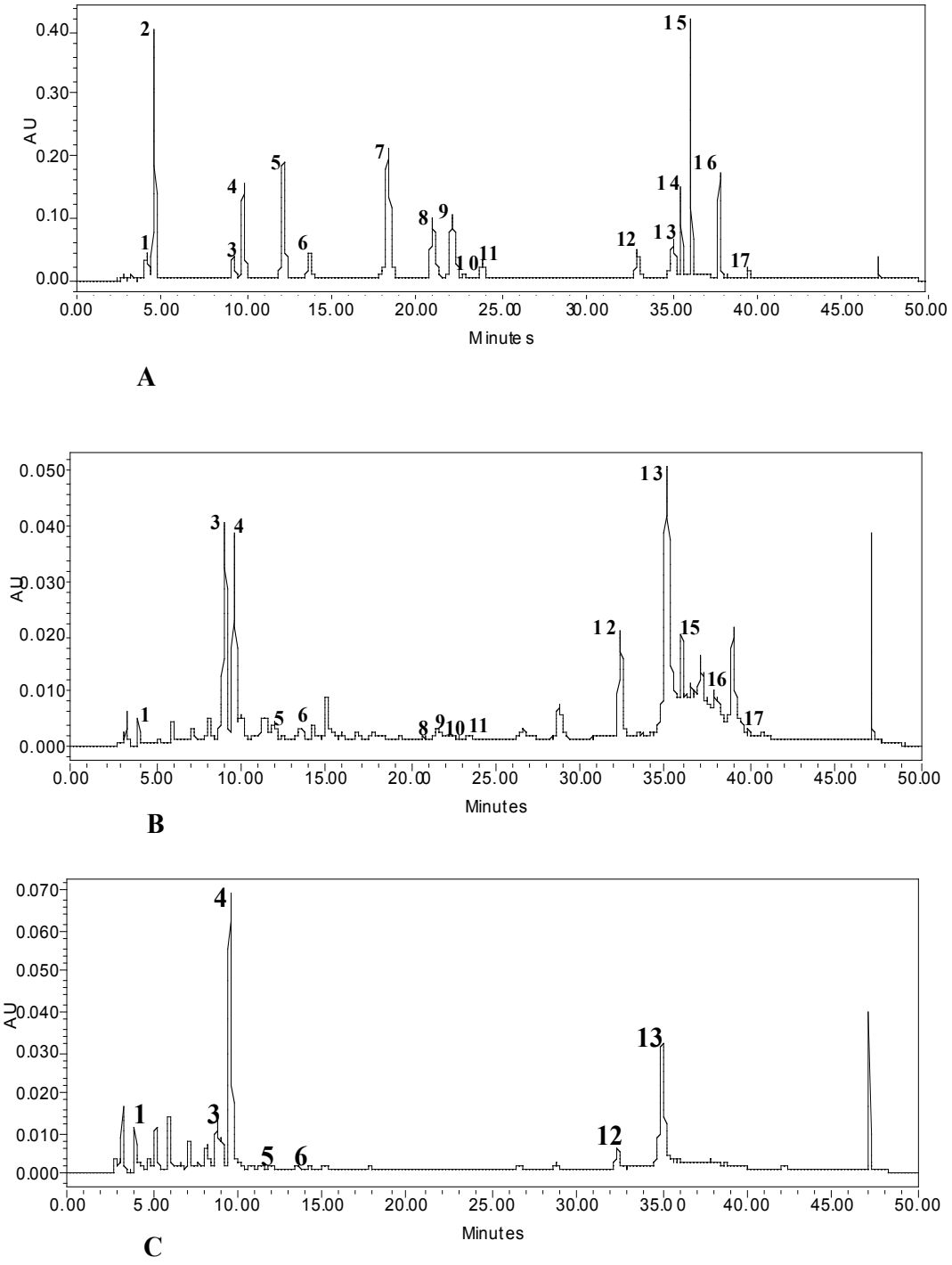

Figure 4.3. A: Mixture of standards. B: Ethyl acetate extract C: water extract. The Compounds in extracts are compared with the known standards (A) and their identification is determined. Detected compounds in ethyl acetate extract (B) are; 1: Gallic acid. 3: (+)-chatechin; 4: Chlorogenic acid. 5: Epicatechin. 6: Caffeic acid. 8: Coumaric acid. 9: Ferulic acid. 10: Taxifolin. 12: Myricetin. 13: Rutin.. 15: Kaemferol. 16: Quercetin. and 17: Emodin. Few of these compounds are found in water extract as well, but not any additional polyphenols are detected in water extract.

CHAPTER 5: CONCLUSION

As discussed in Chapter 1, most drugs are derived from plants, and more than 3000 plants have been identified by the United States National Cancer Institute as having anti-cancer properties and most of the drugs used for cancer either they come from natural products or they are based on natural products (Hort, 1948: Sigerist, 1951: Riddle, 1987). Efforts to synthesize drugs derived from plants is uneconomical since these drugs usually are complex and difficult to synthesize.

Food additives, like antioxidants, when prepared synthetically have to undergo expensive testing in order to meet required safety standards (Franklet, 1995). Additionally, consumers are reluctant to accept synthesized drugs, and the desire for natural food additives has constantly increased (Aruoma, O.I., 1994).. At present phytochemical products are largely obtained from plants grown in commercial plantations which are susceptible to disease and vulnerable to unfavorable changes in environmental conditions such as drought that reduce the quantity and or quality of the products for which they are cultivated. For all these reasons, plant tissue cultures have been considered as alternative sources of useful plant products. Plant cells cultured in vitro have been found capable of producing many of the same compounds that the parent plants produce (Parr et al., 1988; Basile et al., 1993; Nair MSR et al., 1986; Jianyong and Jian-Jiang Zhong, 1999).

In this work, tissue cultures from excised petioles of *Ficus carica* incubated under a variety of carefully controlled conditions were used to produce measurable quantities of antioxidant polyphenols. Polyphenols are secondary plant metabolites, widespread in the

plant kingdom, including fruits and plant parts where they serve a variety of functions in the plants that produce them. In humans, they may help prevent development of cancer by their antioxidant, free radical scavenging, and antiprolifiration, antiprogression activities. They also may possess anti-inflammatory, antiviral and antibacterial properties (Kellogg, et al., 1994; Rijnkels, et al., 1997; Sachiko Okabe et al., 1998).

The fruits of *Ficus carica L*. (Moraceae), the plant that produces the edible fig, is mostly grown in tropical, semitropical, and temperate Mediterranean climates where the best fruits are obtained (Condit 1941, 1947, 1955). The fruits are mostly preserved dry because fresh fruits are very perishable (Kim et al., 1992; Budavari et al., 1996). Fresh or dry, they are very rich in nutrients, especially in polyphenols (Edenharder et al., 1998; Vinson 1999). Because of their vulnerability to spoiling, they are not readily available to many potential consumers and possibly not known to many people living in regions where the plant does not grow. The objective of this work was to determine whether tissues isolated from *Ficus carica* could be cultured under conditions that result in yields of polyphenols comparable to those presently obtained from field grown figs and produced in sufficient quantities to be used as food supplements or other medicinal uses.

Many nutrient media-hormone combinations, and variations in pH, photoperiod, light intensity, temperature, and sucrose concentrations were investigated to optimize culture conditions that would support the best yield of polyphenols. In a series of experiments up to 200 cultures were used resulting in obtaining healthy, friable callus with satisfactory growth in combination with satisfactory phenol concentrations/antioxidant activity. Of the many conditions investigated, it was found that petiole explants manifested the best growth and phenol production on B-5 solid media containing 30 g/L sucrose and

supplemented with 0.5 mg/L NAA and 0.5 mg/L BAP at a 16 hours photoperiod and 29ºC±1. Under these conditions a plateau in growth and phenol production was reached at 45 to 50 days. The total polyphenolic yield of the callus tissue was 4.26±0.96 percent of the dry weight, approximately equal to that found by Solomon et al. (2006) in fig fruit (281mg/100g fresh weight). With an incubation period of 45 to 50 days, the cultures could be predictably "harvested" 7 to 8 times a year.

About 105 suspension cultures were used to investigate the best conditions for good growth and polyphenolic yield. Under the conditions utilized, the increase in tissue mass and phenol production was less that obtained by tissue cultured on solidified media. As had occurred with tissue cultures on solidified media, suspension-cultured tissue reached a growth and phenol producing plateau at about 50 days when incubated in dark at 24ºC±1. A good elicitor for secretion of polyphenols in these media, without affecting the cell viability for 6 days, was a 10µl pectinase of *A.niger* in 50ml medium containing 1g fresh weight callus. More work is needed to obtain better polyphenolic yield in suspension cultures of *Ficus carica* L., with an aim to maintain good cell viability and continuing secretion of phenols in multi-subculturing media.

As mentioned in Chapter 4, callus was extracted in MeOH and separated by using hexane, ethy acetate, and water. In the DPPH assay, the ethyl acetate and water extracts were very active, IC_{50}=28.35±6.1 and IC_{50}=53.32±5.8, respectively, and the water extract eluted with 50:50 MeOH:water in DIAION column was even more active with an antiradical activity of IC_{50}=17.58±2.02

Ethyl acetate and water extracts were further fractionated using column chromatochraphy and analyzed using TLC and HPLC to determine the phenolic

compounds found in solid caltures. Most of the polyphenols found in in fruit, leaves and the rest of the plant (Chapter 4) are present in callus of petiole tissue cultures of *Ficus carica.* As a result of TLC and HPLC analysis, apigenin, caffeic acid, catechin, chlorogenic acid, emodin, epicatechin, gallic acid, isoquercetrin, kaemferol, myricetrin, quercetin, rutin and taxifolin were identified. All these compounds are useful to human health. As we have seen in chapter 2, catechins are found to have a preventive function as antioxidative "radioprotective, anti-mutagenic, anti-tumor, anti-hyper-cholesterolemic, anti-hyperglycemic, fat-reducing, anti-hypertensive, anti-ulcer, anti-bacterial, anti-viral", "inhibit a-amylase and control obesity and diabetes by diminishing the hydrolysis of starch to glucose" (Hara Y, 1995). Kaempferol, prevents atherosclerosis by inhibiting low density lipoprotein (LDL) oxidation and it is effective in to fighting cancer cells mainly when it is used together with quercetin (Ackland ML et al. 2005; Kowalski Jan et al., 2005). Apigenin was shown to prevent over-proliferation of cells by binding to estrogen receptor sites on membranes of these cells, and inhibited the growth of human leukemia cells. In an other study apigenin and luteolin caused leukemia cells to mature into healthy lymphocytes. Rutin, a bioflavonoid found in many fruits and vegetables,is used to treat hemorrhoids and insufficient blood circulation in legs that may cause varicose and leg edema (MacLennan et al., 1994; Titapant V. et al., 2001) while myricetin was found to have protective properties against prostate cancer (Knekt, P. et al., 2002). Studies in *vitro* showed that quercetin decreased primary tumor growth, prevented metastasis in a model of pancreatic cancer, inhibited proliferation of colon cancer cells and blocked the formation of lipid peroxides, while luteolin caused apoptosis to pancreatic cells and inhibited proliferation of thyroid cancer cells (Lee, LT et al., 2002; Yamashita,

N. et al. 2000; Yin F, et al., 1999) and emodin induced apoptosis in human hepatoma cells (Sheih et al., 2004).

It is suggested that polyphenols act synergistically. As mentioned above, kaempferol is more effective in to fighting cancer cells when it is used together with quercetin. It seems that polyphenols are in a unique combination in each plant species, difficult to be synthesized in a lab, since many polyphenols are unknown, and although may be in very little quantities in each plant their activity may be highly significant. Therefore, using compound polyphenols in our diet may be more beneficial than using single ones. In tissue cultures of *Ficus carica*, presumably, there are many unidentified phenolic compounds, and probably new compounds, that may be beneficial to human health. As further work, identification of possible new phenolic and other chemical compounds in these culture and the possibility of using ethyl acetate and water extracts, as a whole, as food supplements is suggested.

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APPENDIX A

(Chapter 3)

Preliminary work. Before the main work of this research was started, different media were tried inoculated with explants collected from differed parts of Ficus carica (stem, leaf and petioles of the leaf) and supplemented with a variety of growth hormones including the auxins IBA, IAA, NAA and 2,4-D, and cytokinins BAP, kinetin and N-6-(delta-2 isopentelnyl) adenine (2iP) as well as the growth factor TDZ and coconut milk. By examining the cultures, it was concluded that combination of more than one auxin with more than one cytokinin did not generate and maintain better callus than the combination of a single auxin with single cytokinin of different concentrations. There was also no conclusive evintance that the different media tried supported callus better than the common media MS or B-5. Stem parts and leaf parts as inocula delayed in generating callus and proved to be inconvenient. Petioles requires less time to be prepared, sterilized and generate callus. There are no report in literature using slide petioles of Ficus carica for tissue cultures. Part of preliminary work is shown in Table A.1, and main media in Table A.2.

Table A.1. *Ficus carica L.*tissue cultures used in preliminary work. Petiole tissue was used as explants. The media additional to ingredients mentioned contain vitamins (MS i-Inositol 100 mg/l, thiamine HCl 0.400 mg/l, nicotinic acid 0.500 mg/l pyridoxine HCl 0.500 mg/l , and glycine 2.00 mg/l), (L&M: inositol 100mg/l, thiamine HCl 1.00 mg/l, nicotinic acid 0.500 mg/l, pyridoxine HCl 0.500 mg/l, and glycine 2.00 mg/l), (A&D :inositol 100 mg/l, and thiamine HCl ;0.400 mg/l),). Abbreviations: ,A&R:Anderson's Rhododendron bas: Basal media. cm: coconut milk. col: color. Cont. context comp: compact friab.:friable gn: greenish. gr: gray Kin.: kinetin half MS1/2: strength MS media ,mo: months : N&N: Nitch and Nitch. pho: photoperio. .sem: semicompact. Sucr. sucrose.wht: white. y: yellow. Cultures were either in petri dishes (about 10 pieces) or in jars of 100 ml (mostly 2 pieces each).

Nur	Medium	Sucr.	cm	PG	Auxin	mg/l		Cytokiniin		TDZ	pH	Gellan	Temp	times	Light	Cont.	col.	Age
		(g/l)	0%	(mg/l)	NAA	IBA	IAA	BAP	Kin.	mg/l		(g/l)	Celcius	subc	Pho lux			mo
1	MS bas.	30		89		1		0.25			5.5	4.5	26	3	16 2000	comp	gn	3
2	MS bas.	30		89		1		0.25			5.5	4.5	26	3	16 50	comp	gn y	3
3	MS bas.	30		89		1		2.5			5.5	4.5	26	3	dark	comp	y gr	3
4	MS bas	30		89			0.3	2			5.5	4.5	26	2	16 2000	friab.	y	18
5	MS bas	30		89		0.6		4			5.5	4.5	26	2	16 2000	friab.	y	18
6	MS bas	30		89		0.25		2			5.5	4.5	26	3	dark	comp	wht	6
7	MS 3/4	40		120	1.2	0.5		0.5		0.01	5.5	4.5	28	4	16 2000	comp	y.b	11
8	MS 3/4	40		120	1.2	0.5		2			5.5	4.5	28	5	16 2000	com	gn y	11
9	ms 1/2	30		89		0.6	0.3	4			5.5	4.5	26	4	16 2000	com	gn	10
10	MS bas	30		89	0.1			1			5.5	4.5	26	2	16 2000	sem	gn y	5
11	MS 1/2	20	15	80	0.6			0.8		0.02	5.5	4.5	26	5	16 2000	com	gn y	4
12	MS 1/2	30	15	100		0.4				0.04	5.5	4.5	26	3	16 2000	comp	gn y	3
13	AR	30		89		0.05		1			5.5	4.5	26	5	16 2000	friab.	gr	2
14	MS bas	30		89	0.2					0.16	5.5	4.5	26	2	16 2000	comp	gn y	1
15	MS bas	30		89		0.5					5.5	4.5	26	2	16 2000	friab.	gr y	10
16	MS bas	30		89	0.1			0.5			5.5	4.5	26	3	16 2000	sem	gn y	1
17	MS bas	30		89	0.1			1			5.5	4.5	26	2	16 2000	sem	gr y	1
18	MS bas	30		89	0.1			1			5.5	4.5	26	2	16 50	sem	gr y	1
19	MS bas	30		89	0.1			1			5.5	4.5	26	2	dark	sem	gr y	1
20	N&N	30	10	89	0.5						5.6	4.5	26	3	16 2000	com	gr y	12
21	MS bas	30		89	1.75	1.75			5		5/5	4/5	26	4	16 2000	comp	gr y	18
22	MS 1/2	30		89	1.75	235		4			5.5	4.5	26	3	16 2000	comp	gr y	17
23	MS bas	30		89		0.2					5.5	4.5	26	5	16 50	comp	wht y	11
24	MS bas	30		89		0.25					5.5	4.5	26	4	dark	COMP	wht y	5
25	MS bas	30		89		0.25					5.5	4.5	26	4	16 2000	comp	gn y	5

Table A.2- Media for Plant Tissue and Cell Cultures (mg/L)

Components	Murashige-Skoog (1962)	White (1963	Gamborg (1968)	Nitsch (1951)	Heller (1953)	Schenk - Hildebrandt (1972)	Nitsch - Nitsch (1967)	Kohlenbach - Schmidt (1975)	Knop (1865)
$(NH_4)_2SO_4$	-	-	134	-	-	-	-	-	-
$MgSO_{4\times}\ 7H_2O$	370	720	500	250	250	400	125	185	250
Na_2SO_4	-	200	-	-	-	-	-	-	-
KC1	-	65	-	1,500	750	-	-	-	-
$CaC1_{2\times}\ 2H_2O$	440	-	150	25	75	200	-	166	-
$NaNO_3$	-	-	-	-	600	-	-	-	-
KNO_3	1,900	80	3,000	2,000	-	2,500	125	950	250
$Ca(NO_3)_{2\times}\ 4H_2O$	-	300	-	-	-	-	500	-	1,000
NH_4NO_3	1,650	-	-	-	-	-	-	720	-
$NaH_2PO_{4\times}\ H_2O$	-	16.5	150	250	125	-	-	-	-
$NH_4H_2PO_4$	-	-	-	-	-	300	-	-	-
KH_2PO_4	170	-	-	-	_	-	125	68	250
$FeSO_{4\times}\ 7H_2)$	27.8	-	27.8	-	-	15	27.85	27.85	-
Na_2EDTA	37.3	-	37.3	-	-	20	37.25	37.25	-
$MnSO_{4\times}\ 4H_2O$	22.3	7	10 (1 H_2O)	3	0.1	10	25	25	-
$ZnSO_{4\times}\ 7H_2O$	8.6	3	2	0.5	1	0.1	10	10	-
$CuSO_{4\times}\ 5H_2O$	0.025	-	0.025	0.025	0.03	0.2	0.025	0.025	-
H_2SO_4	-	-	-	0.5	-	-	-	-	-
$Fe_2(SO_4)_3$	-	2.5	-	-	-	-	-	-	-
$NiC1_{2\times}\ 6H_2O$	-	-	-	-	0.03	-	-	-	-
$CoC1_{2\times}\ 6H_2O$	0.025	-	0.025	-	-	0.1	0.025	-	-
$A1C1_3$	-	-	-	-	0.03	-	-	-	-
$FeC1_{3\times}\ 6H_2O$	-	-	-	-	1	-	-	-	-
$FeC_6O_5H_{7\times}\ 5H_2O$	-	-	-	10	-	-	-	-	-
K1	0.83	0.75	0.75	0.5	0.01	1.0	-	-	-
H_3BO_3	6.2	1.5	3	0.5	1	5	10	10	-
$Na_2M_0O_{4\times}\ 2H_2O$	0.25	-	0.25	0.25	-	0.1	0.25	0.25	-
Sucrose	30,000	20,000	20,000	50,000	20,000	30,000	20,000~30,000	10,000	-
Myo-Inositol	100	-	100	-	-	1,000	100	100	-
Nicotinic Acid	0.5	0.5	1.0	-	-	0.5	5	5	-
Pyridoxine HC1	0.5	0.1	1.0	-	-	0.5	0.5	0.5	-
Thiamine HC1	0.1-1	0.1	10	1	1	5	0.5	0.5	-
Ca-Pantothenate	-	1	-	-	-	-	-	-	-
Biotin	-	-	-	-	-	-	0.05	0.05	-
Glycine	2	3	-	-	-	-	2	2	-
Cysteine HC1	-	1	-	10	-	-	-	-	-
Folic Acid	-	-	-	-	-	-	0.5	0.5	-
Glutamine	-	-	-	-	-	-	-	14.7	-

Cited by other authors

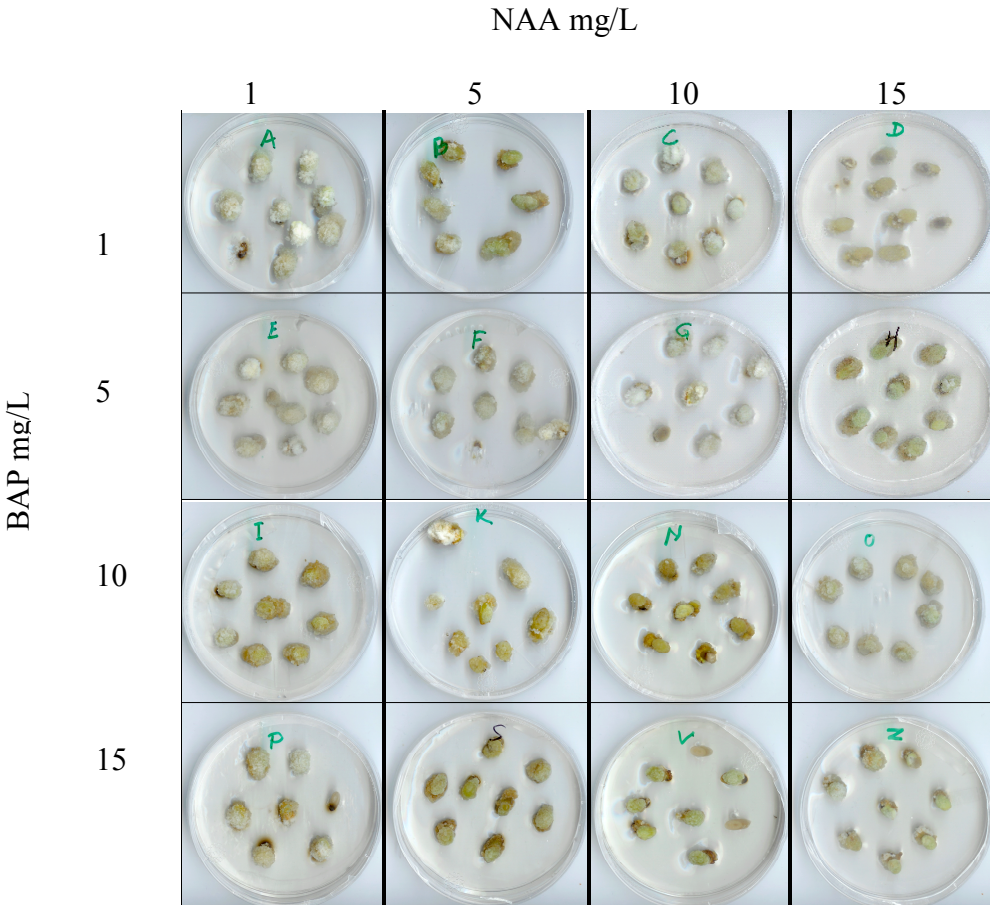


Figure A.1. Sample of 3 weeks old calli on B-5 basal solid media, in petri dishes, supplemented with 1, 5, 10 and 15 mg/L NAA, and 1, 5, 10 and 15 mg/L BAP, incubated in light (85±5% µmol $m^{-2}s^{-1}$) at 25^0C±1. The size of grown calli, including the original explants, is 1-1.5cm in diameter and yellowish to green color. Cultures in plates A, E, I, and F have semifriable callus peripherally and on top of the original expant, while the rest of them have compact rather callus grown around the original explant.

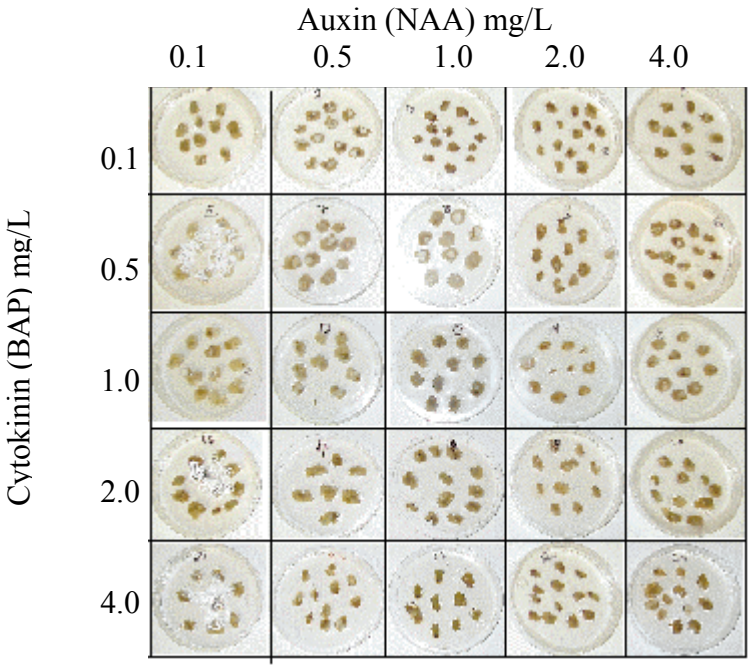


Figure A.2. Samples of cultures incubated on B5 media supplemented with auxin (NAA) and cytokinin (BAP). Cultures 1, 2, 6 and 7 were selected for further experiments

Table A.3. Calli of the same age cultured on MS or B-5 solid media in petri dishes for four weeks under different temperaratures, light intensities, and hormone concentrations. Each treatment consists of five petri dishes containing five pieces of callus of about the same weight. At the end of the fourth week, the weight of each culture was taken and the percent weight increase of each tissue mass was estimated. The calli were freeze dried, extracted in methanol for one hour under argon on shaker and the percent antioxidant polyphenols of dry weight was estimated by using Ciocalteau assay and spectrophotometry. Abriviations. cons.: constant light, Sm.: semi, cm.:compact, fr.: friable, y.: yellow, gr.: green, br.: brown. The calculations and statistical evaluation were perfomed using JMP and Statview for Windows 2000.

Med-ia	Hormones added, mg/l	Photoperiod	Temp. (C±1)	Context	Biomass % increase	Total % antioxidants
MS	0.1 NAA, 0.5 BAP	16hrs	25°C	Sm.cm,y.	372.80±69.64	2.35
MS	0.5 NAA, 0.5 BAP	16hrs	25°C	Sm.cm. y.	484.60±83.44	2.20
MS	0.05 NAA, 0.05 BAP	16hrs	25°C	Sm.cm y.		
MS	0.1 NAA, 0.05 BAP	16hrs	25°C	Sm.cm y.		
MS	0.05 NAA, 0.1 BAP	16hrs	25°C	Sm.cm y.		
MS	0.1 NAA, 0.1 BAP	16hrs	25°C	Sm.cm y.		2.2
MS	0.1 NAA, 0.5 BAP	cons.	25°C	Sm.cm y.gr.	417.80±81.18	2.8
MS	0.5 NAA, 0.5 BAP	cons.	25°C	Sm.cm y.gr	497.00±54.66	
MS	0.05 NAA, 0.05 BAP	cons.	25°C	Sm.cm y.gr	238.13±40.98	2.0
MS	0.1 NAA, 0.05 BAP	cons.	25°C	Sm.cm y.gr	237.18±27.73	
MS	0.05 NAA, 0.1 BAP	cons.	25°C	Sm.cm y.gr	267.09±24.14	
MS	0.1 NAA, 0.1 BAP	cons.	25°C	Sm.cm y.gr	286.42±21.08	
MS	0.1 NAA, 0.5 BAP	dark	25°C	Sm.cm.br.	251.80±50.53	
MS	0.5 NAA, 0.5 BAP	dark	25°C	Sm.cm.br.	281.20±68.87	
MS	0.05 NAA, 0.05 BAP	dark	25°C	Sm.cm.br.	177.78±14.99	4.87
MS	0.1 NAA, 0.05 BAP	dark	25°C	Sm.cm.br.	202.63±19.72	
MS	0.05 NAA, 0.1 BAP	dark	25°C	Sm.cm.br.	168.21±18.89	
MS	0.1 NAA, 0.1 BAP	dark	25°C	Sm.cm.br.	283.14±20.09	3.6
B5	0.1 NAA, 0.5 BAP	16hrs	25°C	.fr.y.	538.40±58.86	
B5	0.5 NAA, 0.5 BAP	16hrs	25°C	.fr.y.	490.20±99.41	2.8
B5	0.05 NAA, 0.05 BAP	16hrs	25°C	.fr.y.		2.2
B5	0.1 NAA, 0.05 BAP	16hrs	25°C	.fr.y.		
B5	0.05 NAA, 0.1 BAP	16hrs	25°C	.fr.y.		
B5	0.1 NAA, 0.1 BAP	16hrs	25°C	.fr.y.		2.6
B5	0.1 NAA, 0.5 BAP	cons.	25°C	fr.y.	560.00±59.73	
B5	0.5 NAA, 0.5 BAP	cons.	25°C	fr.y.	574.80±54.34	2.6
B5	0.05 NAA, 0.05 BAP	cons.	25°C	fr.y.	295.57±23.36	2.4
B5	0.1 NAA, 0.05 BAP	cons.	25°C	fr.y.	528.13±74.76	
B5	0.05 NAA, 0.1 BAP	cons.	25°C	fr.y.	408.91±77.91	
B5	0.1 NAA, 0.1 BAP	cons.	25°C	fr.y.	497.39±81.26	2.13
B5	0.1 NAA, 0.5 BAP	dark	25°C	Sm.cm.br.	410.80±50.30	3.9
B5	0.5 NAA, 0.5 BAP	dark	25°C	Sm.cm.br.		
B5	0.05 NAA, 0.05 BAP	dark	25°C	Sm.cm.br.	283.53±29.02	
B5	0.1 NAA, 0.05 BAP	dark	25°C	Sm.cm.br.	231.84±32.57	4.72
B5	0.05 NAA, 0.1 BAP	dark	25°C	Sm.cm.br.	225.24±42.41	

B5	0.1 NAA, 0.1 BAP	dark	25°C	Sm.cm.br.	318.06±93.13	3.74
MS	0.1 NAA, 0.5 BAP	16hrs	29°C	Sm.cm.y.gr	519.00±93.76	2.7
MS	0.5 NAA, 0.5 BAP	16hrs	29°C	Sm.cm.y.gr	618.22±79.82	2.6
MS	0.05 NAA, 0.05 BAP	16hrs	29°C	Sm.cm.y.gr	317.14±68.434	
MS	0.1 NAA, 0.05 BAP	16hrs	29°C	Sm.cm.y.gr	715.97± 206.87	2.2
MS	0.05 NAA, 0.1 BAP	16hrs	29°C	Sm.cm.y.gr	412.31±103.68	
MS	0.1 NAA, 0.1 BAP	16hrs	29°C	Sm.cm.y.gr	491.56±40.75	2.3
MS	0.1 NAA, 0.5 BAP	dark	29°C	Sm.cm.y.br	343.60±46.92	5.06
MS	0.5 NAA, 0.5 BAP	dark	29°C	Sm.cm.y.br	373.40±69.45	5.0
MS	0.05 NAA, 0.05 BAP	dark	29°C	Sm.cm.y.br	350.78±76.37	4.87
MS	0.1 NAA, 0.05 BAP	dark	29°C	Sm.cm.y.br	313.19±25.59	2.89
MS	0.05 NAA, 0.1 BAP	dark	29°C	Sm.cm.y.br	275.91±35.51	
MS	0.1 NAA, 0.1 BAP	dark	29°C	Sm.cm.y.br	292.56±21.42	4.52
MS	0.1 2,4-D 0.5 BAP	16hrs,	29°C	Sm.cm.y.gr	237±94.08	0.37
MS	0.5 2,4-D 0.5 BAP	16hrs,	29°C	Sm.cm.y.gr	252±36.66	0.44
MS	0.1 IAA 0.5 BAP	16hrs,	29°C	Sm.cm.y.gr		
MS	0.5 IAA 0.5 BAP	16hrs,	29°C	Sm.cm.y.gr		
MS	2.0 IAA 0.5 BAP	16hrs,	29°C	Sm.cm.y.gr		
MS	4.0 IAA 0.5 BAP	16hrs,	29°C	Sm.cm.y.gr		
MS	0.1 2,4-D 0.5 BAP	dark	29°C	Sm.cm.y.br		
MS	0.5 2,4-D 0.5 BAP	dark	29°C	Sm.cm.y.br		
MS	0.1 IAA 0.5 BAP	dark	29°C	Sm.cm.y.br		
MS	0.5 IAA 0.5 BAP	dark	29°C	Sm.cm.y.br		
MS	2.0 IAA 0.5 BAP	dark	29°C	Sm.cm.y.br		
MS	4.0 IAA 0.5 BAP	dark	29°C	Sm.cm.y.br		
B5	0.1 NAA, 0.5 BAP	16hrs	29°C	fr.y.	630.40±93.90	2.31
B5	0.5 NAA, 0.5 BAP	16hrs	29°C	fr.y.	677.80±57.11	2.8
B5	0.05 NAA, 0.05 BAP	16hrs	29°C	fr.y.	654.51±30.42	
B5	0.1 NAA, 0.05 BAP	16hrs	29°C	fr.y.	422.49±65.86	
B5	0.05 NAA, 0.1 BAP	16hrs	29°C	fr.y.	328.33±50.23	2.5
B5	0.1 NAA, 0.1 BAP	16hrs	29°C	fr.y.	793.02±56.91	2.4
B5	0.1 NAA, 0.5 BAP	dark	29°C	Sm.cm.y.br	436.80±76.585	4.2
B5	0.5 NAA, 0.5 BAP	dark	29°C	Sm.cm.y.br	472.40±45.12	
B5	0.05 NAA, 0.05 BAP	dark	29°C	Sm.cm.y.br	443.49±29.81	
B5	0.1 NAA, 0.05 BAP	dark	29°C	Sm.cm.y.br	320.64±24.98	
B5	0.05 NAA, 0.1 BAP	dark	29°C	Sm.cm.y.br	349.66±23.66	4.47
B5	0.1 NAA, 0.1 BAP	dark	29°C	Sm.cm.y.br	564.83±64.33	

Folin-Ciocalteau reagent for estimated total antioxidant phenolics

The amount of total phenolics in the cultures is determined by using Folin-Ciocalteau-phenol reagent (Singleton and Rossi, 1965) containing 2.5% sodium molybdate,10% sodium tungstate, 7.5% sodium carbonate and the sample. Phenolic groups are oxidized with phosphomolybdic- phosphotungstic acid. The color yield depends on hydroxyl groups and the place they have in the molecules. The mixture is allowed to stand for 30 minutes and then the absorbance is taken at 750 nm. The blank is water. Standard curve is prepared using serial dilutions of gallic acid or catechin and the phenolics are expressed as gallic acid equivalen or catechin. Instead of standard curve, the equation of total phenol calculations can be used:

Total Phenols%= $(A_{sample}XW_{standart})/(A_{standard}XW_{sample}\}X100\%$, where A=absorbance, and W=weight. One limitation with Folin-Ciocalteu assay is that compounds such as ascorbic acids or sugars may be interfering in complex samples.

In this lab the Folin-Ciocalteu assay was prepared by using 950 µL distilled water, 50 µL sample or chatechin dilution (1 µg/ µL), and 250 µL Folin-Ciocalteau reagent (Sigma 2N). After vortex, 750 µL of 20% sodium carbonate ($NaCO_3$) was added. After vortex again, 3 mL of distilled water was added, all together in a 5 mL solution in a 15 mL test tube. The black was prepared the same way with the samples with the difference that the 50 µL of sample was replaced with 50 µL of the sample or catechin solvent. It was incubated at room temperature for 2 hours and absorbance was taken at 750 nm with the use of spectrophotometer (BECKMAN DU640B). The weight of chatechin in each dilution is plotted against the abasorbance. The weight is determined by using the dilution factor.

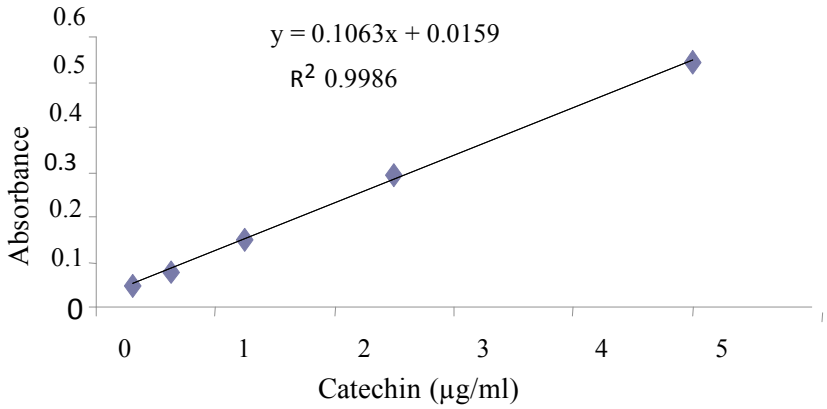


Figure A.3. Catechin calibration curve. Replacing "y:" with the absorbance of a sample found by using Folin Ciocalteu,s assay, the concentration "x" in catechin equivalent in the sample can be calculated. The result is multiplied by the additional to catechin diluting factor of the sample (1.8)

Cell viability assay

The ability of the plant cells to reduce tetrazolium salts has been used by many authors to measure the viability of cells in plant suspension cultures. TTC (2,3,5-triphenyl tetrazolium chloride), a water-soluble salt, is readily reduced by efficient reducing agents such as enzymes of alive cells, mainly hydrogenases, and an orange-red colored product (formazan) is produced. This color can be detected on a spectrophotometer. A sample with a higher number of living cells will produce a stronger color than a sample with fewer viable cells. TTC is dissolved in 0.05 M sodium phosphate buffer (pH 7.5) and incubated with the cells (no shaking to avoid oxidation) in a proportion of 2:1, sodium-phosphate-buffer: cell-medium, in 0.8% (w/v) final concentration of TTC, at room temperature in the dark for 18-20 hours, and then the cells are washed and extracted with 75% ethanol for 30min.at 70^{o}C and the abosrbance of the extracted formazan is taken at 485nm. The percentage of the cell viability is calculated using a standard curve generated with known concentrations of alive cells at known dilutions The appearance of color, however, may be a sufficient indication of viability of the cells in most cases. A strong red color is an indication of high viability.

An even simpler method for roughly determining cell viability is staining cells on a microscope slide by adding a drop of Evans Blue (0.1% w/v aqueous). After the excess of Evans Blue is washed, the slide is examined under a compound microscope. Evans Blue stains the cells that have lost the integrity of their membrane, and they appear blue under the microscope **(**Gaff et al., 1971**).**

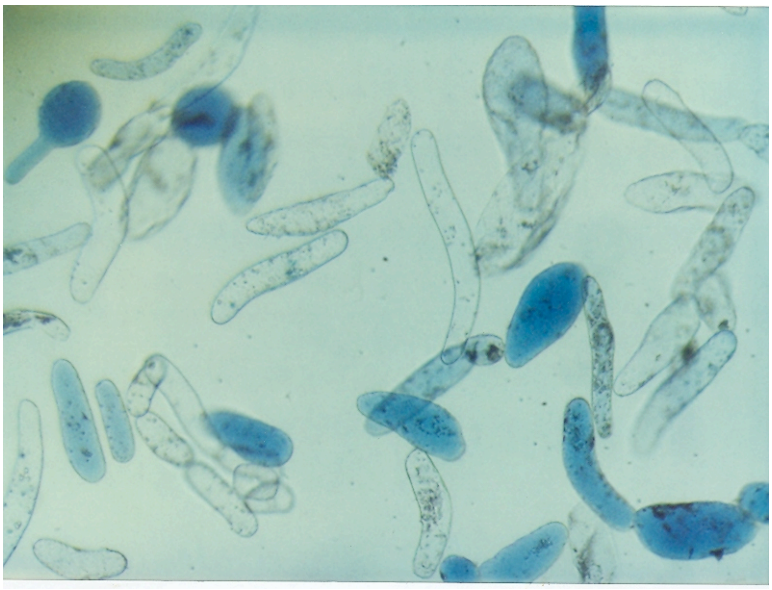


Figure A. 4. Cells in B5 media tissue cultures stained with Evans Blue, as they are viewed under the compound microscope (400X). Blue cells are dead, the light color cells are alive.

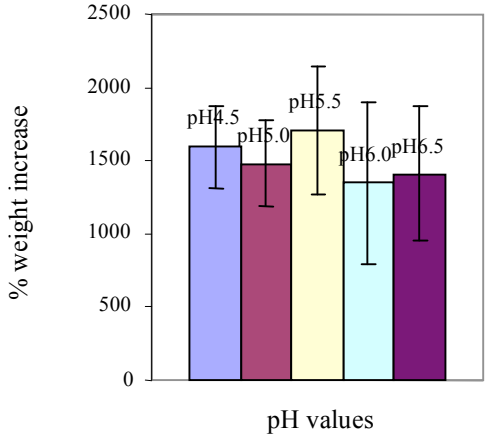


Figure A.5. Histogram showing the effect of pH on callus cultured on solid B5 media supplement with 0.5 mg/L NAA and 0.5 mg/L BAP. The percentage mass (dry weight) growth of cultures of pH 5.5 is significantly higher that this of pH 6.5 (P: 0.01). The bars represent Standard Diviation.

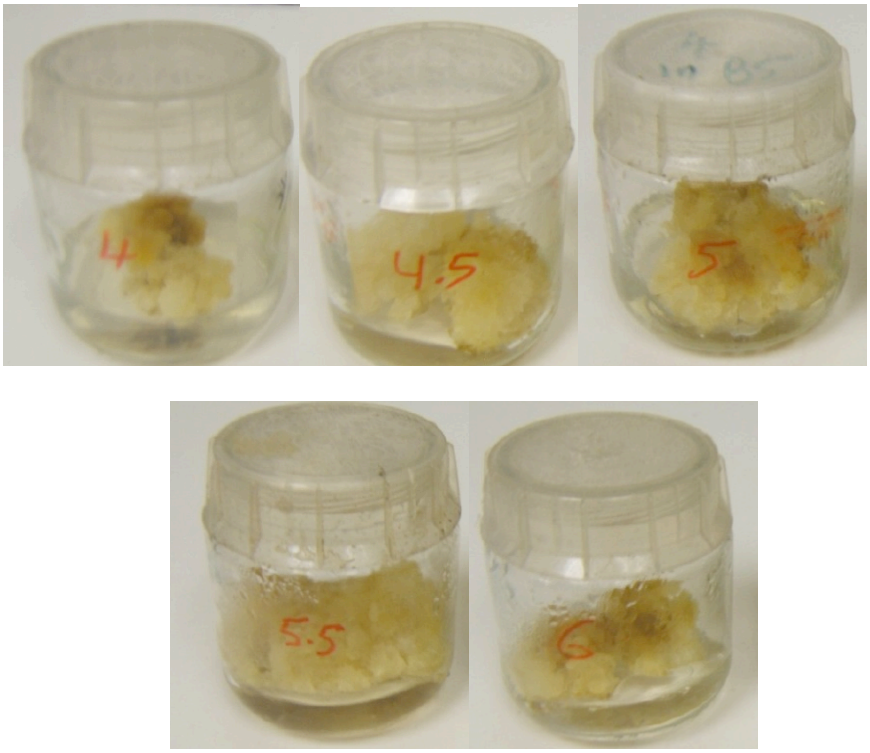


Figure A.6. *Ficus carica* tissue cultures 40 days after subculturing showing the effect of pH on callus growth and context. The different pH values are marked on the jars.

Table A. 4. pH effect on callus polyphenolic content. It is shows that pH 4.5-pH 6.5 has not any significant different effect on phenolic content of callus cultured on solid B5 media supplement with 0.5 mg/L NAA and 0.5 mg/L BAP (P: 0.11 or higher). The mean absorbance is proportional to phenols in callus.

pH	4.5	5.0	5.5	6.0	6.5
Mean absorbance at 750 nm	0.103	0.106	0.121	0.106	0.124
Std Deviation	0.044	0.044	0.048	0.022	0.023
Std Error	0.016	0.016	0.017	0.007	0.008
P value (t-paired test)	(paired with pH 5.5) 0.11	(paired with pH 5.5) 0.15		(paired with pH 5.5) 0.16	(paired with pH 5.5) 0.54
N	8	8	8	8	8

APPENDIX B

(Chapter 4)

DPPH assay

DPPH assay was used to screen tissue cultures for their antioxidant activity. DPPH is a stable free radical compound of purple color and when reduced by an antioxidant turns yellow (bleached) as its absorbance decreases (absorbs at 517 nm) . Depending on the amount of the reduced DPPH the color of ethanol, for example, in which the compounds are dissolved, can be changed from purple to light purple, to light yellow or yellow (Koleva, et al., 2000). In screening samples for antioxidant activity, serial dilutions of the sample in ethanol were mixed with 400 μM of DPPH dissolved in ethanol. They were introduced into 5ml Teflon screw-capped tubes and made up to a volume of 2 ml (500 μl of DPPH , 7-125 μg of samples and ethanol) A parallel dilution series of ascorbic acid were prepared as reference material and a dilution series of the test compound were prepared without DPPH. as a blank. Control tubes were also prepared without the sample (DPPH and ethanol, and ethanol as blank). The tubes were incubated in a water bath at 37ºC for 30 min and then the absorbance of the sample was measured at 750 nm by spectrophotometer. The tests were performed three times. The percent inhibition was found using the equation %In=[C-S)/C]x100 the where C is the absorbance of the control and S the net absorbance of the sample. The result IC_{50} (concentration of sample needed to scavenge 50% of DPPH) by the samples is determined by using Microsoft Excel software to obtain the slope (m)of the linear equation y=mx+b by applying the percent inhibition on the “y” axis and the concentrations on the “x”. Using the equation further by applying the found slope and replacing “y” with 50% inhibition, the concentration (x) is found. IC_{50} less than 50 μg/ml is considered as high antioxidant content. A sample with IC_{50} more than 200 μg/ml is considered as having no significant antioxidant activity. Figures B.1 and Figure B.2 demonstrate the DPPH equation of one

of the three samples of ethyl acetate extract and water extract eluted with water:MeOH, respectively.

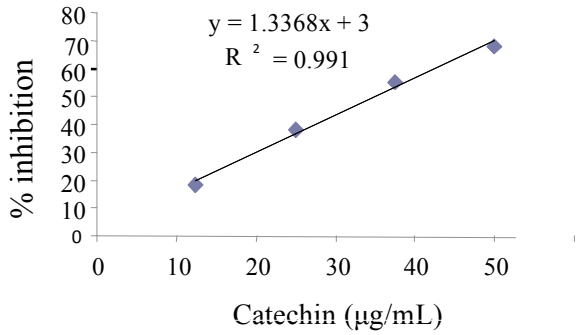


Figure B.1.Example of curve showing the antioxidant activity of an ethyl acetate extract sample IC50=34.

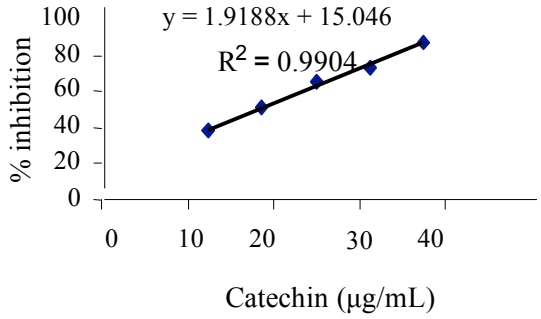
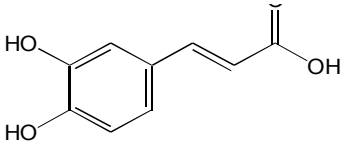
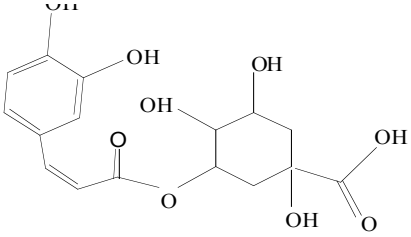


Figure B. 2. Curve showing the antioxidant activity of an water extract sample using DPPH assay.

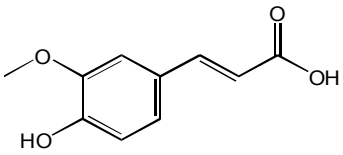
IC50=18



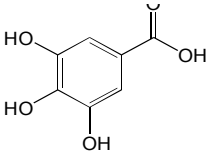
Caffeic acid



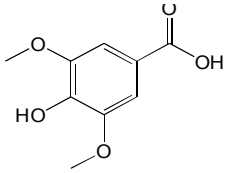
Chlorogenic acid



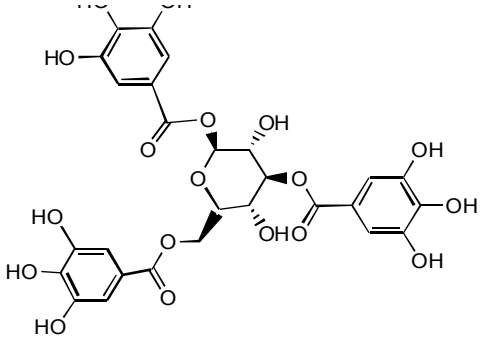
Ferulic acid



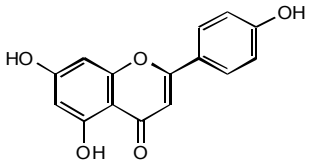
Gallic acid



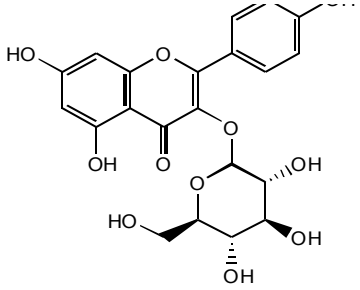
Syringic acid



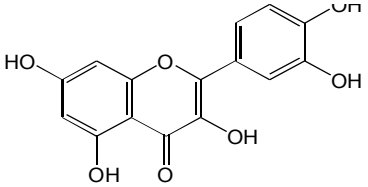
Tannic acid ($C_{27}H_{24}O_{18}$)



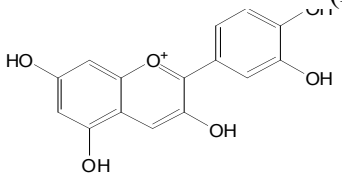
Apigenin



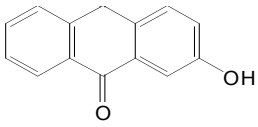
Astragalin
(kaempferol-3-0-glucoside)



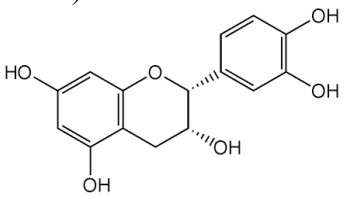
Catechin



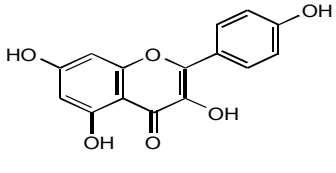
Cyaniding



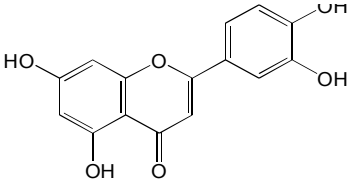
Emodin



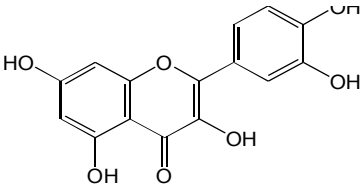
Epicatechin



Kaempferol

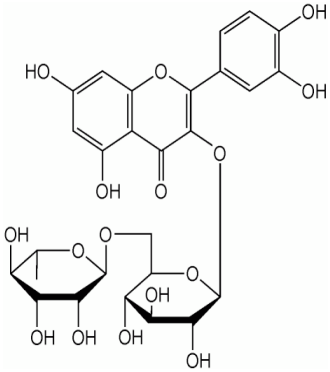


Luteolin

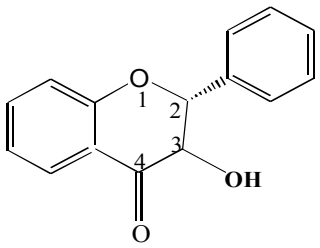


Quercitin

(Continuing on the next page)



Rutin



taxifolin

Figure B. 3. Polyphenols found in *Ficus carica L.*

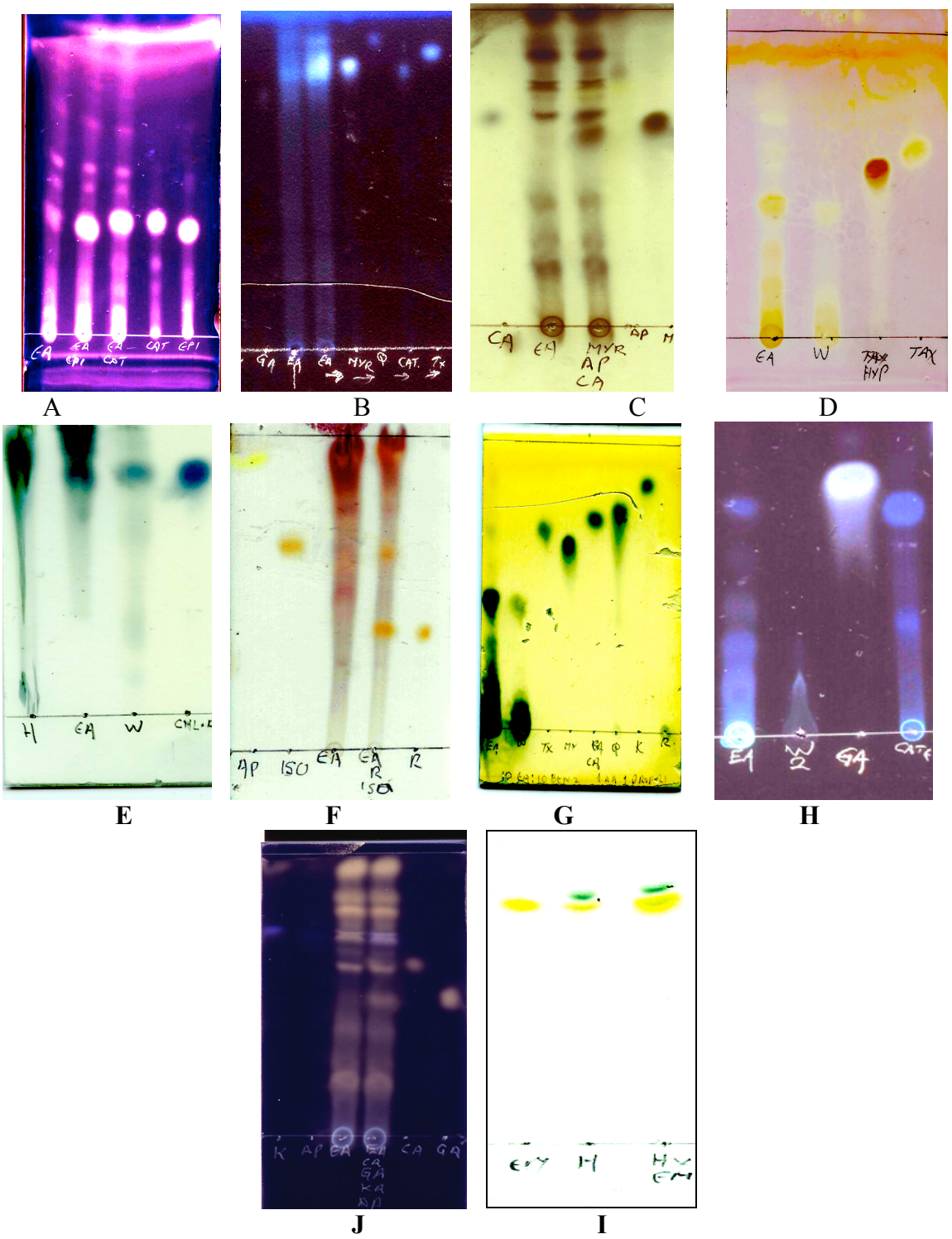
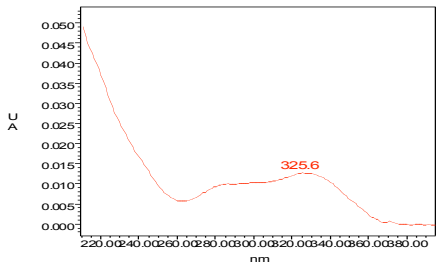


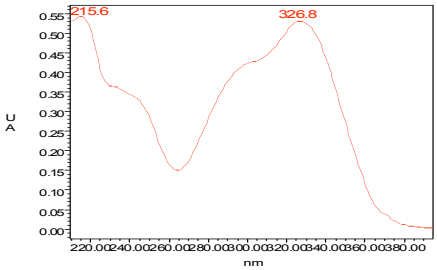
Figure B.4. TLC analysis. (Description on the next page)

A: Ethyl acetate (EA) extract eluted with benzene (BEN): EA: acetic acid (AA): formic acid (FA), 7:3:1:1. B: EA extract eluted with BEN : EA: AA: FA, 5:5:1:1. C: EA extract eluted with BEN : EA: AA: FA, 10:5:1:1, and detected with 5% sulfuric in methanol. D: EA and water (W) extracts, eluted with EA:W:AA: FA, 10: 2.6: 1:1, and detected with DPPH. E. Hexane, EA and water extracts eluted with EA:W:AA: FA, 10: 2.6: 1:1, and sprayed with 50% Folin Ciocalteau reagent. 5% sulfuric acid. F: EA extract eluted with BEN : EA: AA: FA, 10:2:1:1 and detected with DPPH. G: EA and W extracts eluted with EA:W:AA: FA, 10:2:1:1 and visualized with 50% Folin Ciocalteau reagent. H: EA and W2 (fraction of water in Diaion HP20 column chromatography) extracts, eluted with BEN : EA: AA: FA 7:3:1:1 and visualized with 50% Folin Ciocalteau reagent J: Hexane extract, eluted with EA: W: AA: FA, 12:7:1:1 and visualized under UV 254. I: H:EA: AA: FA and visualized under UV 254. Abriviations; AP: apigenin, CA; caffeic acid. CAT or CATE catechin, CHLOR; chlorogenic acid, EM: Emodin. EPI, epecatechin, GA: gallic acid, HYP; hypercentin, ISO: isoquercetrin, K; kaemferol, MYR: myricetrin Q: quercetin R: rutin, TX or TAX: taxifolin . The color of the plates were enhanced using Photo Suite Image to improve contrast.

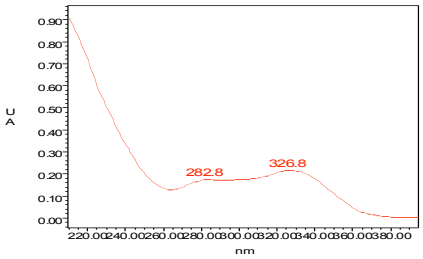
Spectra for polyphenols found in calli of *Ficus carica L.* To verify the identity of the compounds in the samples, the UV vis spectra of the known standards were obtained by the PDA detector simultaneously with the chromatograph analysis, and these spectra were compared with the spectra of compounds in the samples that had the same retention time with that of the standards. The spectra are shown in Figure B.5.



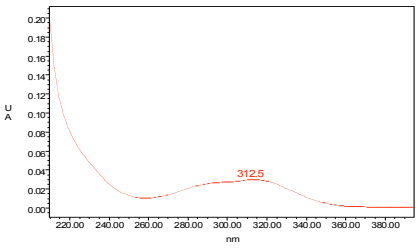
Apigenin



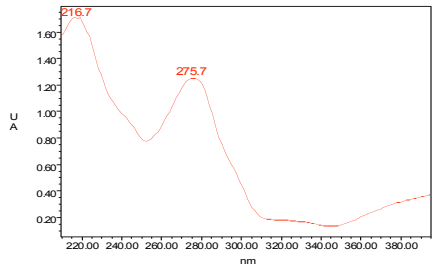
Caffeic acid



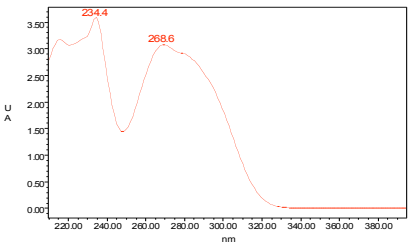
Catechin



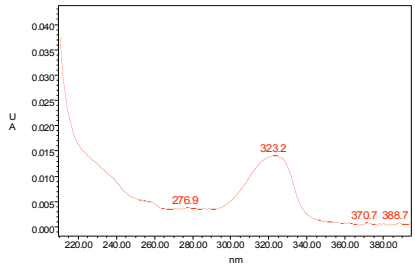
Chlorogenic acid



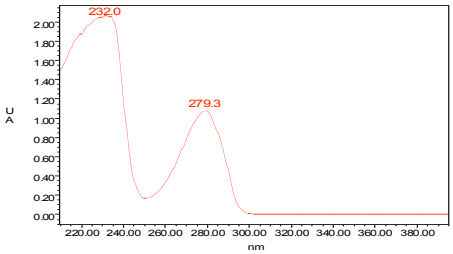
Cyanidin



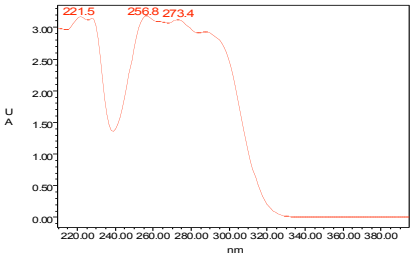
EGCG



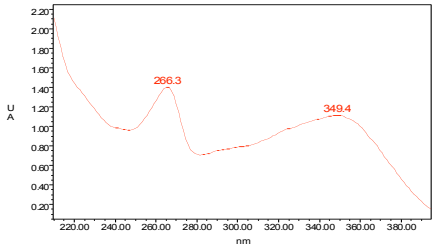
Emodin



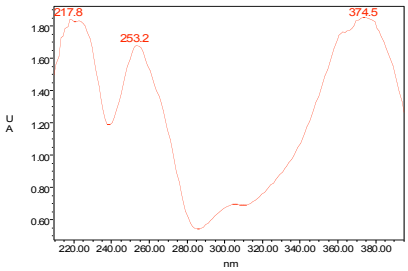
Epicatechin



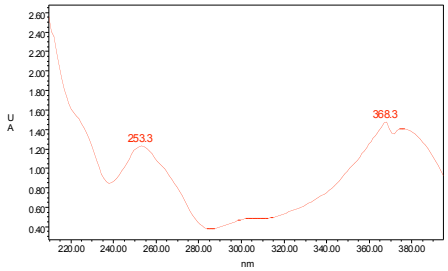
Gallic acid



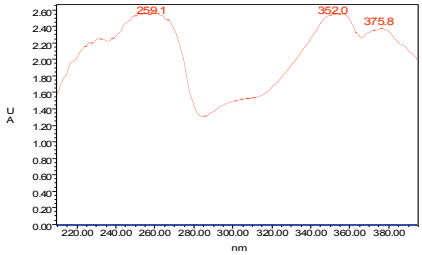
Kaemferol



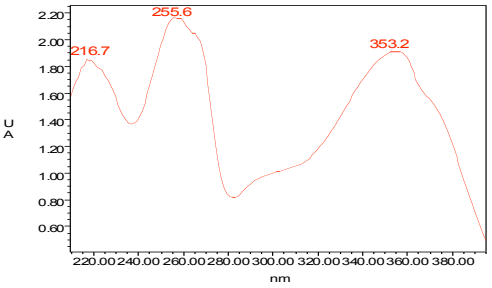
Myricetin



Myricetrin



Quercetin



Rutin

Figure B.5. spectra of phenolic compounds found in tissue cultures of *Ficus carica L.*

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